culture tubes (1 mL/tube). Test compounds were dissolved in growth medium, sterilized by passage through a 0.22- μ m membrane filter and added to tubes of cells (1 mL/tube). Compounds were tested in duplicate at log concentrations ranging from 1 × 10⁻⁷ to 1 × 10⁻⁴ M. Following 48-h incubation at 37 °C, cell counts were determined with a Coulter Model ZF cell counter. Cell growth in the presence of test compounds was expressed as a percentage of growth in untreated control tubes and the concentration of compound producing 50% inhibition of cell growth was determined (ID₅₀).

Acknowledgment. We thank Alexander D. Adams for large-scale syntheses of certain chemical intermediates.

Registry No. 1d, 85426-74-0; **3**, 96555-35-0; **4**, 24521-76-4; **5**, 5334-26-9; **6**, 96555-36-1; **7a**, 96555-37-2; **7b**, 96555-38-3; **8**, 96555-39-4; **9**, 96555-40-7; **10**, 96555-41-8; **11**, 96575-35-8; **12**, 90586-01-9; **13**, 96555-42-9; **14**, 96555-43-0; **15a**, 96555-44-1; **15b**, 96555-45-2; **16a**, 96555-46-3; **16b**, 96555-47-4; **17**, 90914-46-8; **18**, 96555-48-5; **19**, 96575-36-9; **20a**, 96555-49-6; **20b**, 96555-50-9; **1**-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose, 6974-32-9.

Syntheses and Evaluation as Antifolates of MTX Analogues Derived from 2,ω-Diaminoalkanoic Acids

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Methotrexate (MTX) analogues 27a-c bearing 2, ω -diaminoalkanoic acids (ornithine and its two lower homologues) in place of glutamic acid were synthesized by routes proceeding through N^2 -[4-(methylamino)benzoyl]- N^{ω} -[(1,1dimethylethoxy)carbonyl]-2, ω -diaminoalkanoic acid ethyl esters (12a,b) and N²-[4-(methylamino)benzoyl]-N⁵-[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid (13) followed by alkylation with 6-(bromomethyl)-2,4pteridinediamine hydrobromide. Reactions at the terminal amino group of 27-type analogues or of appropriate precursors led to other MTX derivatives whose side chains terminate in ureido (23a,b), methylureido (24), Nmethyl-N-nitrosoureido (30), N-(2-chloroethyl)-N-nitrosoureido (31), and 4-chlorobenzamido (28a-c) groups. Also prepared were unsymmetrically disubstituted ureido types resulting from addition of ethyl isocyanatoacetate and diethyl 2-isocyanatoglutarate to the ethyl esters of 27a,b. Of these ureido adducts (32a,b and 33a,b, respectively), only 33a was successfully hydrolyzed to the corresponding pure acid, in this instance the tricarboxylic acid 34, a pseudo-peptide analogue of the MTX metabolite MTX-7-Glu. Biological evaluations of the prepared compounds affirmed previous findings that the γ -carboxyl is not required for tight binding to dihydrofolate reductase (DHFR) but is operative in the carrier-mediated transport of classical antifolates through cell membranes. High tolerance levels observed in studies against L1210 leukemia in mice suggest the reduced potency may be due not only to lower transport efficacy but also to loss of the function of intracellular γ -polyglutamylation. The N-nitrosoureas 30 and 31 showed appreciable activity in vivo vs. L1210, but the activity did not appear to be due to antifolate action as evidenced by their poor inhibition of both L1210 DHFR and cell growth in vitro.

The venerable anticancer agent methotrexate (MTX) remains the only folic acid antimetabolite currently in established clinical use. Continuing studies of structureactivity relationships have produced information on modifications that may be made on the MTX structure that have little or no effect on inhibition of the intracellular target enzyme [dihydrofolate reductase (DHFR), EC 1.5.1.3] while, in some instances, favorably influencing aspects of membrane transport in normal vs. malignant mammalian cells.¹⁻¹⁰ Such studies have led to promising new MTX analogues now in clinical trials.⁷⁻¹⁰

The findings that MTX analogues modified in the region of the γ -carboxyl group still bind strongly to DHFR^{11,12} prompted interest in analogues in which glutamic acid has been replaced by $2,\omega$ -diaminoalkanoic acids (as in structural type 27 of Scheme III). The terminal amino group of these analogues allows opportunity to prepare varied derivatives. Rosowsky et al. prepared the lysine analogue of MTX via coupling of 4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoic acid with an appropriately protected derivative of the diamino acid.¹³ Kempton et al. prepared both the ornithine and lysine analogues in similar fashion.¹⁴ The products from the coupling reaction were subsequently deprotected and then converted to N^{\u03c6}-substituted derivatives for further studies. Rosowsky et al. converted the lysine analogue to its N^6 -iodoacetyl derivative for study as a potential active site directed irreversible inhibitor¹³ and also added fluorescein isothiocyanate to the N^6 -amino group to give a fluorescent de-

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rivative for use as a reporter ligand for DHFR.¹⁵ Kumar et al. converted the ornithine and lysine analogues to their N^{ω} -dansyl derivatives, which also have proved useful as DHFR probes because of their fluorescent properties and potent inhibition of DHFR.¹⁶⁻¹⁸

Chemistry. In this report we describe alternative syntheses of the ornithine analogue 27c and the two lower homologues 27a,b. From 27a-c or appropriate precursors, we prepared derivatives including types whose side chains terminate in (aminocarbonyl)amino (viz., ureido) groups (as in 23a, b and 24), N-nitrosoureido groups (30 and 31), and 4-chlorobenzamido groups (28a-c). Also prepared were the disubstituted ureido compounds of structural types 32 and 33, one of which (33a) was converted to the deprotected tricarboxylic acid 34, an analogue of the MTX metabolite MTX- γ -Glu bearing a ureido bridge in place of the natural peptide linkage. These compounds were prepared for biological testing in their own right and also

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Scheme II. Syntheses of N^2 -[4-(Methylamino)benzoyl] Derivatives of 2-Amino- ω -[(aminocarbonyl)amino]alkanoic Acids

<u>9a,b</u>	TsNHCHCO₂H
(Scheme I)	(CH₂) nHCONHR
СН ₃	<u>14a</u> : n = 1, R = H
НИС ₆ Н ₄ СОИНСНСО ₂ Н	<u>14b</u> : n = 2, R = H
(СН ₂) _П ИНСОИНК	<u>15</u> : n = 1, R = CH ₃
$\frac{20a,b}{21}: R = H$ $\frac{21}{21}: n = 1, R = CH_3$ (H) (H)	30% HBr-AcOH
$2 \text{ NC}_{6}^{1} \text{H}_{4}^{3} \text{CONHCHCO}_{2} \text{H}$ 2 $(\text{CH}_{2})_{n}^{2} \text{NHCONHR}$	H ₂ NCHCO ₂ H (CH ₂) _n NHCONHR
<u>18a,b</u> : R = H	<u>16a,b</u> :R=H
<u>19</u> : n = 1, R = CH ₃	<u>17</u> :n=1,R=CH ₃

as studies in the further enhancement of synthetic capability in the area of MTX analogues.

A significant part of the recent expansion in synthetic capability in this area is due to the availability of 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (22).¹⁹⁻²¹ The facility with which the (2,4-diamino-6-pteridinyl)methyl grouping may be attached to diverse side chains²² through the use of 22 is further demonstrated in this report. Outlined in Schemes I and II are the synthetic routes used to prepare 4-(methylamino)benzoyl derivatives that served as side-chain precursors. Their conversion to MTX analogues through alkylation with 22 and subsequent manipulations are shown in Scheme III.

