culture tubes (1 mL/tube). Test compounds were dissolved in growth medium, sterilized by passage through a 0.22-μm 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 (ID_{50}).

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

J. R. Piper,*† G. S. McCaleb,† J. A. Montgomery,† F. A. Schmid,‡ and F. M. Sirotnak‡

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35255, and Memorial Sloan-Kettering Cancer Center, New York, New York 10021. *Received November 15, 1984*

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}-[(1,1-dimethylethoxy)carbonyl]-2,ω-diaminoalkanoic acid ethyl esters (**12a,b**) and N^2-[4-(methylamino)benzoyl]-N^5-[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid (**13**) followed by alkylation with 6-(bromomethyl)-2,4-pteridinediamine 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**), *N*-methyl-*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-γ-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 structure–activity 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]

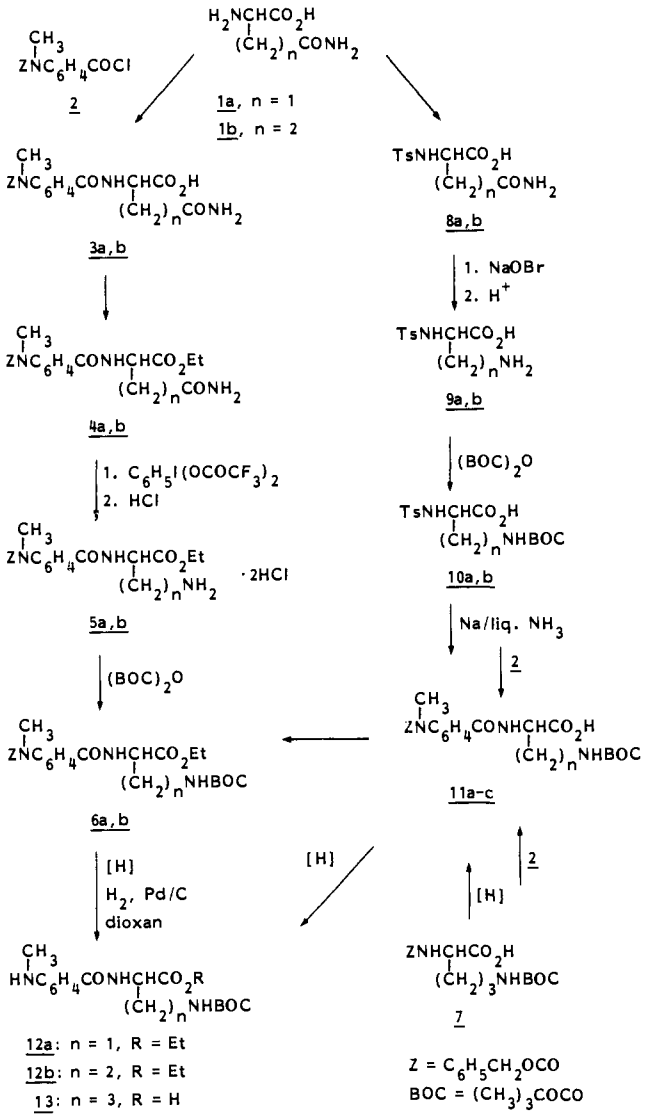
The findings that MTX analogues modified in the region of the γ-carboxyl group still bind strongly to DHFR[11,12] prompted interest in analogues in which glutamic acid has been replaced by 2,ω-diaminoalkanoic acids (as in structural type **27** of Scheme III). The terminal amino group of these analogues 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^{ω}-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 inhibitor[13] and also added fluorescein isothiocyanate to the N^6-amino group to give a fluorescent de-

† Kettering-Meyer Laboratory.
‡ Memorial Sloan-Kettering Cancer Center.

