mmHg)] to yield 0.7 g of a colorless oil. This was dissolved in 3 mL of 2-propanol, titrated with concentrated HCl (12 drops required), and treated with 12 mL of anhydrous ether. The product crystallized spontaneously; it was allowed to stand for several hours and then filtered, and the resulting solids were washed first with 80:20 2-propanol ether and then with ether. After air-drying there resulted 0.7 g of white solids: mp 182–183 °C; yield 29%. The analytical sample was dried at 100 °C for 24 h. Anal. ($C_{13}H_{22}ClNO_2S$) C, H.

In the same manner, the corresponding phenethylamines **2e,f,h** were prepared from the appropriate phthalimides **15b–d**.

Compound **2e** (from **15c**): free base; bp 138–144 °C (0.3 mmHg). **2e**·HCl white crystals; mp 139–140 °C; yield 62%. Anal. ($C_{13}H_{22}ClNO_2S$) C, H.

Compound **2f** (from **15b**): free base; bp 135–155 °C (0.3 mmHg). **2f**·HCl white plates; mp 153.5–154.5 °C; yield 66%. Anal. ($C_{13}H_{22}ClNO_2S$) C, H.

Compound **2h** (from **15d**): free base; bp 140–155 °C (0.25 mmHg). **2h**·HCl: white crystals; mp 161–162 °C; yield 52%. Anal. ($C_{14}H_{24}ClNO_2S$) C, H.

Psychopharmacological Assays. The screening and human potency determinations, the experimental protocols, and the basis of determining effective dosages were essentially those described in detail in earlier studies.[24] Briefly, trials were initiated in normal adult subjects at levels assumed to be inactive (generally 0.5 mg, orally), and the assay levels were increased, at appropriate intervals, in increments of about 1.6:1. With the confirmed establishment of threshold levels (levels at which the chronology of action was certain but the qualitative nature not clearly defined), assays were expanded to a larger group of volunteers, all experienced with a broad spectrum of psychotropic drugs. All potency values were determined in at least two subjects, but five products (1b, **2b**, **2c**, **3c**, and 1j) were sufficiently interesting to warrant broader evaluation. The number of subjects (N) and number of trials (T) are summarized in Table I. The qualitative aspects of these studies are summarized under Results and Discussion.

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Methotrexate Analogues. 23. Synthesis, Dihydrofolate Reductase Affinity, Cytotoxicity, and in Vivo Antitumor Activity of Some Putative Degradation Products of Methotrexate–Poly(L-lysine) Conjugates

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Derivatives of methotrexate (MTX) in which the γ-carboxyl group is joined to the ε-amino group of L-lysine, L-lysyl-L-lysine, or L-lysyl-L-lysyl-L-lysine, respectively, were prepared for evaluation of their dihydrofolate reductase (DHFR) affinity, their ability to retard cell growth in culture, and their antitumor activity in vivo. These small lysine derivatives of MTX are of interest as putative breakdown products of MTX–poly(L-lysine). Inhibition of DHFR in a cell-free assay was decreased only 3-fold relative to MTX, indicating that γ-substitution by up to three lysines is well tolerated for binding. On the other hand, toxicity toward L1210 murine leukemia cells in culture decreased up to 120-fold relative to MTX as the lysines increased in number from one to three, suggesting that uptake across the cell membrane becomes difficult when positively charged lysines are at the γ-position. Growth inhibition of H35 rat hepatoma cells was decreased 40- to 60-fold relative to MTX, but in $H35R_{0.3}$ cells, which have normal DHFR content but are 180-fold MTX resistant by virtue of a transport defect, the lysine derivatives were only 3- to 7-fold less toxic than MTX. When the adducts were given to L1210 leukemic mice by twice-daily injection for 10 days, an increase in life span (ILS) of 80–100% was observed at 40 mg/kg (equivalent to 20–30 mg/kg of MTX). MTX itself, on the same schedule, gave a 100% ILS at 0.5 mg/kg. The low in vivo activity of the mono-, di-, and trilysine adducts suggests minimal systemic hydrolysis to free MTX.

Covalent poly(L-lysine) conjugates of methotrexate (MTX) have been studied in several laboratories[1-10] as a means of achieving drug uptake by pinocytosis as opposed to the usual mechanism of carrier-mediated MTX active transport, and they have given promising therapeutic results against human solid-tumor xenografts in nude mice.[9] From the available evidence in neoplastic[6,8,10] and nonneoplastic[1-5] cell lines in culture, it appears that the conjugates are internalized in micropinocytotic vesicles that coalesce into larger vacuoles and ultimately fuse to protease-rich secondary lysosomes. The conjugates themselves are ineffective as dihydrofolate reductase inhibitors,[2] but degradation of the poly(L-lysine) backbone by the lysosomal proteases yields small fragments that exert typical antifolate effects[6,8] upon being expelled into the cytoplasm. Cellular uptake of the conjugates is much more rapid than the uptake of MTX, especially when the cells are MTX resistant by virtue of a transport defect.[1,6,8] Moreover, the uptake of MTX–poly(L-lysine), unlike that of MTX, is

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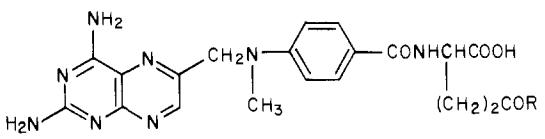
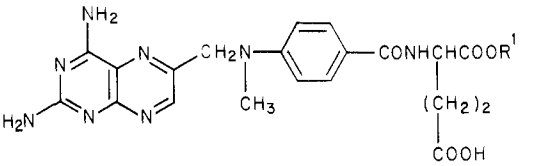
nonsaturable and appears to be unaffected by organic anion inhibitors of MTX transport.[5] Compelling evidence for the lysosomotropic[11] action of the conjugates is provided by the observations that trypsinization diminishes cytotoxicity,[1,3] that the corresponding poly(D-lysine) adducts are inactive even though they are taken up equally well,[2,10] and that addition of lysosomal inhibitors, such as chloroquine or leupeptin, to the culture at the same time as MTX–poly(L-lysine) does not alter the uptake of the conjugate but markedly decreases its activity.[6,10] While there is evidence in at least one instance[10] that MTX and MTX–polyglutamates are formed intracellularly from MTX–poly(L-lysine), the major lysosomal products are believed to be small MTX-linked lysine oligomers.[1-3,8,10] However, neither the molecular structure of these oligomers nor the extent of their contribution to cytotoxicity has been established.