Selective introduction of N-protective groups in the $2,\omega$ -diaminoalkanoic acid series is generally achieved through temporary protection of the 2-amino group in copper complexes. 2,3-Diaminopropanoic acid has been reported to be an exception in that respect because the vicinal amino groups interact with cupric ion to form an alternative coordination compound in dominance over that involving the 2-amino and carboxyl groups. 23 $\,$ Homologous 2,4-diaminobutanoic acid was described as binding with cupric ion in a way that is intermediate between the dominant binding modes of 2,3-diaminopropanoic acid and ornithine.²³ Nevertheless, several preparations of N⁴substituted derivatives of 2,4-diaminobutanoic acid have been successfully achieved via its copper complex.²⁴⁻²⁶ Evidence has also been reported that in solution of pH 5.0 2,3-diaminopropanoic acid forms the copper complex involving its 2-amino and carboxyl groups, leaving its 3amino group unprotected.²⁷ Further, it was reported that

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Scheme III. N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl] Derivatives of $2, \omega$ -Diaminoalkanoic Acids and 2-Amino- ω -[(aminocarbonyl)amino]alkanoic Acids



in solution of pH 6.5–7.0 2,3-diaminopropanoic acid reacts with benzyl chloroformate exclusively at the 3-amino group.²⁷ Later investigators showed, however, that some

of the N²-substituted compound also forms even under proper pH control. $^{\rm 28}$

In the present work, we chose to prepare N^2 -[4-(methylamino)benzoyl]- N^3 -[(1,1-dimethylethoxy)-

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Antifolates of $2,\omega$ -Diaminoalkanoic Acids

carbonyl]-2,3-diaminopropanoic acid ethyl ester (12a) and also the higher homologue 12b by unequivocal routes shown in Scheme I. L-Asparagine and L-glutamine served as starting materials in each of two routes (via 3a,b and 8a,b). In one pathway, a relatively new method for effecting the carboxamide-to-amine rearrangement under mild conditions using *I,I*-bis(trifluoroacetoxy)iodobenzene²⁹ was successfully applied in a situation where the classical Hofmann degradation had failed.³⁰ The ethyl esters 4a,b (but not the free acids 3a,b) were successfully converted by *I,I*-bis(trifluoroacetoxy)iodobenzene to the amines 5a,b. Treatment of 5a,b with bis(1,1-dimethylethyl) dicarbonate then afforded the terminally protected intermediates 6a,b.

The other approach to 6a,b shown in Scheme I proceeds via the previously reported N^2 -tosyl derivatives of L-asparagine and L-glutamine (8a,b),^{31,32} which were prepared by improved procedures. This second approach was developed concurrently with the first and is based on reported conversions of 8a,b to 9a,b using the Hofmann degradation,³³ conditions for which are entirely compatible with the tosylamide group. The two routes are, therefore, fundamentally similar. After the conversion of carboxamides 8a,b to amines 9a,b using the reported procedures, the (1.1-dimethylethoxy)carbonyl derivatives 10a.b were prepared. Reductive removal of tosyl groups of 10a,b using sodium in liquid ammonia left the (1,1-dimethylethoxy)carbonyl groups in place,³⁴ and the deprotected 2-amino intermediates were then treated with 4-[[(benzyloxy)carbonyl]methylamino]benzoyl chloride¹² (2) to provide 11a,b. Treatment of 11a,b with ethyl iodide in N,N-dimethylformamide (DMF) containing N,N-diisopropylethylamine $[(i-Pr)_2NEt]$ led to the pure esters 6a,b, identical with the products obtained by the other route. The protective (benzyloxy)carbonyl function of 6a,b was removed by hydrogenolysis to give the immediate side-chain precursor 12a,b.

The remaining homologous side-chain intermediate 13 shown in Scheme I was readily accessible because of the commercial availability of N^2 -[(benzyloxy)carbonyl]- N^5 -[(1,1-dimethylethoxy)carbonyl]-L-ornithine (7). The (benzyloxy)carbonyl group of 7 was removed by hydrogenolysis and then replaced by the 4-[[(benzyloxy)-carbonyl]methylamino]benzoyl group to afford 11c. Another hydrogenolysis step then gave the side-chain precursor 13.

Outlined in Scheme II are the steps used to prepare the side-chain precursors terminating in ureido groups. Key intermediates 16a,b were prepared by reported procedures³³ and then converted to the immediate precursors 20a,b after coupling with 2. The methyl-substituted compound 21 was similarly prepared via 15, which was obtained by the addition of methyl isocyanate to 9a.

Alkylation of the side-chain precursors with 22 gave the products of structural types 23-26 as shown in Scheme III. Basic hydrolysis of esters 25a,b led to the carboxylic acids 26a,b. Selective removal of the (1,1-dimethylethoxy)-

carbonyl group without affecting the carboxylate ester function was first achieved by treating 25a,b in ethanol solution with excess dry HCl-dioxane solution to give 29a,b dihydrochlorides. Later, 29a,b bis(trifluoroacetates) were prepared by treatment of 25a,b with trifluoroacetic acid alone. Similarly, treatment of the N^{\circ}-protected carboxylic acids 26a-c with trifluoroacetic acid alone gave the deprotected bis(trifluoroacetate) salts 27a-c in high yields and purity.

The order selected for the removal of protective groups from 25a,b depended upon the plans for further use of the respective products. Fully deblocked products 27a-c were used in Schotten-Bauman type aroylations in aqueous sodium hydroxide solution with 4-chlorobenzoyl choride to give the derivatives 28a-c, but the esters 29a,b were used in conversions that were best done in a nonaqueous medium: the dihydrochloride or bis(trifluoroacetate) salt of the ester was used in DMF containing $(i-Pr)_2NEt$. The N-methyl-N-nitrosoureido compounds 30 and the N-(2chloroethyl)-N-nitrosoureido analogue 31 were easily prepared in the nonaqueous medium from 29a and the appropriate 2,4,5-trichlorophenyl N-substituted Nnitrosocarbamate in an application of the excellent general method recently reported by Martinez et al. for the regioselective synthesis of N-nitrosoureas.³⁵ The free carboxylic acid derivatives corresponding to 30 and 31 are, however, to be preferred over the esters in this study because the free α -carboxyl group is required for tight binding to DHFR.¹² We had earlier attempted without success to selectively nitrosate the methylureido compound 24 to produce the carboxylic acid corresponding to 30. To this end we also examined various conditions intended to allow conversion of 27a using 2,4,5-trichlorophenyl Nmethyl-N-nitrosocarbamate, but none sufficed. When 27a bis(trifluoroacetate) was used in nonaqueous medium such as DMF containing 3 equiv of $(i-Pr)_2NEt$ [or 2 equiv of $(i-Pr)_2$ NEt plus 1 equiv of pyridine], no reaction occurred because of insolubility. When 2 equiv of $(i-Pr)_2NEt$ plus 1 equiv of NaOH [or simply 3 equiv of $(i-Pr)_2NEt$] were used in DMF containing enough water to cause solution, no conversion of 27a occurred because the activated Nmethyl-N-nitrosocarbamate underwent hydrolytic decomposition. We were also unable to devise a method for effecting hydrolysis of 30 and 31 without causing concomitant decomposition of their N-alkyl-N-nitrosoureido groups. Even enzymatically promoted ester hydrolysis through treatment with an esterase-rich medium such as fetal calf serum would also be expected to cause rapid decomposition of the N-nitrosoureido function.³⁶

The amines **29a,b**, freed in situ from their dihydrochlorides, underwent smooth additions with ethyl isocyanatoacetate and diethyl 2-isocyanatoglutarate³⁷ to give the ureido compounds **32a,b** and **33a,b**. These compounds may be regarded as ester analogues of previously reported γ -carboxyl peptides of MTX with glycine and glutamic acid;¹² lower homologues **32a** and **33a** are closer in structural analogy to the MTX- γ -peptides in that the NHCO-NH group is present in place of the CH₂CONH group. Ordinary ester hydrolysis conditions (slight excess of NaOH in aqueous ethanol) applied to these compounds

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Table I. L1210 Cell Growth Inhibition Data on 26a-c and 27a-c°

compd	n	IC_{50} , ^b nm
MTX		3.28 ± 1.2
26a	1	440 ± 128
26b	2	333 ± 111
26c	3	97 ± 6.3
27a	1	1030 ± 170
27b	2	1360 ± 410
27c	3	507 ± 146

^a Procedure used is described in ref 40. ^b Average of three to four separate determinations \pm standard deviation.

appeared to produce the intended carboxylic acids as the major products, but unexpected coproducts were also formed as significant contaminants that were not removed. Similar problems were also encountered in unsuccessful attempts to prepare the deprotected ureido-bridged side-chain precursors for alkylation with 22. Eventually, however, we succeeded in preparing the pseudo-peptide analogue of choice, the tricarboxylic acid 34, by mild hydrolysis in aqueous ethanol promoted by $Ba(OH)_2$.

The unwanted coproducts mentioned above apparently resulted from intramolecular cyclodehydrations within the distal portions of the side chains since no similar tendency was observed in the ureido-terminal compounds 23a,b and 24 nor in their side-chain precursors. Ester hydrolyses promoted by NaOH led to product mixtures whose assays by high-performance liquid chromatography revealed impurities having slightly greater retention times in reversed-phase systems than the dominant product. Field desorption mass spectral determinations on the product mixtures revealed molecular ion peaks due to the expected products plus molecular ion peaks of 18 mass units less. The HPLC and mass spectral evidence, as well as precedence, suggests that the troublesome coproducts might be hydantoin³⁸ and pyroglutamic acid³⁹ derivatives.