(1) Sirotnak, F. M.; DeGraw, J. I. "Pharmacology and Experimental Clinical Therapeutics"; Sirotnak, F. M., Ed.; Academic Press: New York, 1984; Vol. 2, pp 43–95.
(2) Sirotnak, F. M.; Chello, P. L.; DeGraw, J. I.; Piper, J. R.; Montgomery, J. A. "Molecular Actions and Targets for Cancer Chemotherapeutic Agents"; Sartorelli, A. C., Ed.; Academic Press: New York, 1981; pp 349–383.
(3) Sirotnak, F. M. *Prog. Cancer Res. Ther.* **1983**, *28*, 77.
(4) Samuels, L. L.; Moccio, D. M.; Sirotnak, F. M. *Proc. Am. Assoc. Cancer Res.* **1983**, *23*, 278.
(5) Sirotnak, F. M.; Moccio, D. M. *Cancer Res.* **1980**, *40*, 1230.
(6) Sirotnak, F. M.; Chello, P. L.; Brockman, R. W. *Methods Cancer Res.* **1979**, *16*, 382.
(7) Sirotnak, F. M.; DeGraw, J. I.; Moccio, D. M.; Dorick, D. M. *Cancer Treat. Rep.* **1978**, *62*, 1047.
(8) Moccio, D. M.; Sirotnak, F. M.; Samuels, L. L.; Ahmed, T.; Yagoda, A.; DeGraw, J. I.; Piper, J. R. *Cancer Res.* **1984**, *44*, 352.
(9) Sirotnak, F. M.; DeGraw, J. I.; Moccio, D. M.; Samuels, L. L.; Goutas, L. G. *Cancer Chemother. Pharmacol.* **1984**, *12*, 18.
(10) Sirotnak, F. M.; DeGraw, J. I.; Schmid, F. A.; Goutas, L. J.; Moccio, D. M. *Cancer Chemother. Pharmacol.* **1984**, *12*, 26.
(11) Sirotnak, F. M.; Chello, P. L.; Piper, J. R.; Montgomery, J. A. *Biochem. Pharmacol.* **1978**, *27*, 1821.
(12) Piper, J. R.; Montgomery, J. A.; Sirotnak, F. M.; Chello, P. L. *J. Med. Chem.* **1982**, *25*, 182.
(13) Rosowsky, A.; Wright, J. E.; Ginty, C.; Uren, J. *J. Med. Chem.* **1982**, *25*, 960.
(14) Kempton, R. J.; Black, A. M.; Anstead, G. M.; Kumar, A. A.; Blankenship, D. T.; Freisheim, J. H. *J. Med. Chem.* **1982**, *25*, 475.

0022-2623/85/1828-1016$01.50/0

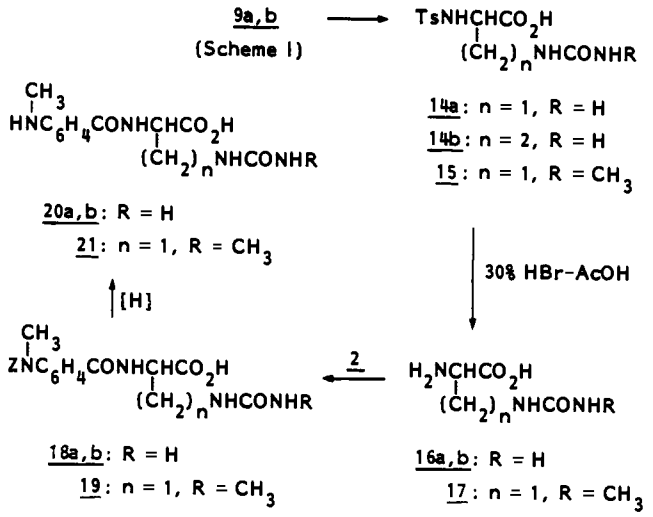
Scheme I. Routes to N^2-[4-(Methylamino)benzoyl]-N^ω-BOC-2,ω-diaminoalkanoic Acids and Their Esters