As part of our overall interest in chemically modified MTX derivatives,[12-15] we have synthesized three γ-linked lysyl peptides (**1–3**) that are potential lysosomal degradation products of MTX–poly(L-lysine). This paper describes the preparation of **1–3**, and compares the dihydrofolate reductase inhibitory activity and in vitro cytotoxicity of these compounds relative to MTX. In agreement with previous reports[16,17] that γ-amide substitution in the glutamate moiety of MTX is relatively well-tolerated by dihydrofolate reductase, the γ-lysyl derivatives showed an IC_{50} only 2- to 3-fold higher than that of the parent drug. Moreover, the IC_{50} was not very sensitive to an increase in number of lysines from one to three, suggesting that when these compounds bind to the enzyme active site, the positively charged oligolysyl side chain remains free and does not interact with groups on the enzyme. In contrast to the 2- to 3-fold decrease in dihydrofolate reductase affinity, on the other hand, there was a 30- to 120-fold decrease in cytotoxicity, which was consistent with the view that these small lysyl derivatives are poorly taken up and probably cannot utilize the pinocytotic mechanism to penetrate cells.

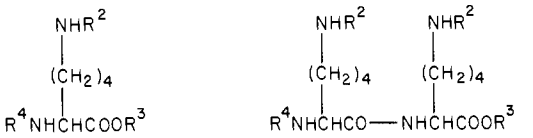


MTX, R = OH
1 [MTX(γ-ϵ)-Lys], R = $NH_2CH[(CH_2)_4NH]COOH$
2 [MTX(γ-ϵ)-$(Lys)_2$],
R = $NH_2CH[(CH_2)_4NH]CONHCH[(CH_2)_4NH_2]COOH$
3 [MTX(γ-ϵ)-$(Lys)_3$],
R = $NH_2CH[(CH_2)_4NH]CONHCH[(CH_2)_4NH_2]$-
$CONHCH[(CH_2)_4NH_2]COOH$

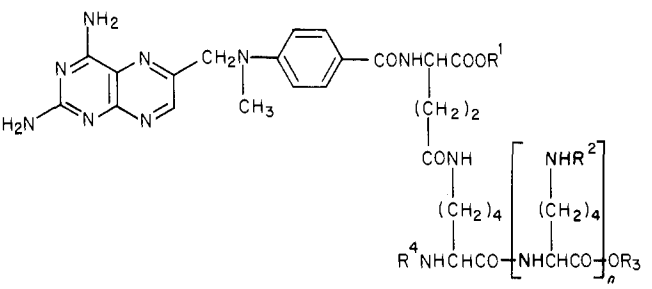
Chemistry. MTX α-*tert*-butyl ester (**4**)[16] was coupled in 76% yield to N^{α}-*t*-Boc-L-Lys methyl ester (**7**) via the diethyl phosphorocyanidate method at room temperature.[13,18] Compound **7** was prepared in 67% yield, as the HCl salt, by reaction of N^{α}-*t*-Boc-N^{ϵ}-Cbz-L-Lys with methyl iodide and cesium carbonate,[14] followed at once by hydrogenolysis of the Cbz group over 5% Pd/C and treatment with HCl. The coupling product, diester **10**, was selectively cleaved with $Ba(OH)_2 \cdot 8H_2O$ in 50% EtOH to obtain the monester **11** (87%). Treatment of **11** with CF_3CO_2H at room temperature for 10 min afforded the desired MTX(γ-ϵ)-Lys (**1**, 57%). The nonpolar diester **10** showed the anticipated solubility in organic solvents and could be purified readily by silica gel chromatography with 95:5 $CHCl_3$–MeOH as the eluent. The diacid **1**, on the other hand, had to be purified on DEAE-cellulose with 3% NH_4HCO_3 as the eluent, after extensive preliminary desalting with distilled water. Freeze-drying of the NH_4HCO_3 eluates left the product as a free acid. In addition to elemental analyses, which indicated a tetrahydrate, **1** gave the expected lysine/glutamic acid ratio of 1:1 by amino acid analysis and showed ultraviolet absorption maxima appropriate for a 2,4-diaminopteroyl derivative. In contrast to the monoacid **11**, which could be precipitated from alkaline solution with AcOH, the diacid **1** remained dissolved over the entire range of pH 4 to 6.



4, R^1 = *t*-Bu
5, R^1 = CH_2Ph



6, R^2 = Cbz; R^3 = Me; R^4 = Boc
7, R^2 = Cbz; R^3 = Me; R^4 = H
8, R^2 = Cbz; R^3 = Me; R^4 = Boc
9, R^2 = Cbz; R^3 = Me; R^4 = H



	n	R^1	R^2	R^3	R^4
10	0	*t*-Bu		Me	Boc
11	0	*t*-Bu		H	Boc
12	0	CH_2Ph		H	Boc
13	1	CH_2Ph	Cbz	Me	Boc
14	1	H	Cbz	H	Boc
15	2	CH_2Ph	Cbz	Me	Boc
16	2	H	Cbz	H	Boc

For the preparation of MTX(γ-ϵ)-$(Lys)_2$ (**2**) and MTX(γ-ϵ)-$(Lys)_3$ (**3**), we chose to employ the previously undescribed MTX α-benzyl ester (**5**), which was obtained in 94% yield (analytically pure without chromatography) from 4-amino-4-deoxy-N^{10}-methylpteroic acid[16] and O^{γ},*N*-bis(trimethylsilyl)-L-glutamic acid α-benzyl ester via diethyl phosphorocyanidate coupling. That silylation of the