Biological Activity. Listed in Table I are the L1210 cell growth inhibition results from the amino acid derivatives 27a-c and their BOC precursors 26a-c. Among the BOC derivatives, 26c is three- to fourfold more inhibitory than the lower homologues. This difference appears to be related to the greater methylene chain length of 26c and might reflect greater lipophilicity plus a higher pK_a for the α -carboxyl group. Both factors affect transport and affinity to DHFR. The N^{ω} -BOC compounds 26a-c are threeto fivefold more inhibitory than the corresponding deprotected 27a-c. Even greater increases in inhibitory potency resulted from introduction of the 4-chlorobenzoyl groups as in compounds 28a-c (Table IV). Ornithinederived 28c is the most potent of the three compounds. The N^{ω} -(4-chlorobenzoyl) derivatives 28a-c are as inhibitory toward isolated DHFR as MTX (Table IV), but only 28c showed cell growth inhibitory activity against L1210 cells and cytotoxicity toward H.Ep.-2 cells near those of MTX. In initial in vivo tests, only 28c produced an increase in life span. In retests at higher dose levels, 28c gave an ILS of 104% at a dose level of 768 mg/kg. Even this high dose did not produce weight loss.

Before results from the ureido derivatives 23a,b and 24 are discussed, findings from earlier studies warrant mention. We earlier obtained evidence that in certain instances 2,4-diaminofolate analogues bind effectively to mammalian cell DHFR only in situ.⁴⁰ During studies with L1210 cells and four analogues (10-benzylaminopterin, 10-phenethylaminopterin, 10-oxaaminopterin, 3-deazamethotrexate), large discrepancies were observed between the data on the inhibition of growth in culture and the data on the inhibition of DHFR from cell-free extract. These observations prompted additional experiments that showed that the binding affinities of these compounds toward L1210 cell DHFR in situ are appreciably greater than indicated by the results of the cell-free enzyme inhibition assay. The in situ results agreed more closely with the extent to which each inhibited L1210 cell growth in culture. These studies showed that the inhibition of cell-free DHFR by various analogues may not always be a valid indication of inhibitory potency.⁴⁰

Each of the three terminal ureido derivatives 23a,b and 24 showed reduced influx compared to MTX, but no differences were observed for efflux (Table II). The large values for K_i for L1210 cell DHFR inhibition with 23a and 24, viewed in light of the prior studies mentioned above⁴⁰ and the data on transport and growth inhibition, are probably aberrancies of the cell-free enzyme system. The IC₅₀ values for growth inhibition can be interpreted as reflecting tight binding (approximately the same as MTX) within the whole cell for 23a but reduced binding for methyl-substituted 24. Both 23a and 23b appear to be effective inhibitors, at least of L1210 cell growth. In the in vivo tests (Table III), 23a was about as efficacious as MTX but only $1/_{30}$ as potent. The other analogues appeared to be relatively ineffective; however, because of the extremely high tolerance of mice to these compounds, only 23a was adequately tested at the LD_{10} dosage. The properties of 23a revealed in the tests summarized in Tables II and III appear to be quite similar to those of the previously reported MTX analogue with L-glutamine replacing L-glutamic acid.¹²

A summary of L1210 data for the N-methyl- and N-(2chloroethyl)-N-nitrosourea derivatives 30 and 31 is included in Table IV. These compounds were not transported well into L1210 cells, and they were relatively poorly toxic in the cell growth test. The high values for the K_i of these esters vs. DHFR are to be expected since the free α -carboxyl group required for tight binding¹² is lacking. In the in vivo tests vs. P388 leukemia in mice, 31 gave an ILS of 113% while 30 produced a 58% increase. It would appear from the available data that the relative activity of these compounds in the in vivo tests is due to action as nitrosoureas instead of as antifolates. Continued pursuit of the free carboxyl form of this type of nitrosourea is warranted. The carboxylic acids corresponding to 30 and 31 conceivably could bind tightly to DHFR while the N-nitrosoureido group is transformed in vivo to an isocyanato group.^{36c} The isocyanate derivative could then combine with a suitably reactive group of the enzyme to form a covalent bond and thereby irreversibly inhibit the enzyme.

The carbonylbisamino acid 34, a pseudo-peptide analogue of MTX- γ -Glu, was found to exert the same inhibitory action toward L1210 cell DHFR as MTX (Table IV). It is not transported into cells as efficiently as MTX, but its efflux rate is the same as that of MTX. This compound is considerably less potent than MTX in the cell growth inhibition test, apparently a reflection of lower influx.

The well-tolerated high doses of MTX analogues with the γ -carboxyl replaced by other groups may be due to the inability of these glutamate-modified analogues to undergo

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Table II. Summary of Biological Studies for 23a,b and 24 with Rodent Neoplastic Cells in Vitro^a

	L1210	cell membrane transport ^b					
	L1210 cell growth inhibn	L1210 cell DHFR growth inhibn inhibn		influx H	<i>K_i</i> , μ M		efflux k , (min ⁻¹)
compd	IC_{50}^{b} nM K_{i}^{b} nM	K_{i} , ^b nM	epithelial	S180	Erlich	L1210	L1210
MTX	2.7	0.006	339	11.4	10.1	3.7	0.23
23a	9.4	35	>500	11.6	12.5	11.6	0.25
23b	21.8	0.004	>500	24.6	15.9	17.3	0.19
24	34.3	14.2	>500	19.7	13.4	16.7	0.27

^a The methods used in these studies are described in refs 40 and 56. ^b Average of three to five determinations (SD ±18%).

Table III. Summary of Therapy Data for 23a,b and 24 vs. L1210 Leukemia^a

compd	Rx, ^b mg/kg	mice (no. × expts)	wt at term., g ±SD	MST (days ±SD)	ILS, %
control		5×12	20.7 ± 1.6	6.8 ± 0.6	
MTX	15	5×12	19.8 ± 3.2	17.1 ± 1.9	152
23a	432	3×2	16.0 ± 1.4	16.3 ± 3.6	129
23b	432	3×2	21.6 ± 1.2	9.3 ± 1.2	33
24	144	3×2	20.0 ± 0.2	7.0 ± 0	12
a b 4 . + 1	1	1		ho hadala	

^a Methods used are described in ref 57. ^b Schedule $q2d \times 5$.

 γ -polyglutamylation. Studies on MTX and aminopterin analogues with L-cysteic and L-homocysteic acid in place of L-glutamic acid produced biological results similar to these discussed here and led Rosowsky and co-workers to conclude that the reduced molar potency of the sulfonic acid derivatives reflected on their inability to form polyglutamates.⁴¹ The higher polyglutamates of folate analogues may be selectively retained in certain tissues,⁴²⁻⁵⁰ but they are also potent inhibitors of secondary enzyme targets, i.e., thymidylate synthase^{51,52} and AICAR transformylase.⁵³ Reduced secondary inhibitory effects may also be factors in the low molar potency of MTX analogues lacking the γ -carboxyl group.

Experimental Section

¹H NMR spectra (determined in Me₂SO- d_6 with a Varian XL-100-15 spectrometer) and mass spectra (determined on a Varian MAT 311A mass spectrometer equipped with electron impact field ionization/field desorption and fast atom bombardment ion sources) on compounds so designated in Tables V-VII were consistent with assigned structures. Analytical results

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indicated by element symbols in Tables V–VII were within ±0.4% of the theoretical values except where indicated otherwise. Spectral determinations and most of the elemental analyses were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. Some of the elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Examinations by TLC were performed on Analtech precoated (250 μ m) silica gel G(F) plates. Except where other conditions are specified, evaporations were performed with a rotary evaporator and a H₂O aspirator. Compounds listed in Table VII were dried in vacuo (<1 mm) at room temperature over P₂O₅ to constant weight and were then allowed to equilibrate with ambient conditions of the laboratory before analysis.

Compounds of Scheme I (See Table V). N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-asparagine (3a). A cold (0-5 °C) stirred solution of 1a-H₂O (3.96 g, 26.4 mmol) in 1 N NaOH (26.4 mL) containing dioxane (12 mL) was treated dropwise and simultaneously over a 30-min period with two solutions: (a) 1 N NaOH (26.4 mL) and (b) 4-[[(benzyloxy)carbonyl]methylamino]benzoyl chloride¹² (2; 8.00 g, 26.4 mmol) in dioxane (25 mL). The resulting solution was kept cold for 1 h longer and then at 20-25 °C for 1 h before it was diluted with H₂O (85 mL). Treatment with 12 N HCl (about 2.4 mL) to pH 4 produced a white solid that was recrystallized from EtOH to give pure 3a (7.22 g).