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

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



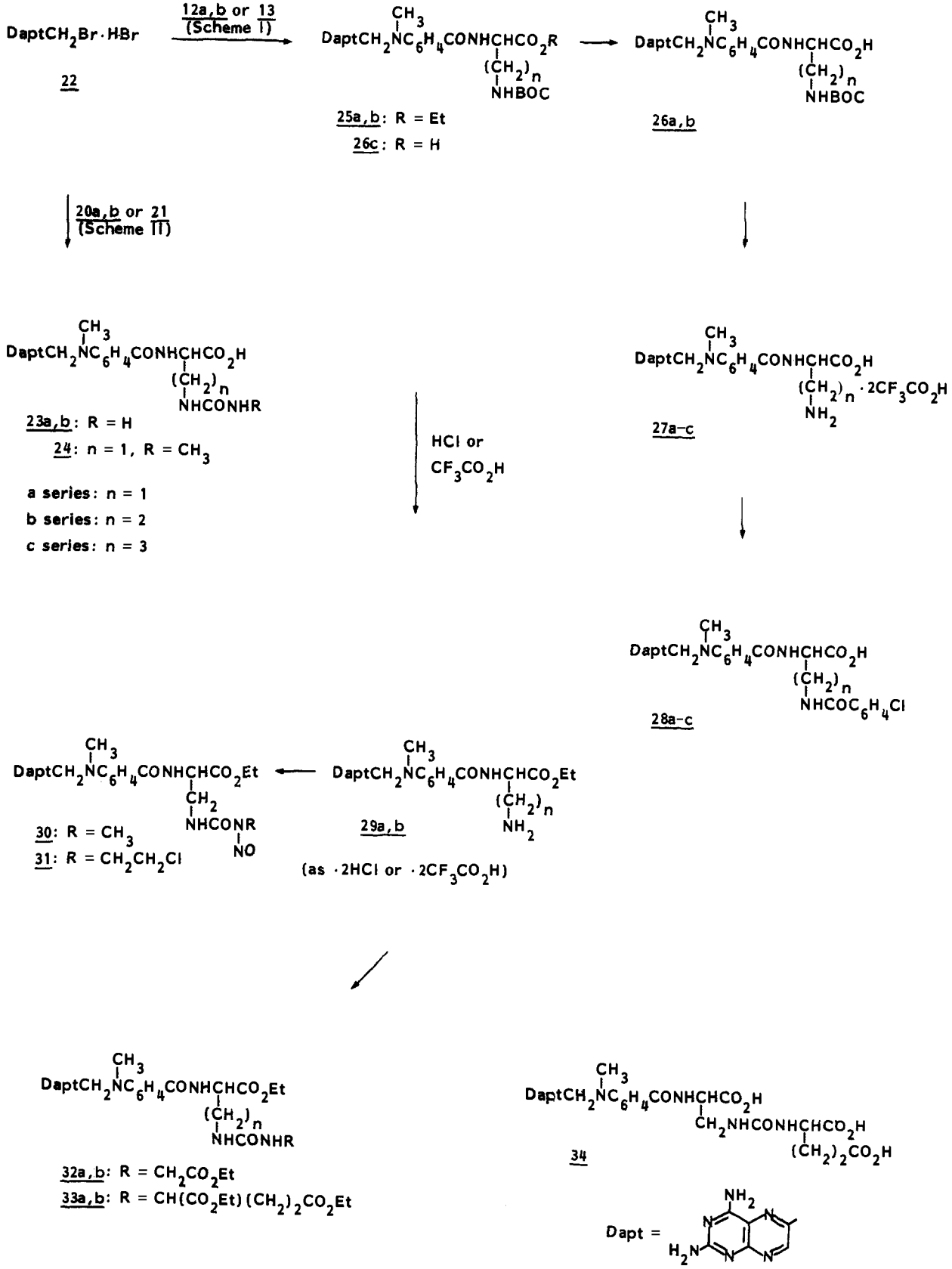
as studies in the further enhancement of synthetic capability in the area of MTX analogues.

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 chains[22] 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,ω-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 N^4-substituted 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 3-amino group unprotected.[27] Further, it was reported that

(15) Rosowsky, A.; Wright, J. E.; Shapiro, H.; Beardsley, P.; Lazarus, H. *J. Biol. Chem.* **1982**, *257*, 14162.
(16) Kumar, A. A.; Freisheim, J. H.; Kempton, R. J.; Anstead, G. M.; Black, A. M.; Judge, L. *J. Med. Chem.* **1983**, *26*, 111.
(17) Kumar, A. A.; Kempton, R. J.; Anstead, G. M.; Freisheim, J. H. *Biochemistry* **1983**, 22, 390.
(18) Kumar, A. A.; Kempton, R. J.; Anstead, G. M.; Price, E. M.; Freisheim, J. H. *Anal. Biochem.* **1983**, *128*, 191.
(19) Piper, J. R.; Montgomery, J. A. *J. Org. Chem.* **1977**, *42*, 208.
(20) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. *J. Med. Chem.* **1982**, *25*, 877.
(21) Piper, J. R.; Montgomery, J. A. *J. Heterocycl. Chem.* **1974**, *11*, 279.
(22) (a) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A. *J. Med. Chem.* **1983**, *26*, 291. (b) Rosowsky, A.; Forsch, R. *J. Med. Chem.* **1982**, *25*, 1454. (c) Henkin, J.; Washtien, W. L. *J. Med. Chem.* **1983**, *26*, 1193. (d) Tang, K. C.; Coward, J. K. *J. Org. Chem.* **1983**, *48*, 5001. (e) Antonjuk, D. J.; Boadle, D. K.; Cheung, H. T. A.; Tran, T. Q. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1989.
(23) Albert, A. *Biochem. J.* **1952**, *50*, 690.
(24) Smith, L. H. *J. Am. Chem. Soc.* **1955**, *77*, 6691.
(25) British Patent 957 892, 1964; *Chem. Abstr.* **1964**, *61*, 8406*d*.
(26) Rosowsky, A.; Wright, J. E. *J. Org. Chem.* **1983**, *48*, 1539.