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Table I. Dihydrofolate Reductase Inhibitory Activity and Cytotoxicity of MTX(γ-ϵ)-Lys (1), MTX(γ-ϵ)-$(Lys)_2$ (2), and MTX(γ-ϵ)-$(Lys)_3$ (3)

	L1210 DHFR[a]		L1210 cells[b]		H35 cells[c]		$H35R_{0.3}$ cells[c,d]	
compd	IC_{50}, nM	rel act.	IC_{50}, μM	rel act.	IC_{50}, μM	rel act.	IC_{50}, μM	rel act.
MTX	50	1.0	0.024[e]	1.0	0.010	1.0	1.8 (180)	1.0
1	87	0.58	0.76	0.032	0.40	0.025	6.0 (15)	0.30
2	86	0.59	1.8	0.013	0.50	0.020	9.5 (19)	0.19
3	140[f]	0.36	2.9	0.0083	0.56	0.018	12 (21)	0.15

[a] Spectrophotometric assay at 340 nm as previously described,[27] with purified enzyme (98 nM) in 0.1 M potassium phosphate, pH 7.0. [b] Cells were incubated for 48 h, and percent cell growth relative to controls was plotted to obtain an IC_{50}; see ref 12. [c] Cells were incubated for 72 h; see ref 8. [d] Numbers in parentheses are the ratios of the IC_{50} values for the $H35R_{0.3}$ cells relative to the H35 cells and are a measure of the degree of cross-resistance between MTX and the MTX-lysine adducts. [e] Mean of two separate experiments. [f] The enzyme concentration in this experiment was 80 nM; the IC_{50} was corrected for this difference for purpose of comparison.

amino ester gave *N*-silylation in addition to silylation of the γ-carboxyl was evident from the NMR spectrum, which contained two well-separated trimethylsilyl singlets. Ester **5** was coupled directly to the N^{ϵ},*O*-bis(trimethylsilyl) derivative of N^{α}-*t*-Boc-L-Lys via the mixed carboxylic carbonic anhydride technique. The yield of coupling product (**12**) after column chromatography (silica gel; 85:15 $CHCl_3$–MeOH) was only 52%. However, another 41% of slightly impure product was obtained by subsequent addition of some Et_3N to the eluent, thus raising the yield to ca. 90%. Condensation of **12** with N^{ϵ}-Cbz-L-Lys methyl ester to form the blocked dilysyl adduct **13** was performed in 79% yield with diphenylphosphoryl azide as the coupling reagent, the benzyl and methyl ester groups in **13** were cleaved with $Ba(OH)_2 \cdot 8H_2O$ in 50% EtOH to give the diacid **14** (86%), and the N^{α}-*t*-Boc and N^{ϵ}-Cbz groups were removed simultaneously with CF_3CO_2H–thioanisole[19] or HBr–AcOH at room temperature to obtain **2** in 77 and 42% yield, respectively. Use of CF_3CO_2H for deblocking in this instance (compare **1**) gave a hydrated trifluoroacetate salt, even after DEAE-cellulose chromatography with 3% NH_4HCO_3 as the eluent, whereas HBr led only to a simple hexahydrate. As in the characterization of **1**, the dilysyl adduct **2** gave the expected lysine/glutamic acid ratio of 2:1 by amino acid analysis, and its UV absorption was consistent with intact 2,4-diamino substitution on the pteridine ring.

For the preparation of MTX(γ-ϵ)-$(Lys)_3$ (**3**), we chose to couple the partially protected monolysyl intermediate **12** with N^{ϵ}-Cbz-L-Lys-N^{ϵ}-Cbz-L-Lys methyl ester (**9**), rather than to add a third lysine residue to a suitably blocked derivative of **2**. This route was chosen to avoid possible racemization of the carbon adjacent to the activated dipeptide C terminal, a well-known phenomenon in peptide chemistry.[20] The required lysyllysine derivative (**9**) was obtained in about 80% yield (two steps) by condensation of N^{α}-*t*-Boc-N^{ϵ}-Cbz-L-Lys and N^{ϵ}-Cbz-L-Lys methyl ester in the presence of diphenylphosphoryl azide, followed by removal of the N^{α}-Boc group with *p*-toluenesulfonic acid in boiling benzene. A crystalline tosylate salt was isolated, which was coupled directly to **12** in the presence of diphenylphosphoryl azide to form the diester **15** in 74% yield. The methyl and benzyl ester groups in **15** were removed with $Ba(OH)_2 \cdot 8H_2O$ in 60% EtOH (a higher than usual amount of EtOH was used in order to achieve solubility), and the N^{α}-*t*-Boc group in **16** was removed with HBr–AcOH. The yields in these two steps were 94 and 81%, respectively. The final product (**3**) was found to be satisfactorily desalted on a Sephadex G-10 column (this procedure was also used in the case of **2**), and acceptable microanalytical and spectrophotometric data were obtained. Amino acid analysis gave the desired lysine/glutamic acid ratio of 3:1.

Biological Activity. The dihydrofolate reductase inhibitory activity of compounds **1–3** was compared with that of MTX against purified enzyme from L1210 murine leukemia cells.[12] Enzyme activity was measured spectrophotometrically at 340 nm in 0.1 M phosphate buffer, pH 7.0. As shown in Table I, the IC_{50} for MTX under these assay conditions was 50 nM, whereas that of the lysine derivatives ranged from 86 to 140 nM. Thus, there was a 1.5- to 3-fold decline in binding affinity as the number of lysine residues increased from one to three.