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-Lglutamine (3b). A stirred solution of 1b (10.9 g, 74.6 mmol) in 2 N NaOH (40 mL) and dioxane (40 mL) at 0-5 °C was treated dropwise at the same time with a solution of 2 N NaOH (38 mL) and a solution of 2^{12} (22.6 g, 75.0 mmol) in dioxane (80 mL) over a period of about 30 min. The resulting solution was left in a refrigerator overnight and then evaporated until about 80 mL had been removed. The remaining solution was then treated with 1 N HCl (80 mL). The gummy precipitate that formed solidified after about 1 h, and the collected solid was reprecipitated from a solution in EtOH (150 mL) by addition of H₂O (600 mL) to give crystalline 3b (27.6 g), homogeneous according to TLC (CHCl₃-MeOH, 9:1).

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-Lasparagine Ethyl Ester (4a). A solution of 3a (7.57 g, 18.9 mmol), EtI (3.28 g, 21.0 mmol), (*i*-Pr)₂NEt (2.71 g, 21.0 mmol), and DMF (30 mL) was kept at 20-25 °C for 4 days and then evaporated (<1 mm, bath to 35 °C). The semisolid residue was dissolved in CHCl₃, and the solution was washed successively with H₂O and 2% NH₄OH solution. Evaporation of the dried (MgSO₄) and filtered solution left a white solid that was recrystallized from EtOH to give pure 4a (7.1 g).

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-Lglutamine Ethyl Ester (4b). A solution of 3b (27.0 g, 65.5 mmol), EtI (11.3 g, 72.5 mmol), (*i*-Pr)₂NEt (9.35 g, 72.5 mmol), and DMF (100 mL) was kept at 20–25 °C for 4 days and then evaporated (<1 mm, bath to 35 °C), and the residue was stirred with H₂O to give a white solid. Recrystallization from EtOH afforded pure 4b (23.0 g).

Ethyl N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-2,3-diaminopropanoic Acid Hydrochloride (5a). A solution of 4a (6.50 g, 15.2 mmol) and *I,I*-bis(trifluoroacetoxy)iodobenzene²⁸ (9.80 g, 22.8 mmol) in MeCN (100 mL) and H₂O (25 mL) was kept at 20-25 °C for 16 h and then refluxed 30 min. At this time TLC (CHCl₃-MeOH, 9:1) showed disappearance of 4a. The solution was evaporated until most of the CH₃CN had been removed. Treatment with 12 N HCl (8.0 mL) followed, and the mixture that formed was extracted with Et₂O (twice with

Table IV. Summary of Biological Test Results for 28a-c, 30, 31, and 34

	L1210 cell	L1210 cell	L1210 cell tran	membrane sport ^a	in vivo act. in mice.	
compd	growth inhibn IC ₅₀ , ^{a,b} nM	DHFR inhibn <i>K_i,^a</i> pM	influx $K_i, \mu \mathbf{M}$	efflux k, min ⁻¹	% ILS (opt dose)	
MTX	3.28 ± 0.5	5.81 ± 0.6	4.57 ± 0.6	0.203 ± 0.03	173 (12) ^c	
28a	11.9 ± 0.7	4.98 ± 0.5	7.43 ± 1.3	0.255 ± 0.01		
28b	27.3 ± 1.5	6.42 ± 0.7	4.67 ± 0.8	1.21 ± 0.8		
28c	1.71 ± 0.6	5.31 ± 0.4	4.32 ± 1.3	0.437 ± 0.08	104 (768) ^c	
30	3650 ± 1335	6350 ± 780	42.4 ± 7.8	0.298 ± 0.06	58 (200) ^d	
31	2900 ± 593	3240 ± 420	46.9 ± 11	0.202 ± 0.03	113 $(400)^d$	
34	200 ± 49	4.71 ± 0.8	16.4 ± 2.4	0.234 ± 0.03	· ·	

^a Methods described in ref 40. ^bCytotoxicity to H.Ep.-2 cells (ED₅₀, nM): MTX, 2.4; **28a**, 470; **28c**, 4.0 (method described in ref 58). ^cL1210 10⁶ tumor cell implant (ip), q2d × 5 (ref 57). ^dP388 10⁶ tumor cell implant (ip), qd 1-5.⁵⁹

Table V. Data for Compounds Shown in Scheme I

no.	yield, %	mp, °C	molec formula
3a	68	175ª	$C_{20}H_{21}N_{3}O_{6}^{c}$
3b	90	168°	$C_{21}H_{23}N_3O_6^{\circ}$
4a	88	169ª	$C_{22}H_{25}N_3O_6^c$
4b	79	$159 - 160^{b}$	$C_{23}H_{27}N_3O_6^{c}$
5a	55	glass	$C_{21}H_{25}N_3O_5 \cdot HCl \cdot H_2O^{c,d}$
5b	85	glass	$C_{22}H_{27}N_3O_5 \cdot HCl^d$
6a	91 ^e	oil	$C_{26}H_{33}N_3O_7^d$
6b	35/	103^{a}	$C_{27}H_{35}N_3O_7^{c,d}$
8a	90	189–191 ^{5,g}	$C_{11}H_{14}N_2O_5S$
8b	73	$163 - 164^{b,h}$	$C_{12}H_{16}N_2O_5S$
9a	53	210-212 dec ^{b,i}	$C_{10}H_{14}N_2O_4S$
9b	95	238-239 dec ^b	$C_{11}H_{16}N_2O_4S$
10a	77	$127-128 \ dec^{b,k}$	$C_{15}H_{22}N_2O_6S$
10b	73	$147-148 \ dec^{b,i}$	$C_{16}H_{24}N_2O_6S$
11 a	56	$149 - 150^{b}$	$C_{24}H_{29}N_3O_7^{c,m}$
1 1b	96	glass	$C_{25}H_{31}N_3O_7{}^m$
11c	85	glass	$C_{26}H_{33}N_3O_7{}^m$
12a	п	glass	$C_{18}H_{27}N_3O_5{}^m$
12b	n	glass	$C_{19}H_{29}N_3O_5^m$
13	n	glass	$C_{18}N_{27}N_3O_5{}^m$

^a Observed on a Kofler Heizbank. ^b Mel-Temp apparatus. ^c Anal. C, H, N. ^d Mass spectrum consistent with assigned structure. ^e From **5a**; yield from **11a** was quantitative. ^f From **5b**; yield from **11b** was 83%. ^g Lit.³¹ mp 191 °C, prepared similarly but using Et₂O-aqueous NaHCO₃. ^hLit.³² mp 161-163 °C, prepared from tosyl-L-pyroglutamic acid and NH₄OH. ⁱ Rudinger et al.³³ reported 56% yield, mp 218-219 °C dec varying with time of heating. ^jRudinger et al.³³ reported 94% yield, mp 235-238 °C dec varying with time of heating. ^kLit. mp (variable) 125-126 °C dec to 128-129 °C dec (effervescenc)³⁴ and 129-131 °C dec,⁶⁰ both reported procedures used (1,1-dimethylethoxy)carbonyl azide. ^lLit.⁶¹ mp 149.5-150 °C dec for product from (1,1-dimethylethoxy)carbonyl azide. ^m¹H NMR spectrum consistent with assigned structure. ⁿ Obtained in essentially theoretical yield and used directly in the conversion shown in Scheme III.

Table VI. Data for Compounds Shown in Scheme II

no.	yield, %	mp, °C	molec formula ^{a,b}
1 4a	60	174 dec ^{<i>a,b</i>}	$C_{11}H_{15}N_3O_5S$
1 4b	74	190–191 dec ^{c,d}	$C_{12}H_{17}N_3O_5S$
15	78	182–184°	$C_{12}H_{17}N_3O_5S^e$
16a	70	$220 \ dec^{a,f}$	$C_4H_9N_3O_3$
16 b	67	209–211 dec ^{c,g}	$C_5H_{11}N_3O_3$
17·HBr	100^{h}	205–207 dec ^{i,c}	C5H11N3O3·HBre
18a	58	173–174°	$C_{20}H_{22}N_4O_6^{e}$
18 b	74	$157 - 158^{\circ}$	$C_{21}H_{24}N_4O_4^e$
19	50	173–175°	$C_{21}H_{24}N_4O_6^e$
20a	j		$C_{12}H_{16}N_4O_4{}^k$
20b	j		$C_{13}H_{18}N_4O_4{}^k$
21	j		$C_{13}H_{18}N_4O_4{}^k$

^aObserved on a Kofler Heizbank. ^bLit.³³ mp 174-179 °C dec. ^cMel-Temp apparatus. ^dLit.³³ mp 185-188 °C dec. ^eAnal. C, H, N. ^fLit.³³ mp 218-220 °C dec. ^gLit.³³ mp 203-206 °C dec and 207-208 °C. ^hYield of product suitable for use in the preparation of 18. ⁱAfter recrystallization from MeCN. ^jObtained in theoretical yields as glasslike solid and used directly in the conversion in dicated in Scheme III. ^k¹H NMR spectrum consistent with assigned structure except for retention of small amounts of AcOH and Et₂O.