Scheme III. N^2-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl] Derivatives of 2,ω-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 N^2-substituted compound also forms even under proper pH control.[28]

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

(27) Kjaer, A.; Larsen, P. O. *Acta Chem. Scand.* **1959**, *13*, 1565.

(28) Mokotoff, M.; Logue, L. W. *J. Med. Chem.* **1981**, *24*, 554.

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)iodobenzene[29] 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 chloride[12] (**2**) to provide **11a**,b. Treatment of **11a**,b with ethyl iodide in *N,N*-dimethylformamide (DMF) containing *N,N*-diisopropylethylamine [$(i\text{-Pr})_2$NEt] 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 procedures[33] 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^{ω}-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 $(i\text{-Pr})_2$NEt. The *N*-methyl-*N*-nitrosoureido compounds **30** and the *N*-(2-chloroethyl)-*N*-nitrosoureido analogue **31** were easily prepared in the nonaqueous medium from **29a** and the appropriate 2,4,5-trichlorophenyl N-substituted *N*-nitrosocarbamate 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 *N*-methyl-*N*-nitrosocarbamate, but none sufficed. When **27a** bis(trifluoroacetate) was used in nonaqueous medium such as DMF containing 3 equiv of $(i\text{-Pr})_2$NEt [or 2 equiv of $(i\text{-Pr})_2$NEt plus 1 equiv of pyridine], no reaction occurred because of insolubility. When 2 equiv of $(i\text{-Pr})_2$NEt plus 1 equiv of NaOH [or simply 3 equiv of $(i\text{-Pr})_2$NEt] were used in DMF containing enough water to cause solution, no conversion of **27a** occurred because the activated *N*-methyl-*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-isocyanatoglutarate[37] 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 NHCONH group is present in place of the CH_2CONH group. Ordinary ester hydrolysis conditions (slight excess of NaOH in aqueous ethanol) applied to these compounds

(29) (a) Radhakrishna, A. S.; Parham, M. E.; Riggs, R. M.; Loudon, G. M. *J. Org. Chem.* **1979**, *44*, 1746. (b) Loudon, G. M.; Radhakrishna, A. S.; Almond, M. R.; Blodgett, J. K.; Boutin, R. H. *J. Org. Chem.* **1984**, *49*, 4272.
(30) Cf: Schneider, F. *Justus Leibigs Ann. Chem.* **1937**, *529*, 1.
(31) Zaoral, M.; Rudinger, J. *Collect. Czech. Chem. Commun.* **1959**, *24*, 1993.
(32) Rudinger, J.; Poduska, K.; Zaoral, M.; Jost, K. *Collect. Czech. Chem. Commun.* **1959**, *24*, 2013.
(33) Rudinger, J.; Poduska, K.; Zaoral, M. *Collect. Czech. Chem. Commun.* **1960**, *25*, 2022.
(34) Broadbent, W.; Morley, J. S.; Stone, B. E. *J. Chem. Soc. C* **1967**, 2632.
(35) Martinez, J.; Oiry, J.; Imbach, J. L.; Winternitz, F. *J. Med. Chem.* **1982**, *25*, 178.
(36) (a) Montgomery, J. A.; James, R.; McCaleb, G. S.; Johnston, T. P. *J. Med. Chem.* **1967**, *10*, 668. (b) Montgomery, J. A.; James, R.; McCaleb, G. S.; Kirk, M. C.; Johnston, T. P. *J. Med. Chem.* **1975**, *18*, 568. (c) Wheeler, G. P.; Bowdon, B. J.; Struck, R. F. *Cancer Res.* **1975**, *35*, 2974.
(37) Humphlett, W. J.; Wilson, C. V. *J. Org. Chem.* **1961**, *26*, 2507.

Table I. L1210 Cell Growth Inhibition Data on **26a–c** and **27a–c**[a]

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 ± 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 hydantoin[38] and pyroglutamic acid[39] 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 three- to 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). Ornithine-derived **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 above[40] and the data on transport and growth inhibition, are probably aberrancies of the cell-free enzyme system. The IC_{50} 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*-(2-chloroethyl)-*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 binding[12] 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

(38) Ware, E. *Chem. Rev.* **1950**, *46*, 403.
(39) Schroder, E.; Lubke, K. "The Peptides"; Gross, E., Translator; Academic Press: New York, 1965; Vol. 1, pp 193–194.
(40) Sirotnak, F. M.; Chello, P. L.; Moccio, D. M.; Piper, J. R.; Montgomery, J. A.; Parham, J. C. *Biochem. Pharmacol.* **1980**, *29*, 3293.