Examination of the full titration curves (not shown) revealed that, in contrast to MTX, the lysine derivatives are characterized by a curvilinear pattern of inhibition suggestive of an increased rate of dissociation for the enzyme–inhibitor complex. Deviation from linearity began at 65% inhibition with one lysine residue present and 60% inhibition with two lysines. In the case of the trilysine derivative, the inhibition curve was virtually devoid of linearity. Enzyme inhibition did not exceed 90% with any of the three lysine derivatives at concentrations up to 350 nM, whereas with MTX there was >95% inhibition at 100 nM. It thus appears that the introduction of positively charged groups, in this case lysines, at the γ-position of the glutamate side chain has an unfavorable influence on dihydrofolate reductase binding and that this type of electrostatic interaction should be kept in mind in the design of MTX analogues.

To determine what effect an increasing number of lysine residues at the γ-position of MTX has on cytotoxicity, we tested compounds **1–3** as inhibitors of L1210 cell growth in culture. As shown in Table I, the addition of one lysine residue (**1**) caused a 31-fold reduction in cytotoxicity, which contrasted sharply with the less than 2-fold reduction in dihydrofolate reductase affinity. With two lysines (**2**) there was a 77-fold decrease in toxicity, and with three lysines (**3**) the decrease was 120-fold. Thus, the incremental change in cytotoxicity was much more substantial from MTX to **1** than from **1** to **2**, or from **2** to **3**. The most likely explanation for the fact that compounds **1–3** were less cytotoxic than would be expected from the dihydrofolate reductase binding data is low uptake into the cells. From this we conclude that if MTX–poly(L-lysine) were given in vivo, it is unlikely that small oligomeric fragments, such as **1–3**, in the circulation, arising, for example, via the action of proteolytic liver enzymes, could contribute much to antitumor activity. This conclusion reinforces the proposed mode of action of MTX–poly(L-lysine)[1-3,6,10] via selective lysosomal activation within the tumor itself.

Because of previous reports from one of our laboratories that MTX–poly(L-lysine) is approximately equitoxic toward an MTX-sensitive rat hepatoma (H35) and an

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Table II. Antitumor Activity of Mono-, Di-, and Trilysine Conjugates of Methotrexate

compd	dose,[a] mg/kg	7-day wt change, %	survival, days		% ILS
			range	T/C, median	
MTX(γ–ε)–Lys (1)	10 (7)	+11	9–13	11/9	+22
	20 (14)	+5	14–16	14/9	+55
	40 (28)	+2	15–18	17/9	+89
MTX(γ–ε)–$(Lys)_2$ (2)	10 (6)	+8	10–12	11/9	+11
	20 (12)	+2	12–15	13/9	+44
	40 (24)	−2	14–17	15/9	+67
MTX(γ–ε)–$(Lys)_3$ (3)	10 (5)	+6	10–13	12/9	+33
	20 (10)	+10	12–13[b]	12/9	+33
	40 (20)	+10	13–17	14/9	+55
MTX	0.5	0	14–20	18/9	+100
	1.0	−9	17–22	19/9	+111

[a] Groups of five $B6D2F_1J$ male mice were inoculated ip with 10^5 L1210 cells on day 0, and treatment was started on day 1. Animals were injected ip with drugs made up in sterile 0.9% NaCl solution. A b.i.d.(1–4), qd(5,6), b.i.d.(7–10) schedule was used, with one double dose given on days 5 and 6. Numbers in parentheses are MTX-equivalent doses rounded off to the nearest milligram per kilogram. [b] One early death occurred in this group on day 3.

MTX-resistant subline ($H35R_{0.3}$),[8-10] it was of interest to compare MTX and compounds **1–3** in this system. As shown in Table I, the toxicity of all three analogues toward H35 cells in culture was ca. 50-fold lower than that of MTX itself, in qualitative agreement with the results for L1210 cells. Moreover, the IC_{50} appeared to vary as a function of the number of lysine residues, although the difference was smaller than with L1210 cells. It thus appears that H35 cells may be somewhat less discriminating in their structural requirements for cell-membrane transport. Another point that emerges from Table I is that, while there was a 180-fold decrease in toxicity for MTX in the $H35R_{0.3}$ cells relative to the H35 cells, the difference for the lysine adducts was only 15- to 20-fold. As a result, when the IC_{50}'s for compounds **1–3** were compared with that of MTX in the $H35R_{0.3}$ cells, the toxicity difference came to less than 7-fold.[21] Overall, the data suggest that lysine adducts **1–3** probably contain too few lysines to be taken up into cells by pinocytosis and that, in contrast to MTX–poly(L-lysine), these compounds are unlikely to find use in the treatment of tumors resistant to MTX by virtue of a transport defect.

Since it was conceivable that compounds **1–3** would show activity in vivo despite their low toxicity in vitro, perhaps by acting as MTX prodrugs capable of being bioactivated by the host, we tested them against L1210 leukemia in mice. A modified b.i.d.×10 schedule was employed, on the basis that these compounds presumably cannot form polyglutamates (because of their γ-glutamyl substitution) and, therefore, ought to be given by frequent injection.[12,13,15] As shown in Table II, an increase in life span (ILS) exceeding 50% was achieved with each of the lysine derivatives, with no weight loss indicative of host toxicity. However, the lysine derivatives were clearly less potent on a molar basis than MTX itself. Thus, while MTX at 0.5 mg/kg gave a +100% ILS, a dose of 40 mg/kg (equivalent to 28 mg/kg of MTX) had to be given in the case of the most potent lysine derivative (**1**) to achieve a roughly comparable +89% ILS. Thus, there was at least an 80-fold decrease in potency between MTX and the lysine derivatives in vivo. These results were consistent with the in vitro data and suggested that bioconversion to free MTX by the host, if it occurs at all, must be minimal.

It should be noted that while compounds **1–3** are potential lysosomal breakdown products of MTX–poly(L-lysine), they are by no means the only ones that might arise from the random conjugates studied to date.[1-10] Since all the reported preparations of MTX–poly(L-lysine) thus far have used the carbodiimide method, with no attempt to fractionate the conjugated products except by size exclusion of unchanged MTX, it is very likely that attachment of the MTX in these preparations involves either or both carboxyl groups of the glutamate moiety. Furthermore, there is the possibility of intrastrand and interstrand cross-linking. Thus, it is conceivable that, in some of the postulated oligomeric species arising via lysosomal degradation, the MTX is α-linked or α,γ-linked. The present studies do not address themselves to the possible activity of such species, but we believe their contribution should be minimal, since a free α-carboxyl group is very important for tight dihydrofolate reductase binding.[16,17] It is possible, on the other hand, that lysosomally formed oligomers containing α- or α,γ-linked MTX could still be toxic if they were degraded in the cytoplasm all the way to MTX itself.