Table	VII.	Data for Comp	pounds	Shown in	Scheme III	
	no	vield	%	mole	o formula ^{a,b}	

no.	yield, %	molec formula ^{a,b}
23a	68	$C_{19}H_{22}N_{10}O_4 \cdot 3H_2O$
23b	49	$C_{20}H_{24}N_{10}O_{4}\cdot 3H_{2}O$
24	50	$C_{20}H_{24}N_{10}O_{4}\cdot 3H_{2}O$
25a	79	$C_{25}H_{33}N_9O_5 \cdot 0.25H_2O$
25b	79	$C_{26}H_{35}N_9O_5 \cdot H_2O$
26a	86	$C_{23}H_{29}N_9O_5 \cdot 1.8H_2O$
26b	93	$C_{24}H_{31}N_9O_5 \cdot 1.67H_2O$
26c	95	$C_{25}H_{33}N_9O_5 \cdot 3.5H_2O$
27a	99	$C_{18}H_{21}N_9O_3 \cdot 2CF_3CO_2H \cdot 0.8H_2O$
27b	94	$C_{19}H_{23}N_9O_3 \cdot 2CF_3CO_2H \cdot H_2O$
27c	96	$C_{20}H_{25}N_9O_3 \cdot 2CF_3CO_2H \cdot H_2O$
28a	99	$C_{25}H_{24}ClN_9O_4 \cdot 1.8H_2O^c$
28b	99	$C_{26}H_{26}ClN_9O_4 \cdot 1.5H_2O$
28c	98	$C_{27}H_{28}ClN_9O_4\cdot 2.6H_2O$
29a ·2HCl	98	$C_{20}H_{25}N_9O_3$ ·2HCl·2.3H ₂ O ^d
$29a \cdot 2CF_3CO_2H$	95	$C_{20}H_{25}N_9O_3\cdot 2CF_3CO_2H\cdot 1.5H_2O$
29b-2HCl	95	$C_{21}H_{27}N_{11}O_5 H_2O$
30	84	$C_{22}H_{27}N_{11}O_5 \cdot H_2O^e$
31	74	$C_{23}H_{28}ClN_{11}O_5 \cdot H_2O$
32a	45	$C_{25}H_{32}N_{10}O_6 \cdot 2.5H_2O$
32b	61	$C_{26}H_{34}N_{10}O_6 \cdot 2H_2O$
33 a	72	$C_{30}H_{42}N_{10}O_8 \cdot 2H_2O$
33b	79	$C_{31}H_{42}N_{10}O_8 \cdot 2H_2O$
34	46	$C_{24}H_{28}N_{10}O_8 \cdot 3H_2O$

^aAnal. C, H, N with exceptions noted for 28a, 29a·2HCl, and 30. ^b¹H NMR spectra were obtained on all compounds except 23a,b, 27a-c, and 29a·2CF₃CO₂H and were consistent with the assigned structures. ^cAnal. N: calcd, 21.91; found, 20.76. ^dAnal. N: calcd, 22.76; found, 21.46. ^eAnal. N: calcd, 28.34; found, 27.90.

50-mL portions). The aqueous solution was then evaporated (final conditions <1 mm, bath to 30 °C) to give $5a \cdot H_2O$ as an amorphous solid (3.81 g), homogeneous by TLC (CHCl₃-MeOH, 9:1; UV and ninhydrin detection).

Ethyl N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-2,4-diaminobutanoic Acid Hydrochloride (5b). Treatment of 4b (23.0 g, 52.3 mmol) with *I*,*I*-bis(trifluoroacetoxy)iodobenzene²⁹ (33.8 g, 78.5 mmol) in MeCN (780 mL)-H₂O (260 mL) as described above (under the preparation of 5a) led to 5b as a glasslike solid (20.0 g), homogeneous by TLC-(CHCl₃MeOH, 9:1). This material was used without further characterzation for the preparation of 6b.

Ethyl N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]- N^3 -[(1,1-dimethylethoxy)carbonyl]-2,3-diaminopropanoate (6a). Method A. From 5a. A solution of 5a-H₂O (454 mg, 1.00 mmol) in DMF (2.5 mL) was treated with Et₃N (101 mg, 1.00 mmol) followed by bis(1,1-dimethylethyl) dicarbonate⁵⁴ (240 mg, 1.10 mmol). After 20 min, DMF was removed (<1 mm, bath to 30 °C), and the residue was stirred with EtOAc (25 mL). The EtOAc solution was washed once with H₂O and then dried (Na₂SO₄) and evaporated to give 6a as a colorless oil (456 mg), homogeneous by TLC (CHCl₃-MeOH, 95:5).

Method B. From 11a. A solution of 11a (10.0 g, 21.2 mmol) in DMF containing EtI (3.64 g, 23.3 mmol) and $(i-Pr)_2NEt$ (3.01 g, 23.3 mmol) was kept at 20–25 °C for 5 days. Evaporation (<1

 ⁽⁵⁴⁾ Procedure used is an adaptation of a general method described by: Moroder, L.; Hallett, A.; Wunsch, E.; Keller, O.; Wersin, G. Hoppe-Seyler's Z. Physiol. Chem. 1976, 357, 1651.

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mm, bath to 30 °C) was followed by stirring the residue with C_6H_6 (100 mL), and the C_6H_6 solution was washed successively with 0.1 N HCl (two 25-mL portions), H_2O , 0.1 N NaHCO₃, and H_2O before it was dried (Na₂SO₄) and evaporated to give **6a** as a colorless oil (10.5 g). This sample was homogeneous according to TLC and identical with that prepared by method A according to side-by-side and deliberate mixture chromatograms.

Ethyl N²-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-N⁴-[(1,1-dimethylethoxy)carbonyl]-2,4-diaminobutanoate (6b). Method A. From 5b. A solution of the sample of 5b described above (20.0 g, 44.5 mmol) in DMF (100 mL) was treated with Et_3N (4.57 g, 45.3 mmol) followed by bis(1,1-di-methylethyl) dicarbonate⁵⁴ (10.8 g, 49.0 mmol); and the resulting solution was stirred at 25 °C for 1 h. The DMF was evaporated (<1 mm, 30 °C), and the residue was stirred with EtOAc (300 mL). The EtOAc solution was washed twice with H_2O , dried (Na_2SO_4), and evaporated to give an oil (22 g). A solution of the oil in Et_2O (300 mL) was washed with 0.05 N NaOH (200 mL) followed by H_2O (200 mL) and then dried (Na₂SO₄) and evaporated to give an oil (13.5 g, 59% yield) virtually homogeneous according to TLC (CHCl₃-MeOH, 95:5). The oil solidified when stirred with nhexane (200 mL)-EtOAc (25 mL), and the collected solid was reprecipitated from a clarified solution in EtOAc (25 mL) by addition of ligroin (bp 37-58 °C) to give pure 6b (8.10 g).

Method B. From 11b. A solution of 11b (3.31 g, 6.83 mmol) in DMF (10 mL) containing $(i-Pr)_2NEt$ (1.00 g, 7.74 mmol) and EtI (1.19 g, 7.65 mmol) was kept at 20–25 °C for 5 days and then evaporated (<1 mm, 30 °C). The residue was dissolved in CHCl₃, and the solution was washed with H₂O and 5% NH₄OH solution and then dried (Na₂SO₄) and evaporated to give a colorless, viscous oil that solidified when stirred with *n*-hexane. The product (2.9 g) had melting point, mixture melting point, IR spectra, and thin-layer chromatogram identical with those of **6b** prepared by method A.

 N^2 -[(4-Methylphenyl)sulfonyl]-L-asparagine (8a). A solution of 1a·H₂O (15.0 g, 0.100 mol) in 2 N NaOH (50 mL)-dioxane (125 mL) was treated dropwise with stirring and ice bath cooling with 2 N NaOH (60 mL) while pulverized tosyl chloride (21.0 g, 0.110 mol) was added in portions during 15 min. The mixture was stirred 1 h in the cold and then 1.5 h longer after the cooling bath had been removed. The nearly clear mixture was clarified by filtration, then diluted with H₂O (to 425 mL), and treated with 12 N HCl (10 mL) to cause precipitation of crystalline 8a (25.6 g).

 N^2 -[(4-Methylphenyl)sulfonyl]-L-glutamine (8b). The procedure described above applied to 1b (0.446 mol) readily afforded 8b (96.9 g).

 N^2 -[(4-Methylphenyl)sulfonyl]-2, ω -diaminoalkanoic acids 9a,b were prepared from 8a,b by the Hofmann rearrangement according to reported procedures.³³ Yields and properties of 9a,b were in agreement with those reported (see Table V).