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

compd	L1210 cell growth inhibn IC_{50},[b] nM	L1210 DHFR inhibn K_i,[b] nM	cell membrane transport[b] influx K_i, μM epithelial	S180	Erlich	L1210	efflux k, (min^{-1}) 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] Methods used are described in ref 57. [b] Schedule q2d × 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 Me_2SO-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 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 μm) silica gel G(F) plates. Except where other conditions are specified, evaporations were performed with a rotary evaporator and a H_2O aspirator. Compounds listed in Table VII were dried in vacuo (<1 mm) at room temperature over P_2O_5 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**·H_2O (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 chloride[12] (**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 H_2O (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]-L-glutamine (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 H_2O (600 mL) to give crystalline **3b** (27.6 g), homogeneous according to TLC ($CHCl_3$–MeOH, 9:1).

N^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-asparagine Ethyl Ester (4a). A solution of **3a** (7.57 g, 18.9 mmol), EtI (3.28 g, 21.0 mmol), $(i\text{-Pr})_2NEt$ (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 $CHCl_3$, and the solution was washed successively with H_2O and 2% NH_4OH solution. Evaporation of the dried ($MgSO_4$) 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]-L-glutamine Ethyl Ester (4b). A solution of **3b** (27.0 g, 65.5 mmol), EtI (11.3 g, 72.5 mmol), $(i\text{-Pr})_2NEt$ (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 H_2O 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)iodobenzene[29] (9.80 g, 22.8 mmol) in MeCN (100 mL) and H_2O (25 mL) was kept at 20–25 °C for 16 h and then refluxed 30 min. At this time TLC ($CHCl_3$–MeOH, 9:1) showed disappearance of **4a**. The solution was evaporated until most of the CH_3CN had been removed. Treatment with 12 N HCl (8.0 mL) followed, and the mixture that formed was extracted with Et_2O (twice with

(41) (a) Rosowsky, A.; Moran, R. G.; Forsch, R.; Colman, P.; Uren, J.; Wick, M. *Biochem. Pharmacol.* **1984**, *33*, 155. (b) Rosowsky, A.; Forsch, R. A.; Freisheim, J. H.; Moran, R. G.; Wick, M. *J. Med. Chem.* **1984**, *27*, 600.
(42) (a) Baugh, C. M.; Krumdieck, C. L.; Nair, M. G. *Biochem. Biophys. Res. Commun.* **1973**, *52*, 27. (b) Baugh, C. M.; Nair, M. G. *Biochemistry* **1973**, *12*, 3923.
(43) Fry, D. W.; Yalowich, J. C.; Goldman, I. D. *J. Biol. Chem.* **1982**, *257*, 1870.
(44) Balinska, M.; Galivan, J.; Coward, J. K. *Cancer Res.* **1981**, *41*, 2751.
(45) Gewirtz, D. A.; White, J. C.; Randolph, J. K.; Goldman, I. D. *Cancer Res.* **1980**, *40*, 573.
(46) Rosenblatt, D. S.; Whitehead, V. M.; Dupont, M. M.; Vuchich, M. J.; Vera, N. *Mol. Pharmacol.* **1978**, *14*, 210.
(47) Schilsky, R. L.; Bailey, B. D.; Chabner, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2919.
(48) Whitehead, V. M. *Cancer Res.* **1977**, *37*, 408.
(49) Covey, J. M. *Life Sci.* **1980**, *26*, 665.
(50) Jolivet, J.; Schilsky, R. L.; Bailey, B. D.; Drake, R. C.; Chabner, B. A. *J. Clin. Invest.* **1982**, *70*, 351.
(51) Kisliuk, R. L.; Gaumont, Y.; Baugh, C. M.; Galivan, J. H.; Maley, G. F.; Maley, F. Proceedings of the 6th International Symposium on the Chemistry and Biology of Pteridines, La Jolla, CA, 1978; *Dev. Biochem.* **1979**, *4*, 431.
(52) Szeto, D. W.; Cheng, Y.-C.; Rosowsky, A.; Yu, C.-S.; Modest, E. J.; Piper, J. R.; Temple, C., Jr.; Elliott, R. D.; Rose, J. D.; Montgomery, J. A. *Biochem. Pharmacol.* **1979**, *28*, 2633.
(53) Baggott, J. E. *Fed. Proc.* **1983**, *42*, 667.