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 tetramethylsilane as the internal reference. Quantitative UV absorption spectra were measured on a Cary Model 210 instrument. TLC was carried out on Eastman 13181 silica gel sheets, Analabs silica gel plates, or Eastman 13254 cellulose sheets (all containing a fluorescent indicator), and spots were visualized in a viewing chamber under 254-nm illumination or with ninhydrin spray as appropriate. Column chromatography was performed on silica gel (Baker 3405, 60–200 mesh), DEAE-cellulose (Whatman DE-52, preswollen), or Sephadex G-10 (Pharmacia). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Elemental

(21) A similar effect has been previously noted in our laboratory with MTX and γ-substituted MTX derivatives when these were compared against MTX-sensitive cells and cells resistant to MTX by virtue of a transport defect.[22,23] We believe the relatively small difference in the IC_{50} for the γ-substituted compounds reflects the fact that they cannot form nonefflux-ing γ-polyglutamate conjugates in either the wild-type cells or the resistant mutants. For recent papers on the role of γ-polyglutamation as a determinant of MTX toxicity, see ref 10 and 24–26.

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microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of the theoretical values unless otherwise noted.

L-Glutamic acid α-benzyl ester, N^{ϵ}-(benzyloxycarbonyl)-L-lysine methyl ester, and N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysine were obtained from Chemical Dynamics Corp., South Plainfield, NJ. 4-Amino-4-deoxy-N^{10}-methylpteroic acid, methotrexate α-*tert*-butyl ester, and diethyl phosphorocyanidate were prepared as previously described.[16,18] Cesium carbonate was from Alfa, Beverly, MA, and diphenylphosphoryl azide was from Aldrich Chemical Co., Milwaukee, WI. DMF used in coupling reactions was dried over Linde 4A molecular sieves.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysine *tert*-Butyl (α-Glu) Methyl (Lys) Ester (10). A solution of N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysine (3.80 g, 0.01 mmol) in dry DMF (50 mL) was stirred overnight at room temperature with Cs_2CO_3 (3.26 g, 0.01 mol) and MeI (1.25 mL, 2.84 g, 0.02 mol). The solvent was evaporated, and the residue was partitioned between C_6H_6 and H_2O. The C_6H_6 layer was washed with H_2O and evaporated to give the methyl ester **6** as an oil (3.98 g, 100%): IR (neat) ν 3340, 2940, 1710 (br) (ester C=O), 1520; NMR ($CDCl_3$) δ 1.4–1.9 [m, 15 H, $(CH_3)_3CO$ and $CH_2CH_2CH_2$], 2.9–3.4 (m, 2 H, CH_2N), 3.70 (s, 3 H, CH_3O), 4.2–4.5 (br m, 1 H, α-CH), 4.8–5.1 (br m, 1 H, NH), 5.08 (s, 2 H, benzylic CH_2), 7.30 (s, 5 H, aryl protons). The oily ester was dissolved directly in MeOH (200 mL) and glacial AcOH (5 mL), and the solution was shaken overnight in a Parr hydrogenation apparatus in the presence of 5% Pd/C (0.4 g). After removal of the catalyst and evaporation of the solvent, the residue was dissolved in H_2O, a small amount of solid K_2CO_3 was added, and the product was extracted into $CHCl_3$. The $CHCl_3$ solution was washed with H_2O and evaporated to dryness, and the residue (2.3 g) was dissolved in cold 1 N HCl (10 mL). The pH was adjusted to 3–4 with dilute NH_4OH, the solution was freeze-dried, the residue was taken up in a small volume of EtOAc, a small amount of insoluble NH_4Cl was filtered off, and the solvent was removed to give **7**·HCl as a foam (1.99 g, 67%). A portion of this material (296 mg, 1.0 mmol), from which final traces of moisture were removed by repeated coevaporation with DMF, was dissolved in dry DMF (10 mL), and **4** (270 mg, 0.5 mmol) was added, followed by diethyl phosphorocyanidate (326 mg, 2.0 mmol) and Et_3N (250 mg, 2.5 mmol). After being kept at 0 °C for 1 h and at room temperature overnight, the solvent was evaporated, and the residue was partitioned between $CHCl_3$ and dilute NH_4OHt. The $CHCl_3$ layer was evaporated, and the residue was purified by silica gel column chromatography, with 95:5 $CHCl_3$–MeOH as the eluent. Evaporation of pooled TLC-homogeneous fractions gave 290 mg (76%) of yellow solid: IR (KBr) ν 3300, 2930, 1715 (ester C=O), 1600, 1500 cm^{-1}. Anal. ($C_{36}H_{52}N_{10}O_8 \cdot 0.5H_2O$) C, H, N.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysine *tert*-Butyl (α-Glu) Ester (11). $Ba(OH)_2 \cdot 8H_2O$ (48 mg, 0.15 mmol) was added to a solution of the diester **10** (152 mg, 0.2 mmol) in 1:1 EtOH–H_2O (4 mL), and the mixture was stirred at room temperature overnight. The suspension was treated with Na_2SO_4 (22 mg, 0.15 mmol) in a minimum of H_2O, and after the solution was stirred for 5 min, the $BaSO_4$ precipitate was collected. Acidification of the filtrate with 10% AcOH and refrigeration produced a solid, which was filtered, washed with cold H_2O, and dried in a freeze-drying apparatus: yield 136 mg (87%); IR (KBr) ν 3350, 2940, 1700 (sh), 1630, 1600, 1505 cm^{-1}. Anal. ($C_{35}H_{50}N_{10}O_8 \cdot 2.5H_2O$) C, H, N.