N²-[(1,1-Dimethylethoxy)carbonyl]-N³-[(4-methylphenyl)sulfonyl]-2,3-diaminopropanoic Acid (10a). A cold solution of 9a (41.9 g, 0.162 mol) in 1 N NaOH (162 mL), H₂O (160 mL), and dioxane (160 mL) was treated dropwise with stirring and cooling (ice-H₂O bath) over a period of about 30 min with a solution of bis(1,1-dimethylethyl) dicarbonate⁵⁴ (38.8 g, 0.178)mol) in dioxane (160 mL). The resulting mixture was stirred in the cold for 2 h while smooth evolution of CO_2 occurred. Then, the ice-H₂O bath was removed and stirring at 20-25 °C was continued for about 2.5 h or until CO₂ evolution had ceased and a clear solution had formed. Most of the dioxane was removed by evaporation. The remaining aqueous solution of the Na salt of 10a was extracted once with EtOAc (100 mL), and the EtOAc phase was discarded. The aqueous solution was then treated with stirring with 20% citric acid in H₂O until the mixture was acidic (pH about 3). The oily crude product that precipitated was extracted with EtOAc (800 mL), and the H₂O-washed and dried (Na₂SO₄) EtOAc solution was evaporated to give crude 10a as a mostly solid residue. Two recrystallizations from 2:1 (v/v)EtOAc-ligroin (bp 37-58 °C) afforded pure 10a (44.9 g).

 N^2 -[(1,1-Dimethylethoxy)carbonyl]- N^4 -[(4-methylphenyl)sulfonyl]-2,4-diaminobutanoic Acid (10b). A stirred solution of 9b (40.0 g, 0.147 mol) in 1 N NaOH (220 mL), H₂O (150 mL), and dioxane (150 mL) was treated at 20-25 °C with a solution of bis(1,1-dimethylethyl) dicarbonate⁵⁴ (48.0 g, 0.220

mol) in dioxane (220 mL) added in a thin stream during 15 min. The stirred mixture was then gradually warmed to 50-55 °C and kept in this range for about 1 h or until CO₂ evolution had ceased. The isolation of pure **10b** (39.5 g) was essentially the same as that described above for **10a** with a final recrystallization from 1:1 EtOAc-ligroin (bp 37-58 °C).

N²-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]- N^{3} -[(1,1-dimethylethoxy)carbonyl]-2,3-diaminopropanoic Acid (11a). A rapidly stirred solution of 10a (15.0 g, 41.9 mmol) in liquid NH₃ (about 200 mL) was cooled in a dry ice-Me₂CO bath at -60 °C and treated with small pieces of clean Na until the blue color persisted for 10 min. The color was discharged by addition of a very small amount of glacial AcOH delivered from a syringe. The NH₃ was then allowed to evaporate (vented through a soda lime packed drying tube) with the aid of N_2 flow. When the mixture reached room temperature, the flask was placed on a rotary evaporator and evacuated to complete removal of NH₃. The solid residue was stirred with cold 0.1 N AcOH (150 mL) to give a still basic, cloudy mixture that was immediately treated with glacial AcOH as required to produce pH 7. This mixture, which contained oily droplets of *p*-toluenethiol, was extracted with Et₂O (three 25-mL portions), and the ethereal extracts were discarded. The aqueous solution was then combined with a solution of (i-Pr)₂NEt (11.0 g, 85.0 mmol) in dioxane (60 mL), and the resulting solution was cooled in an ice-H₂O bath while it was treated with stirring with a solution of 2^{12} (12.7 g, 41.8 mmol) in dioxane (100 mL). After the ice- H_2O bath had thawed (2-3 h), the solution was left at 20-25 °C overnight (15-17 h). Nearly all the dioxane was then removed by evaporation. The solution that remained (pH 8) was treated with 20% citric acid solution until precipitation had ceased (pH about 4). The white gumlike precipitate was extracted with EtOAc, and the H₂O-washed and dried (Na_2SO_4) EtOAc solution was evaporated to give a frothy residue (21 g). This crude material was redissolved in EtOAc (150 mL), and the stirred solution was treated in portions with ligroin (bp 37-58 °C) (about 450 mL). The white solid that separated (15.6 g) had a wide melting point range, beginning about 130 °C. A thorough trituration with Et_2O (100 mL) followed, and the Et_2O -insoluble portion (14.9 g) had a melting point of 140-143 °C. Recrystallization from EtOAc (about 90 mL) then gave pure 11a (11.1 g).

N²-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]- N^{4} -[(1,1-dimethylethoxy)carbonyl]-2,4-diaminobutanoic Acid (11b). In a 10.0-mmol run, 10b was detosylated by treatment with Na in liquid NH_3 as described under the preparation of 11a. The residue that remained following removal of NH₃ was dissolved in cold H_2O (20 mL), and the solution was treated with stirring with acidic ion-exchange resin (Amberlite IR-120) until the pH reached 7. After filtration, the solution was evaporated (<1 mm, bath to 25 °C) to give a white solid residue (2.15 g) whose thinlayer chromatogram developed with H₂O produced one spot (UV and ninhydrin detection). The residue was dissolved in H_2O (20 mL) and dioxane (20 mL) containing (i-Pr)₂NEt (2.58 g, 20.0 mmol), and the stirred solution was chilled in an ice- H_2O bath while a solution of 2^{12} (2.28 g, 7.50 mmol) in dioxane (100 mL) was added dropwise during 30 min. The solution was left in the cooling bath while it was allowed to warm to room temperature and then left overnight. Evaporation of most of the dioxane was followed by treatment with 20% citric acid to pH 4 and extraction of the gummy precipitate with EtOAc. The H₂O-washed and dried (Na_2SO_4) solution was evaporated to give 11b as a semisolid residue (3.50 g). This material was indicated by TLC (EtOAc-MeOH, 9:1) to be virtually homogeneous; it proved to be of suitable purity for the preparation of pure 6b (by method B) in high yield as described above.

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]- N^5 -[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic Acid (11c). Hydrogenolysis of 7 (18.3 g, 50.0 mmol) in MeOH (150 mL)-H₂O (50 mL) containing 5% Pd on C (1.0 g) was carried out at 3.16-3.52 kg/cm² (45-50 psi) at 25 °C with a Parr shaker over a period of 7 h. During that time the pressure flask was purged with N₂ and recharged with H₂ three times. The filtered solution was evaporated to give the solid N⁵-(1,1-dimethylethoxy)carbonyl intermediate. After trituration with Et₂O, the yield of collected solid was 92% (10.7 g), homogeneous by TLC (H₂O, ninhydrin). Most of this product (10.0 g, 43.1 mmol) was converted to 11c by treatment with 2^{12} (13.0 g, 42.8 mmol) in dioxane-H₂O containing (*i*-Pr)₂NEt (11.6 g, 90.0 mmol) as described under the preparation of 11b. The isolation procedure was also similar, and 11c was obtained as a glasslike solid (18.3 g).

Ethyl N²-[4-(Methylamino)benzoyl]- N° -[(1,1-dimethylethoxy)carbonyl]-2, ω -diaminoalkanoates (12a,b). Compound 12a was prepared by hydrogenolysis of 6a (10.0 g, 20.0 mmol) in dioxane (200 mL) containing 5% Pd on C (1.0 g) at atmospheric pressure and room temperature with H₂ over H₂O in a gas buret. The filtered solution was evaporated (final conditions <1 mm, bath to 35 °C) to 12a (7.1 g, 97% yield) as a foam, homogeneous according to TLC (CHCl₃-MeOH, 95:5). Similarly, hydrogenolysis of 6b (8.00 g, 15.6 mmol) in EtOH (120 mL) produced 12b in 97% yield (5.75 g) as a glassy residue. These samples proved satisfactory for use in the preparation of 25a,b described below.

 N^2 -[4-(Methylamino)benzoyl]- N^5 -[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid (13) was prepared by hydrogenolysis of 11c (8.00 g, 16.0 mmol) in dioxane (50 mL) containing 5% Pd on C at 25 °C and 3.16-3.52 kg/cm² in a Parr shaker as described under the preparation of 11c for the deprotection of 7 to give the intermediate N^5 -[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid. The yield of 13 as a colorless glass was quantitative (5.84 g), homogeneous by TLC in two systems (H₂O and CHCl₃-MeOH, 1:1). This material was used directly for conversion to 26c as described below.

Compounds of Scheme II (See Table VI). N^2 -[(4-Methylphenyl)sulfonyl]-2-amino- ω -[(aminocarbonyl)amino]alkanoic acids (14a,b) were prepared by treatment of 9a,b with KOCN in H₂O essentially as described by Rudinger et al.³³ The yield of 14b was greatly improved (from 13% to 74%) by adding KOCN (35.4 g, 0.436 mol) during 15 min to the stirred mixture of 9b (30.0 g, 0.110 mol) in H₂O (800 mL) at 50 °C and then heating at 100 °C for 50 min. Acidification of the chilled solution then readily afforded 14b (25.8 g).