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

compd	L1210 cell growth inhibn IC_{50},[a,b] nM	L1210 cell DHFR inhibn K_i,[a] pM	L1210 cell membrane transport[a] influx K_i, μM	L1210 cell membrane transport[a] efflux k, min^{-1}	in vivo act. in mice, % 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. [b] Cytotoxicity to H.Ep.-2 cells (ED_{50}, nM): MTX, 2.4; **28a**, 470; **28c**, 4.0 (method described in ref 58). [c] L1210 10^6 tumor cell implant (ip), q2d × 5 (ref 57). [d] P388 10^6 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	175[a]	$C_{20}H_{21}N_3O_6$[c]
3b	90	168[a]	$C_{21}H_{23}N_3O_6$[c]
4a	88	169[a]	$C_{22}H_{25}N_3O_6$[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[f]	103[a]	$C_{27}H_{35}N_3O_7$[c,d]
8a	90	189–191[b,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,j]	$C_{11}H_{16}N_2O_4S$
10a	77	127–128 dec[b,k]	$C_{15}H_{22}N_2O_6S$
10b	73	147–148 dec[b,l]	$C_{16}H_{24}N_2O_6S$
11a	56	149–150[b]	$C_{24}H_{29}N_3O_7$[c,m]
11b	96	glass	$C_{25}H_{31}N_3O_7$[m]
11c	85	glass	$C_{26}H_{33}N_3O_7$[m]
12a	*n*	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 Et_2O–aqueous $NaHCO_3$. [h] Lit.[32] mp 161–163 °C, prepared from tosyl-L-pyroglutamic acid and NH_4OH. [i] Rudinger et al.[33] reported 56% yield, mp 218–219 °C dec varying with time of heating. [j] Rudinger et al.[33] reported 94% yield, mp 235–238 °C dec varying with time of heating. [k] Lit. mp (variable) 125–126 °C dec to 128–129 °C dec (effervescence)[34] and 129–131 °C dec,[60] both reported procedures used (1,1-dimethylethoxy)carbonyl azide. [l] Lit.[61] mp 149.5–150 °C dec for product from (1,1-dimethylethoxy)-carbonyl azide. [m] ^1H 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]
14a	60	174 dec[a,b]	$C_{11}H_{15}N_3O_5S$
14b	74	190–191 dec[c,d]	$C_{12}H_{17}N_3O_5S$
15	78	182–184[c]	$C_{12}H_{17}N_3O_5S$[e]
16a	70	220 dec[a,f]	$C_4H_9N_3O_3$
16b	67	209–211 dec[c,g]	$C_5H_{11}N_3O_3$
17·HBr	100[h]	205–207 dec[i,c]	$C_5H_{11}N_3O_3 \cdot HBr$[e]
18a	58	173–174[c]	$C_{20}H_{22}N_4O_6$[e]
18b	74	157–158[c]	$C_{21}H_{24}N_4O_4$[e]
19	50	173–175[c]	$C_{21}H_{24}N_4O_6$[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]

[a] Observed on a Kofler Heizbank. [b] Lit.[33] mp 174–179 °C dec. [c] Mel-Temp apparatus. [d] Lit.[33] mp 185–188 °C dec. [e] Anal. C, H, N. [f] Lit.[33] mp 218–220 °C dec. [g] Lit.[33] mp 203–206 °C dec and 207–208 °C. [h] Yield of product suitable for use in the preparation of 18. [i] After recrystallization from MeCN. [j] Obtained in theoretical yields as glasslike solid and used directly in the conversion indicated in Scheme III. [k] ^1H NMR spectrum consistent with assigned structure except for retention of small amounts of AcOH and Et_2O.

Table VII. Data for Compounds Shown in Scheme III

no.	yield, %	molec formula[a,b]
23a	68	$C_{19}H_{22}N_{10}O_4 \cdot 3H_2O$
23b	49	$C_{20}H_{24}N_{10}O_4 \cdot 3H_2O$
24	50	$C_{20}H_{24}N_{10}O_4 \cdot 3H_2O$
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	$C_{20}H_{25}N_9O_3 \cdot 2HCl \cdot 2.3H_2O$[d]
29a·2CF$_3$CO$_2$H	95	$C_{20}H_{25}N_9O_3 \cdot 2CF_3CO_2H \cdot 1.5H_2O$
29b·2HCl	95	$C_{21}H_{27}N_{11}O_5 \cdot H_2O$
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	$C_{26}H_{34}N_{10}O_6 \cdot 2H_2O$
33a	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	$C_{24}H_{28}N_{10}O_8 \cdot 3H_2O$