N^{ϵ}-[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-γ-L-glutamyl]-L-lysine [MTX(γ–ε)-Lys, 1]. A solution of the ester **11** (1.47 g, 2.0 mmol) in CF_3CO_2H (7 mL) was kept at room temperature for 10 min, after which the CF_3CO_2H was partially removed by rotary evaporation, and H_2O (20 mL) was added. Solid K_2CO_3 was added carefully to the acidic mixture in small portions to avoid foaming, and when a clear alkaline solution was obtained, it was applied onto a DEAE-cellulose column that had been previously equilibrated with 3% NH_4HCO_3 and washed to neutrality with H_2O. The column was eluted with a large volume of H_2O until all the salts were removed, and the product was taken off with 3% NH_4HCO_3 (note: this desalting procedure may have to be performed more than once, depending on how much K_2CO_3 is needed to neutralize the residual CF_3CO_2H). TLC-homogeneous fractions of the NH_4HCO_3 eluate were pooled and freeze-dried to obtain **1** as a bright-yellow solid (0.70 g, 57%): IR (KBr) ν 3390, 1605 (br) cm^{-1}; UV λ_{max} (0.1 N HCl) 243 nm (ε 20 100), 306 (23 600); UV λ_{max} (pH 7.4) 259 nm (ε 27 300), 302 (25 400), 372 (11 100). Anal. ($C_{26}H_{34}N_{10}O_6 \cdot 4H_2O$) C, H, N. Lys/Glu = 1.03.

Methotrexate α-Benzyl Ester (5). A suspension of α-benzyl L-glutamate (1.42 g, 6 mmol) in dry C_6H_6 (10 mL) was treated with Et_3N (1.33 g, 13.2 mmol) and trimethylsilyl chloride (1.66 mL, 1.42 g, 13.2 mmol), stirred at room temperature overnight, diluted with hexane (20–30 mL), and filtered quickly under suction. The filter cake was washed with hexane, and the combined filtrates were evaporated to obtain α-benzyl N,O^{γ}-bis-(trimethylsilyl)-L-glutamate (2.25 g, 99%) as a moisture-sensitive oil: NMR (CCl_4) δ −0.03 [s, 9 H, $(CH_3)_3SiN$], 0.20 [s, 9 H, $(CH_3)_3SiO$], 1.4–2.4 (m, 4 H, CH_2CH_2), 3.35 (m, 1 H, α-CH), 5.04 (s, 2 H, benzylic CH_2), 7.26 (s, 5 H, aryl protons).

4-Amino-4-deoxy-N^{10}-methylpteroic acid (1.44 g, 4 mmol) was added in small portions to a stirred solution of diethyl phosphorocyanidate (1.63 g, 10 mmol) and Et_3N (1 g, 10 mmol) in dry DMF (140 mL) at room temperature, and the mixture was left to stand overnight [an extra 20% of diethyl phosphorocyanidate and Et_3N may be added at this point if TLC (silica gel; 4:1 $CHCl_3$–MeOH) still shows some unchanged starting material]. When no starting material remained according to TLC analysis, the freshly made silylated amino ester (2.25 g, 6 mmol) was added in a little DMF, and the solution was left to stand at room temperature for 2–3 days. A small amount of H_2O was then added, and the mixture was evaporated to dryness by rotary evaporation. The residue was resuspended in H_2O, and the pH was adjusted to 9 with NH_4OH to dissolve the product. A small amount of insoluble material, consisting mainly of recovered starting material (TLC), was filtered off. The filtrate was acidified with 10% AcOH, and the precipitated solid was collected, washed with cold H_2O, and dried in a lyophilizer to obtain **5** as a yellow solid (2.15 g, 94%): R_f 0.43 (cellulose; pH 7.4 phosphate buffer), 0.62 (silica gel; 15:5:1 $CHCl_3$–MeOH–AcOH); IR (KBr) ν 3700, 1735 (ester C=O), 1605–1635 cm^{-1}. Anal. ($C_{27}H_{28}N_8O_5 \cdot 1.5H_2O$) C, H, N.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-γ-L-glutamyl]-L-lysine Benzyl (α-Glu) Ester (12). A suspension of N^{α}-(*tert*-butyloxycarbonyl)-L-lysine (738 mg, 3 mmol) in dry C_6H_6 (6 mL) was treated with Et_3N (666 mg, 6.6 mmol) and trimethylsilyl chloride (840 μL, 714 mg, 6.6 mmol), stirred at room temperature overnight, diluted with a large volume of hexane, and quickly filtered under suction. The filter cake was washed with hexane, and the combined filtrates were evaported to obtain the moisture-sensitive N^{ϵ},O-bis(trimethylsilyl) derivative as an oil (1.16 g, 99%): NMR (CCl_4) δ 0.01 [s, 9 H, $(CH_3)_3SiN$], 0.25 [s, 9 H, $(CH_3)_3SiO$], 1.05–1.93 (m, 6 H, $CH_2CH_2CH_2$), 1.38 [s, 9 H, $(CH_3)_3CO$], 2.80 (m, 2 H, CH_2N), 4.10 (m, 1 H, α-CH), 6.20 (m, 2 H, NH).