 N^2 -[(4-Methylphenyl)sulfonyl]-2-amino-3-[[(methylamino)carbonyl]amino]propanoic Acid (15). A cold (0-5 °C) solution of 9a (10.3 g, 40.0 mmol) in 1 N NaOH (44 mL) was treated with stirring during 10-15 min with MeNCO (2.50 g, 43.6 mmol). The mixture was stirred at about 5 °C for 1.5 h and then filtered (Celite) and treated with 12 N HCl to pH 2. The oily precipitate solidified with stirring (11.0 g, mp 180-182 °C) and was recrystallized from EtOH to give pure 15.

2-Amino- ω -[(aminocarbonyl)amino]alkanoic Acids (16a,b). Detosylation of 14a,b was carried out as described below for the conversion of 15 to 17-HBr. The procedure is essentially the same as that reported by Rudinger et al.³³ The compounds were freed from their hydrobromides by neutralization with NH₄OH in H₂O-MeOH as reported.

2-Amino-3-[[(methylamino)carbonyl]amino]propanoic Acid (17) Hydrobromide. A solution of 15 (9.00 g, 28.6 mmol) in 30% dry HBr-AcOH (80 mL) containing phenol (9.0 g) was kept in a closed flask for 4 days. A solid began separating after the first day. The mixture was combined with Et_2O (250 mL), and the solid was collected and washed thoroughly with Et_2O to give 17.HBr (7.0 g) of purity suitable for the preparation of 19 described below.

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-2amino- ω -[(aminocarbonyl)amino]alkanoic acids (18a,b) were prepared by coupling of 2 with 16a,b in H₂O-dioxane containing (*i*-Pr)₂NEt (2.5 mol equiv with respect to 2) using the procedure described below for the preparation of 19. Each product was obtained pure, following a recrystallization from MeCN.

 N^2 -[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-2amino-3-[[(methylamino)carbonyl]amino]propanoic Acid (19). A solution of 2 (2.86 g, 9.42 mmol) in dioxane (25 mL) was added dropwise during 30 min to a cold (0-5 °C), stirred solution prepared from 17-HBr (2.42 g, 10.0 mmol) and (*i*-Pr)₂NEt (4.27 g, 33.0 mmol) in H₂O (30 mL) and dioxane (30 mL). The resulting solution was stirred in the cold 1 h longer, left overnight in a refrigerator, and then kept at 20-25 °C for 2 h before it was concentrated (rotary evaporator, H₂O aspirator, bath to 35 °C) in order to remove the dioxane. The remaining solution was diluted with H₂O (50 mL), and the resulting solution was acidified by treatment with 12 N HCl to give a gummy precipitate that solidified within 1 h with stirring and cooling. Recrystallization from MeCN gave pure 19.

 N^{2} -[4-(Methylamino)benzoyl]-2-amino- ω -[(aminocarbonyl)amino]alkanoic Acids (20a,b) and N²-[4-(Methylamino)benzoyl]-2-amino-3-{[(methylamino)carbonyl]amino]propanoic Acid (21). The procedure for the preparation of 21 is illustrative. A solution of 19 (5.10 g, 11.9 mmol) in glacial AcOH (150 mL) containing suspended 30% Pd on C (1.0 g) was hydrogenated in a Parr shaker at 3.16 kg/cm^2 (45 psi) at 25 °C for 3.5 h. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo (rotary evaporator, final pressure <1 mm, bath to 30 °C). The residue was stirred with Et_2O (150 mL) to give 21 as a white amorphous solid in essentially theoretical yield (3.20 g). Examination by TLC showed one spot (H₂O, detection by UV and ninhydrin), and its ¹H NMR spectrum is as expected for 21 except for weak signals indicating retention of small amounts of AcOH and Et₂O. Similar results were obtained in preparations of 20a,b from 18a,b. These materials proved suitable for direct conversions to 23a,b and 24 as described below

Compounds of Scheme III (See Table VII). N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-ω-[(aminocarbonyl)amino]alkanoic Acids (23a,b) and N^2 -[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-3-[[(methylamino)carbonyl]amino]propanoic Acid (24). Pulverized 22.C₃H₇OH¹⁹ (2.1 g of 95% purity, 5.0 mmol) was added in portions during 10-15 min to a stirred solution of the appropriate 20a,b or 21 (5.5 mmol) in Me₂NAc (30 mL). The mixture was stirred at 20-25 °C in a stoppered flask protected from light for 5 days. The solution that formed was combined with cold H_2O (200 mL), and the pH of the resulting solution was adjusted (from 3.0) to 4.5 by addition of 1 N NaOH to cause precipitation of the product. After a refrigeration period, the solid was collected and then reprecipitated from a solution of its Na salt as follows. A stirred suspension of the solid in H_2O (100 mL) was treated with 1 N NaOH (1 equiv), and the resulting solution was carefully treated with 1 N HCl to pH 4.5. The yellow precipitate was collected after the mixture had been kept at 0-5 °C for several h. Compound 23b did not receive further purification. Compound 23a was finally recrystallized from H_2O (1.85 g from 350 mL gave 1.66 g). Compound 24 (2.5 g) was dissolved in 1 N NaOH (6 mL), and the solution was combined with EtOH (180 mL) to cause precipitation of the Na salt of 24, which was collected and dissolved in H₂O (100 mL). The Norit-treated and filtered (Celite mat) solution was then treated with 1 N HCl to pH 4.6 to give pure 24 (1.95 g).

Ethyl N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]- N^{ω} -[(1,1-dimethylethoxy)carbonyl]-2, ω -diaminoalkanoates (25a,b). The procedure for the preparation of 24 is typical. A mixture of 22 C₃H₇OH¹⁹ (7.00 g of 95% purity, 16.8 mmol) and the sample of 12a described above (7.10 g, 19.4 mmol) in Me₂NAc (100 mL) was stirred for 6 days in a stoppered flask wrapped in Al foil. The solution that formed was then chilled (ice-H₂O bath), and $(i-Pr)_2NEt$ (4.5 g, 35 mmol) was added dropwise during 10 min. The resulting mixture was added dropwise during 30 min to rapidly stirred 0.5 N NH₄OH (700 mL) with external cooling (ice-H₂O bath). Solid 25 separated readily as a yellow precipitate (7.30 g), homogeneous according to TLC (CHCl₃-MeOH, 3:1). A sample for elemental analysis was recrystallized from MeCN. Compound 25 (5.34 g) was prepared from 12b (5.53 g, 14.6 mmol) and 22 (11.8 mmol) in the same manner.

 N^2 -[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]- N^{ω} -[(1,1-dimethylethoxy)carbonyl]-2, ω -diaminoalkanoic Acids (26a,b). In a typical procedure, the ester 25a or 25b (3.0 mmol) was dissolved in EtOH (45 mL), and the solution was treated with NaOH (3.6 mL of 1.0 N). After 4 h at 20-25 °C, the conversion was complete according to TLC. The mixture, which contained precipitated Na salt, was treated with H₂O (25 mL), and the solution that formed was concentrated by evaporation to about 15 mL to remove EtOH. Dilution with H₂O (to 100 mL) followed, and the solution was treated dropwise with 1 N HCl to pH 3.8 (meter) to precipitate the product.

 N^2 -[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]- N^5 -[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic Acid (26c). A mixture of 22-C₃H₇OH¹⁹ (5.13 g of 95%, 12.3 mmol) and the sample of 13 described above (5.84 g, 16.0 mmol) in Me₂NAc (80 mL) was stirred at 20-25 °C for 5 days in

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a stoppered flask wrapped in Al foil. The solution that formed was added dropwise to NH4OH solution (360 mL of approximately 0.3 N), and the resulting solution was treated with Norit, then filtered (Celite mat), and carefully treated with stirring with 1 N HCl to pH 3.8-4.0 to cause precipitation of 26c.

N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2,ω-diaminoalkanoic Acid Bis(trifluoroacetates) (27a-c). The procedure for the preparation of 27a is illustrative of that used for the conversions of 26a-c to 27a-c. A solution of 26a-1.8H₂O (1.78 g, 3.27 mmol) in CF₃CO₂H (25 mL) was stirred at 0-5 °C (bath temperature) for 1 h and was then poured in a thin stream into stirred Et₂O (250 mL). The yellow precipitate of 27a (2.11 g) that formed was collected and washed with Et₂O. The homologues 27b,c were prepared on similar scales.