[a] Anal. C, H, N with exceptions noted for **28a**, **29a·2HCl**, and **30**. [b] ^1H NMR spectra were obtained on all compounds except **23a,b**, **27a–c**, and **29a·2CF$_3$CO$_2$H** and were consistent with the assigned structures. [c] Anal. N: calcd, 21.91; found, 20.76. [d] Anal. N: calcd, 22.76; found, 21.46. [e] Anal. 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·H$_2$O** as an amorphous solid (3.81 g), homogeneous by TLC ($CHCl_3$–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)iodobenzene[29] (33.8 g, 78.5 mmol) in MeCN (780 mL)–H_2O (260 mL) as described above (under the preparation of **5a**) led to **5b** as a glasslike solid (20.0 g), homogeneous by TLC ($CHCl_3$MeOH, 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·H$_2$O** (454 mg, 1.00 mmol) in DMF (2.5 mL) was treated with Et_3N (101 mg, 1.00 mmol) followed by bis(1,1-dimethylethyl) dicarbonate[54] (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 H_2O and then dried (Na_2SO_4) and evaporated to give **6a** as a colorless oil (456 mg), homogeneous by TLC ($CHCl_3$–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)$_2$NEt (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.

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 $NaHCO_3$, and H_2O before it was dried (Na_2SO_4) 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 N^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-N^4-[(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-dimethylethyl) dicarbonate[54] (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 (Na_2SO_4) and evaporated to give an oil (13.5 g, 59% yield) virtually homogeneous according to TLC ($CHCl_3$–MeOH, 95:5). The oil solidified when stirred with *n*-hexane (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 $(i\text{-Pr})_2NEt$ (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 $CHCl_3$, and the solution was washed with H_2O and 5% NH_4OH solution and then dried (Na_2SO_4) 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**·H_2O (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 H_2O (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).

N^2-[(1,1-Dimethylethoxy)carbonyl]-N^3-[(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), H_2O (160 mL), and dioxane (160 mL) was treated dropwise with stirring and cooling (ice–H_2O bath) over a period of about 30 min with a solution of bis(1,1-dimethylethyl) dicarbonate[54] (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-H_2O bath was removed and stirring at 20–25 °C was continued for about 2.5 h or until CO_2 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 H_2O until the mixture was acidic (pH about 3). The oily crude product that precipitated was extracted with EtOAc (800 mL), and the H_2O-washed and dried (Na_2SO_4) 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), H_2O (150 mL), and dioxane (150 mL) was treated at 20–25 °C with a solution of bis(1,1-dimethylethyl) dicarbonate[54] (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 CO_2 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).

N^2-[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 NH_3 (about 200 mL) was cooled in a dry ice–Me_2CO 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 NH_3 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 NH_3. 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 Et_2O (three 25-mL portions), and the ethereal extracts were discarded. The aqueous solution was then combined with a solution of $(i\text{-Pr})_2NEt$ (11.0 g, 85.0 mmol) in dioxane (60 mL), and the resulting solution was cooled in an ice–H_2O 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 H_2O-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).

N^2-[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 NH_3 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 thin-layer chromatogram developed with H_2O produced one spot (UV and ninhydrin detection). The residue was dissolved in H_2O (20 mL) and dioxane (20 mL) containing $(i\text{-Pr})_2NEt$ (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 H_2O-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)–H_2O (50 mL) containing 5% Pd on C (1.0 g) was carried out at 3.16–3.52 kg/cm^2 (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 N_2 and recharged with H_2 three times. The filtered solution was evaporated to give the solid N^5-(1,1-dimethylethoxy)carbonyl intermediate. After trituration with Et_2O, the yield of collected solid was 92% (10.7 g), homogeneous by TLC (H_2O, 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–H_2O containing $(i\text{-Pr})_2$NEt (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 N^2-[4-(Methylamino)benzoyl]-N^ω-[(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 H_2 over H_2O 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 ($CHCl_3$–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 (H_2O and $CHCl_3$–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 H_2O 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 H_2O (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 NH_4OH in H_2O–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]-2-amino-ω-[(aminocarbonyl)amino]alkanoic acids (18a,b) were prepared by coupling of 2 with 16a,b in H_2O–dioxane containing $(i\text{-Pr})_2$NEt (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]-2-amino-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 $(i\text{-Pr})_2$NEt (4.27 g, 33.0 mmol) in H_2O (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, H_2O aspirator, bath to 35 °C) in order to remove the dioxane. The remaining solution was diluted with H_2O (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 N^2-[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/cm² (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 (H_2O, 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 Et_2O. 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). N^2-[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·C_3H_7OH[19] (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 Me_2NAc (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 H_2O (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 N^2-[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·C_3H_7OH[19] (7.00 g of 95% purity, 16.8 mmol) and the sample of 12a described above (7.10 g, 19.4 mmol) in Me_2NAc (100 mL) was stirred for 6 days in a stoppered flask wrapped in Al foil. The solution that formed was then chilled (ice–H_2O bath), and $(i\text{-Pr})_2$NEt (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 NH_4OH (700 mL) with external cooling (ice–H_2O bath). Solid 25 separated readily as a yellow precipitate (7.30 g), homogeneous according to TLC ($CHCl_3$–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 H_2O (25 mL), andd the solution that formed was concentrated by evaporation to about 15 mL to remove EtOH. Dilution with H_2O (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·C_3H_7OH[19] (5.13 g of 95%, 12.3 mmol) and the sample of 13 described above (5.84 g, 16.0 mmol) in Me_2NAc (80 mL) was stirred at 20–25 °C for 5 days in