A solution of the MTX ester **5** (1.42 g, 2.5 mmol) in dry DMF (50 mL) was treated with Et_3N (303 mg, 3 mmol) and *i*-BuOCOCl (390 μL, 408 mg, 3 mmol), and the mixture was stirred at room temperature for 20 min [formation of the mixed anhydride is conveniently verified by treating an aliquot with benzylamine in DMF and observing the mobile MTX γ-benzyl amide α-benzyl ester by TLC (silica gel, 9:1 $CHCl_3$–MeOH)]. When mixed anhydride formation was complete, the freshly made silylated lysine derivative (1.16 g, 3 mmol) was added in a small volume of DMF, and the solution was left at room temperature overnight. A small amount of H_2O was added, the mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in dilute NH_4OH. The solution was acidified with 10% AcOH, and the precipitated solid was collected, dried, and chromatographed on silica gel with 85:15 $CHCl_3$–MeOH as the eluent. Appropriately pooled TLC-homogeneous fractions of the eluate yielded **12** as a yellow solid (1.06 g, 52%); additional material of lesser purity (0.84 g) was recovered from the column by subsequent elution with 85:15 $CHCl_3$–MeOH containing 5% Et_3N: IR (KBr) ν 3390, 1755 (sh), 1690 (sh), 1605–1640 cm^{-1}. Anal. ($C_{38}H_{48}N_{10}O_8 \cdot 2.5H_2O$) C, H, N.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysine Benzyl (α-Glu) Methyl (Lys) Ester (13). To a solution of the monolysyl derivative **12** (1.02 g, 1.25 mmol)

and N^{ϵ}-(benzyloxycarbonyl)-L-lysine methyl ester hydrochloride salt (0.454 g, 1.37 mmol) in dry DMF (25 mL) at 0 °C was added diphenylphosphoryl azide (377 mg, 1.37 mmol) in the same solvent, followed by Et_3N (378 mg, 3.75 mmol). After 3 h at 0 °C, the mixture was left at room temperature overnight, the solvent was evaporated, and the residue was taken up in $CHCl_3$. The $CHCl_3$ solution was washed with dilute NH_4OH and evaporated, and the product was applied onto a silica gel column, which was eluted with 95:5 $CHCl_3$–MeOH. Appropriate fractions were combined and evaporated, and the residue was triturated with Et_2O, collected, and dried in vacuo (P_2O_5, 60 °C) to obtain **13** as a yellow solid (1.02 g, 79%): IR (KBr) ν 3350, 1745 (sh) (ester C=O), 1700 (sh), 1615 cm^{-1}. Anal. ($C_{53}H_{68}N_{12}O_{11}\cdot 0.5H_2O$) C, H, N.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysine (**14**). A suspension of the diester **13** (420 mg, 0.4 mmol) in 1:1 EtOH–H_2O (50 mL) was stirred overnight at room temperature with $Ba(OH)_2\cdot 8H_2O$ (252 mg, 0.8 mmol). Aqueous NH_4HCO_3 (400 mg) was added, the mixture was stirred vigorously, the $BaCO_3$ precipitate was filtered off, and the filtrate was reduced to a small volume by rotary evaporation. Acidification with 10% AcOH gave a yellow precipitate, which was collected and dried on a lyophilizer to obtain the diacid **14** (343 mg, 86%): IR (KBr) ν 3280, 2890, 1680 (sh), 1615 cm^{-1}. Anal. ($C_{45}H_{60}N_{12}O_{11}\cdot 3H_2O$) C, H, N.

N^{ϵ}-[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysyl-L-lysine [MXT(γ–ϵ)–(Lys)$_2$, 2]. **A.** To a solution of **14** (100 mg, 0.1 mmol) in glacial AcOH (10 mL) was added 30% HBr–AcOH (10 mL). A precipitate formed but quickly dissolved. After 1 h at room temperature, the solution was concentrated to dryness by rotary evaporation and further dried with the aid of a vacuum pump (KOH trap). The residue was triturated twice with Et_2O to remove any benzyl bromide (a well-ventilated hood should be used) and then dissolved in H_2O 1–2 mL). After pH adjustment to 10 with 1 N NaOH and saturation with CO_2 (dry ice may be used), the solution was passed through a Sephadex G-10 column (10 g, 14 × 1.5 cm). The product was eluted with 0.1 M NH_4HCO_3, and appropriately pooled TLC-homogeneous fractions were freeze-dried to obtain **2** as a bright-yellow solid (34 mg, 42%): IR (KBr) ν 3290, 2960, 1715 (sh), 1540–1625 cm^{-1}; UV (0.1 N HCl) λ_{max} 306 nm (ϵ 21 700), 242 (16 100); UV (pH 7.4) λ_{max} 258 nm (ϵ 21 400), 304 (22 100), 366 8540). Anal. ($C_{32}H_{46}N_{12}O_7\cdot 6H_2O$) C, H, N. Lys/Glu = 1.92.