N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]- N^{ω} -(4-chlorobenzoyl)-2, ω -diaminoalkanoic Acids (28a-c). Samples of 27a-c were prepared from 26a-c (1.00 mmol) as described above and then used directly in conversions to 28a-c. The procedure for the preparation of 28a is typical. The sample of 27a prepared from 26a (1.0 mmol) was dissolved in H_2O (15 mL), and the resulting solution of pH 2.2 was carefully treated with 1 N NaOH until the pH reached 12. The solution was then diluted with dioxane (10 mL). A solution of freshly distilled 4-chlorobenzoyl chloride (192 mg, 1.1 mmol) in dioxane (3 mL) was then added dropwise and simultaneously with 1 N NaOH as required to keep the pH from becoming less than 10. The solution was stirred 1 h at 20-25 °C and was then evaporated in order to remove most of the dioxane. The remaining aqueous solution was then diluted with H_2O (to 40 mL) and treated with 1 N HCl to pH 3.8 and to cause precipitation of 28a. The yellow precipitate was dried and then triturated with Et₂O and finally dried in vacuo at 58 °C for 1 h. This procedure was also used in preparations of 28b,c.

Ethyl N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoy1]-2, ω -diaminoalkanoates (29a,b). A solution of 25a,b (1.00 mmol) in EtOH (25 mL) was treated with a solution of dry HCl in dioxane (4 mL of 4.4 N). Evolution of CO_2 was observed by means of a bubble counter attached to the system through a Drierite-packed drying tube. After 16 h at 20-25 °C, gas evolution had nearly stopped and yellow solid was present, but TLC (CHCl₃-MeOH, 9:1) showed some 25a,b still present. The conversion was completed during 20 min in a 50 °C bath; TLC then revealed only the ninhydrin-positive product that remained at the origin. Solvents were evaporated, and the residue was stirred with Et₂O and then collected to give 29a,b as their dihydrochlorides.

Treatment of 25a (3.14 g, 5.8 mmol) with CF_3CO_2H (30 mL) at 0-5 °C for 1 h and then at 20-25 °C for 0.5 h followed by slow addition of the solution to stirred Et₂O (400 mL) gave pure 29a bis(trifluoroacetate).

Ethyl N²-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-N³-(methylnitrosocarbamoyl)-2,3-diaminopropanoate (30). 2,4,5-Trichlorophenyl N-methyl-N-nitrosocarbamate³⁵ (170 mg, 0.60 mmol) was added to a cold (0-5 °C), stirred solution of 29a.2CF₃CO₂H.1.5H₂O (330 mg, 0.48 mmol) and (i-Pr)₂NEt (130 mg, 1.0 mmol) in DMF (5 mL). The solution was kept at 0-5 °C for 1.5 h. A thin-layer chromatogram (CHCl₃-MeOH, 3:1) run on a specimen of the reaction solution during the final minutes of a 1.5-h reaction period revealed all 29a had been converted to a single product, which had a greater R_{t} than 29a. The reaction solution was then added slowly to cold. stirred H_2O (50 mL) to give a yellow solid. The collected and H₂O-washed solid was dried and then stirred with several portions of Et_2O (which removed the excess N-nitrosocarbamate) to give pure 30, homogeneous according to TLC.

Ethyl N³-[(2-chloroethyl)nitrosocarbamoyl]-N²-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2,3-diaminopropanoate (31) was prepared from 29a.2CF₃CO₂H and 2,4,5-trichlorophenyl N-(2-chloroethyl)-Nnitrosocarbamate³⁵ in DMF containing $(i-Pr)_2$ NEt in the manner and scale described above for analogous 30.

N-[[[ω-Carboxy-ω-[[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]amino]alkyl]amino]carbonyl]glycine Diethyl Esters (32a,b) and N-[[[ω -Carboxy- ω -[[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]amino]alkyl]amino]carbonyl]glutamic Acid

Triethyl Esters (33a,b). The procedure for the preparation of 33a is illustrative. A solution of 29.2HCl $\cdot 3.5$ H₂O (520 mg, 0.90 mmol) in DMF (8 mL) containing (i-Pr)₂NEt (390 mg, 3.0 mmol) was combined with a solution of diethyl 2-isocyanatoglutarate³⁷ (250 mg, 1.1 mmol) in DMF (1 mL), and the solution was kept at 20-25 °C for 18 h. Evaporation in vacuo (<1 mm, bath to 35 °C) gave an oil that solidified when stirred with H_2O (25 mL). The collected solid was dried and then dissolved in EtOH (20 mL). and the Norit-treated and filtered (Celite) solution was added slowly to stirred Et₂O (35 mL) to cause precipitation of 33a (457 mg). Additional data are given in Table VII along with results from similar preparations of 33b and 32a,b (using commercial ethyl isocyanatoacetate).

N-[[[2-Carboxy-2-[[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]amino]ethyl]amino]carbonyl]-L-glutamic Acid (34). Compound 33a-2H₂O (270 mg, 0.38 mmol) was dissolved in EtOH (8 mL), and the stirred solution was combined with H₂O (8 mL) at 20-25 °C and then treated with Ba(OH)₂·8H₂O (368 mg, 1.16 mmol). A clear solution formed momentarily before yellow solid began precipitating. Stirring at 20-25 °C was continued for 20 h at which time HPLC indicated complete hydrolysis of the ester groups.⁵⁵ Cold H₂O (10 mL) was added, and the stirred mixture was treated with AcOH to pH 4.2. The mixture was chilled before the yellow precipitate was collected and dried (140 mg). The sample was then stirred with H_2O (1 mL), and the suspension was treated with barely enough 0.1 N NH₄OH to cause solution. Addition of $(NH_4)_2SO_4$ (200 mg) caused slight turbidity. Clarification through Celite followed, and the filtrate was treated with 0.1 N HCl to pH 4 to cause precipitation of 34. After refrigeration (18 h), the solid was collected. Assay by HPLC indicated a purity of 97%.^{5t}

Acknowledgment. This investigation was supported by PHS Grants CA08748, 18856, 22764 (F.M.S.), and CA25236 (J.R.P.), awarded by the National Cancer Institute. DHHS.

Registry No. 1a, 70-47-3; 1b, 56-85-9; 2, 66891-86-9; 3a, 96845-84-0; 3b, 96845-85-1; 4a, 96845-86-2; 4b, 96845-87-3; 5a, 96845-88-4; 5b, 96845-89-5; 6a, 96845-90-8; 6b, 96845-91-9; 7, 7733-29-1; 8a, 36212-66-5; 8b, 42749-49-5; 9a, 90899-93-7; 9b, 96845-92-0; 10a, 62778-08-9; 10b, 96845-93-1; 11a, 96845-94-2; 11b, 96845-95-3; 11c, 96845-96-4; 12a, 96845-97-5; 12b, 96845-98-6; 13, 96845-99-7; 14a, 91338-47-5; 14b, 96846-00-3; 15, 96846-01-4; 16a, 585-23-9; 16b, 7419-40-1; 17·HBr, 96846-02-5; 18a, 96846-03-6; 18b, 96846-04-7; 19, 96846-05-8; 20a, 96846-06-9; 20b, 96846-07-0; 21, 96846-08-1; 22, 52853-40-4; 23a, 96846-09-2; 23b, 96846-10-5; 24, 96846-11-6; 25a, 96846-12-7; 25b, 96846-13-8; 26a, 96846-14-9; 26b, 96846-15-0; 26c, 96846-16-1; 27a, 96865-77-9; 27a free base, 96846-17-2; 27b, 96846-19-4; 27b free base, 96846-18-3; 27c, 96846-20-7; 27c free base, 80407-73-4; 28a, 96846-21-8; 28b, 96846-22-9; 28c, 96846-23-0; 29a-2HCl, 96846-24-1; 29a-2CF₃CO₂H, 96846-26-3; 29b-2HCl, 96846-27-4; 30, 96846-28-5; 31, 96846-29-6; 32a, 96846-30-9; 32b, 96846-31-0; 33a, 96846-32-1; 33b, 96846-33-2; 34, 96846-34-3; bis(1,1-dimethylethyl) dicarbonate, 24424-99-5; tosyl chloride, 98-59-9; 4-chlorobenzyl chloride, 122-01-0; 2,4,5trichlorophenyl N-methyl-N-nitrosocarbamate, 80354-50-3; 2,4,5-trichlorophenyl N-(2-chloroethyl)-N-nitrosocarbamate, 80354-51-4; diethyl 2-isocyanatoglutarate, 17046-23-0; ethyl isocyanatoacetate, 2949-22-6; N³-[(1,1-dimethylethoxy)carbonyl]-2,3-diaminopropanoic acid, 96895-04-4; N⁴-[(1,1-dimethylethoxy)carbonyl]-2,4-diaminobutanoic acid, 93387-94-1; N⁵-[(1,1dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid, 13650-49-2; dihydrofolate reductase, 9002-03-3.

- (55) HPLC conditions were described in ref 22a.
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