a stoppered flask wrapped in Al foil. The solution that formed was added dropwise to NH_4OH 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**.

N^2-[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**·1.8H_2O (1.78 g, 3.27 mmol) in CF_3CO_2H (25 mL) was stirred at 0–5 °C (bath temperature) for 1 h and was then poured in a thin stream into stirred Et_2O (250 mL). The yellow precipitate of **27a** (2.11 g) that formed was collected and washed with Et_2O. The homologues **27b,c** were prepared on similar scales.

N^2-[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 Et_2O and finally dried in vacuo at 58 °C for 1 h. This procedure was also used in preparations of **28b,c**.

Ethyl N^2-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-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 ($CHCl_3$–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 Et_2O 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 Et_2O (400 mL) gave pure **29a** bis(trifluoroacetate).

Ethyl N^2-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-N^3-(methylnitrosocarbamoyl)-2,3-diaminopropanoate (30). 2,4,5-Trichlorophenyl *N*-methyl-*N*-nitrosocarbamate[35] (170 mg, 0.60 mmol) was added to a cold (0–5 °C), stirred solution of **29a**·2CF_3CO_2H·1.5H_2O (330 mg, 0.48 mmol) and $(i\text{-Pr})_2NEt$ (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 ($CHCl_3$–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_f than **29a**. The reaction solution was then added slowly to cold, stirred H_2O (50 mL) to give a yellow solid. The collected and H_2O-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 N^3-[(2-chloroethyl)nitrosocarbamoyl]-N^2-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2,3-diaminopropanoate (31) was prepared from **29a**·2CF_3CO_2H and 2,4,5-trichlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate[35] in DMF containing $(i\text{-Pr})_2NEt$ 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·3.5H_2O (520 mg, 0.90 mmol) in DMF (8 mL) containing $(i\text{-Pr})_2NEt$ (390 mg, 3.0 mmol) was combined with a solution of diethyl 2-isocyanatoglutarate[37] (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 Et_2O (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**·2H_2O (270 mg, 0.38 mmol) was dissolved in EtOH (8 mL), and the stirred solution was combined with H_2O (8 mL) at 20–25 °C and then treated with $Ba(OH)_2$·8H_2O (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 H_2O (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 NH_4OH 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%.[55]

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**·2CF_3CO_2H, 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; 2,4,5-trichlorophenyl *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; N^5-[(1,1-dimethylethoxy)carbonyl]-2,3-diaminopropanoic acid, 96895-04-4; N^4-[(1,1-dimethylethoxy)carbonyl]-2,4-diaminobutanoic acid, 93387-94-1; N^5-[(1,1-dimethylethoxy)carbonyl]-2,5-diaminopentanoic acid, 13650-49-2; dihydrofolate reductase, 9002-03-3.

(55) HPLC conditions were described in ref 22a.
(56) Sirotnak, F. M.; Donsbach, R. C. *Cancer Res.* **1976**, *36*, 1151.
(57) Sirotnak, F. M.; Donsbach, R. C. *Cancer Res.* **1975**, *35*, 1737.
(58) Bennett, L. L., Jr.; Vail, M. H.; Allan, P. W.; Shaddix, S. C. *Biochem. Pharmacol.* **1973**, 22, 1221.
(59) Geran, R. I.; Greenberg, N. H.; Macdonald, W. M.; Schumacher, A. M.; Abbott, B. J. *Cancer Chemother. Rep., Part 3* **1972**, *3* (2).
(60) Brtnik, F.; Zaoral, M. *Collect. Czech. Chem. Commun.* **1976**, *41*, 2969.
(61) Poduska, K. *Collect. Czech. Chem. Commun.* **1966**, *31*, 2955.