B. A solution of **14** (94 mg, 0.1 mmol) in a mixture of CF_3CO_2H (2.1 mL) and thioanisole (0.6 mL) was stirred at room temperature for 3.5 h. Most of the CF_3CO_2H was removed by rotary evaporation, and the residue was triturated three times with Et_2O. The product was taken up in a small volume of H_2O, the pH was adjusted to 8 with NH_4OH, and the product was chromatographed on a DEAE-cellulose column as described in the synthesis of **1**. Freeze-drying of appropriately pooled fractions gave **2** as a hydrated trifluoroacetate salt (83 mg, 77%): IR (KBr) ν 3300, 1615–1665 cm^{-1}. Anal. ($C_{32}H_{46}N_{12}O_7\cdot 2.8CF_3CO_2H\cdot 2.75H_2O$) C, H, N, F.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysine Benzyl (α-Glu) Methyl (Lys) Ester (**15**). A solution of N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysine (1.90 g, 5 mmol) and N^{ϵ}-(benzyloxycarbonyl)-L-lysine methyl ester hydrochloride salt (1.65 g, 5 mmol) in dry DMF (25 mL) was stirred at 0 °C while diphenylphosphoryl azide (1.38 g, 5 mmol) and Et_3N (1.51 g, 15 mmol) were added. After 3 h at 0 °C, the reaction mixture was left overnight at room temperature. The residue after rotary evaporation was taken up in EtOAc, and the solution was washed successively with H_2O, 5% $NaHCO_3$, and H_2O. After being dried over K_2CO_3, the solution was evaporated to a glassy solid (3.04 g), which was purified on a silica gel column (95:5 $CHCl_3$–MeOH) to obtain **8** as an oil (2.89 g, 88%): R_f 0.8 (silica gel; 9:1 $CHCl_3$–MeOH); IR (NaCl) ν 3240, 2875, 1645–1680, 1485–1515 cm^{-1}; NMR ($CDCl_3$) δ 1.40 [m, 21 H, $(CH_3)_3CO$ and 2 $CH_2CH_2CH_2$], 3.08 (m, 4 H, 2 CH_2N), 3.67 (s, 2 H, 2 NH), 5.07 (m, 6 H, 2 benzylic CH_2 and 2 α-CH), 6.9 (br m, 1 H, NH), 7.30 (m, 10 H, aryl protons). This material (2.89 g, 4.4 mmol) was dissolved directly in C_6H_6 (50 mL), *p*-TsOH·H_2O (950 mg, 5 mmol) was added, and the mixture was refluxed for 1 h. Evaporation, trituration with hexane, filtration of the solid, and drying in vacuo at 50 °C over P_2O_5 afforded the dilysyl salt **9**·TsOH as a white solid (2.91 g, 90%): mp 92–95 °C; IR (KBr) ν 3330, 2915, 1735 (sh), 1715 (sh), 1685, 1520 cm^{-1}; NMR ($CDCl_3$) δ 1.1–1.9 (m, 12 H, 2 $CH_2CH_2CH_2$), 2.27 (s, 3 H, aryl CH_3), 2.97 (m, 4 H, 2 CH_2N), 3.57 (s, 2 H, 2 NH), 4.37 (m, 2 H, 2 α-CH), 5.00 (s, 4 H, 2 benzylic CH_2), 5.93 (m, 2 H, 2 NH), 7.00 (d, J = 8 Hz, 2 H, aryl protons ortho to Me), 7.29 (s, 10 H, aryl protons), 7.67 (d, J = 8 Hz, 2 H, aryl protons ortho to SO_3^-, overlapping m, 3 H, NH_3^+). This material was used without further purification for the next step.

To a solution of the monolysyl derivative **12** (409 mg, 0.5 mmol) and the dilysyl salt **9**·TsOH (365 mg, 0.5 mmol) in dry DMF (10 mL) at 0 °C was added diphenylphosphoryl azide (138 mg, 0.5 mmol) in the same solvent, followed by Et_3N (152 mg, 1.5 mmol). The reaction was worked up as in the preparation of **13** to obtain **15** as a yellow solid (490 mg, 74%): mp 99–110 °C; IR (KBr) ν 3250, 2900, 1725 (sh), 1675 (sh), 1620 cm^{-1}. Anal. ($C_{67}H_{86}N_{14}O_{14}\cdot 1.5H_2O$) C, H, N.

N^{α}-(*tert*-Butyloxycarbonyl)-N^{ϵ}-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-L-lysyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-L-lysine (**16**). A mixture of the diester **15** (432 mg, 0.33 mmol) and 60% EtOH (60 mL) was warmed gently until dissolution occurred and then cooled to room temperature and treated with $Ba(OH)_2\cdot 8H_2O$ (208 mg, 0.66 mmol). After being stirred overnight, the mixture was treated with NH_4HCO_3 (200 mg) in a little H_2O and vigorously agitated for 5 min. The $BaCO_3$ was filtered off and rinsed with EtOH until colorless, and the combined filtrate and wash was acidified with 10% AcOH. After evaporation of most of the solvent, the product was filtered and dried in a lyophilizer to obtain **16** (377 mg, 94%): IR (KBr) ν 3280, 2875, 1685 (sh), 1625 cm^{-1}. Anal. ($C_{59}H_{78}N_{14}O_{16}\cdot 2H_2O$) C, H, N.

N^{ϵ}-[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-γ-L-glutamyl]-L-lysyl-L-lysyl-L-lysine [MTX(γ–ϵ)–(Lys)$_3$, 3). A solution of the trilysyl derivative **16** (785 mg, 0.63 mmol) in glacial AcOH (60 mL) was treated with 30% HBr–AcOH (60 mL) and sonicated until all the initially formed solid dissolved. After 1 h at room temperature, the reaction mixture was worked up as in the preparation of **2**, except that in this case complete desalting required that the product be passed twice through Sephadex G-10: yield 507 mg (81%); IR (KBr) ν 3280, 1600 cm^{-1}; UV (0.1 N HCl) λ_{max} 306 nm (ϵ 23 100), 242 (17 700); UV (pH 7.4) λ_{max} 258 nm (ϵ 23 000), 304 (23 700), 365 (8860). Anal. ($C_{38}H_{58}N_{14}O_8\cdot CH_3CO_2H\cdot 5.5H_2O$) C, H, N. Lys/Glu = 3.18.

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Registry No. 1, 89637-85-4; **2**, 89637-86-5; **2** trifluoroacetate salt, 89637-87-6; **3**, 89637-88-7; **4**, 79640-70-3; **5**, 89106-05-8; **6**, 73548-77-3; **7**·HCl, 27894-50-4; **8**, 27317-68-6; **9**·TsOH, 89637-89-8; **10**, 89637-90-1; **11**, 89637-95-6; **12**, 89106-06-9; **13**, 89637-91-2; **14**, 89637-92-3; **15**, 89637-93-4; **16**, 89637-94-5; N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysine, 2389-45-9; L-glutamic acid α-benzyl ester, 13030-09-6; trimethylsilyl chloride, 75-77-4; α-benzyl N,O^{γ}-bis(trimethylsilyl)-L-glutamate, 89106-10-5; 4-amino-4-deoxy-N^{10}-methylpteroic acid, 19741-14-1; N^{α}-(*tert*-butyloxycarbonyl)-L-lysine, 13734-28-6; N^{α}-(*tert*-butyloxycarbonyl)-N^{ϵ},*O*-bis(trimethylsilyl)-L-lysine, 89106-11-6; N^{ϵ}-(benzyloxycarbonyl)-L-lsyine methyl ester hydrochloride, 2389-48-2; dihydrofolate reductase, 9002-03-3.