scribed for peptide 2. The yields of purified peptides were 48 mg of peptide 9 and 10 mg of peptide 16. Amino acid compositions were as follows. 9: Tyr, 0.86; Met, 2.01; Phe, 0.98; His, 0.88; Leu, 1.0; Asp, 0.97. 16: Tyr, 0.95; Met, 1.89; Phe, 0.98; Leu, 1.0; Asp, 0.95.

[D-Ala^2]- and [D-Ala^2,des-His^4]deltorphin A (3 and 12, Respectively). N^{α}-Fmoc-D-Ala was substituted for N^{α}-Fmoc-D-Met in the method for the synthesis of peptide 1.[7,13] The yields were 63 mg for peptide 3 and 11 mg for peptide 12. Amino acid compositions gave the following. 3: Tyr, 0.91; Ala, 1.01; Phe, 0.99; His, 0.94; Leu, 1.0; Met, 0.96; Asp, 0.95. 12: Tyr, 0.94; Ala, 0.97; Phe, 0.98; Leu, 1.0; Met, 0.91; Asp 1.02.

[D-Nle^2]deltorphin A (4). N^{α}-Fmoc-D-Nle was substituted for N^{α}-Fmoc-D-Met during synthesis.[7,13] The deprotected peptide was purified by two additional chromatographic steps to yield 21 mg of product. Final amino acid composition was Tyr, 0.87; Nle, 1.02; Phe, 0.97; His, 0.89; Leu, 1.0; Met, 0.95; Asp, 0.97.

[D-Met^2]- and Deltorphin B (18 and 17, Respectively). Deltorphin B was synthesized as described.[7] N^{α}-Fmoc-D-Met replaced N^{α}-Fmoc-D-Ala in the synthesis of [D-Met^2]deltorphin B (18). The amino acid composition, normalized after a 72-h hydrolysis, was Tyr, 0.91; Met, 0.95; Phe, 0.99; Glu, 0.91; Gly, 1.0; Val, 1.67.

Receptor Assays. Rat brain synaptosomes were obtained from Sprague-Dawley male rats, based on the method of Chang and Cuatrecasas,[48] as previously described.[6-8,49,50] The binding assays for δ receptors utilized 0.62 pmol [^{3}H]DPDPE (DuPont/NEN) in a reaction mixture containing 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 μM bestatin, 4 μg of bacitracin, 8% glycerol, 32 μg/mL soybean trypsin inhibitor, and 100 μM (phenylmethyl)sulfonyl fluoride; μ receptor assays used 1.28 nM [^{3}H]DAGO (Amersham) with 1 M $MgCl_2$ in addition to the above buffer and protease inhibitors. After a 2-h incubation at 22 °C, the duplicate samples were rapidly filtered through wetted Whatman GF/C glass microfilters and washed with 3 × 2 mL 50 mM Tris-HCl, pH 7.5, containing 1 mg/mL BSA; the filters were dried at 75 °C and counted in a β counter using CytoScint (ICN) fluorophore. Peptides were assayed at 4-9 concentrations covering 2-3 orders of magnitude with n = 3-8; 3-5 synaptosomal preparations were used to ensure the statistical reliability of SEM. The equation of Cheng and Prusoff[51] was employed to calculate K_i values.

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Synthesis and Biological Activity of Novel Folic Acid Analogues: Pteroyl-*S*-alkylhomocysteine Sulfoximines

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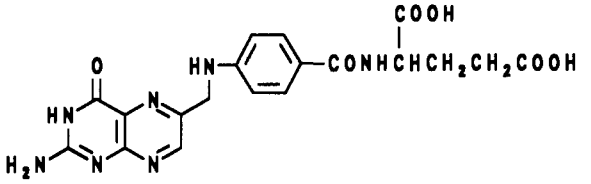
The synthesis of a novel series of γ-substituted folic acid analogues, pteroyl-*S*-alkyl-DL-homocysteine (*RS*)-sulfoximines, and the corresponding *S*-methylhomocysteine sulfone is described. Side reactions of the sulfoximine groups of the amino acid ester reactants were considered. The correct structures of the isolated target compounds were confirmed by NMR and FAB/MS excluding other alternatives. The replacement of the γ-COOH of the glutamate moiety of folic acid with *S*-alkylsulfoximine groups or *S*-methylsulfone did not affect the substrate activity of the vitamin for dihydrofolate reductase. The resulting tetrahydrofolate analogues could serve as cofactors for the thymidylate synthase cycle of murine leukemia L1210 cells in situ. The analogues inhibited the growth of these cells in culture with 2 orders of magnitude lower IC_{50} values [(2-4) × 10^{-4} M] than the parent folic acid.

Folic acid coenzymes play an important role in amino acid metabolism and are essential for the biosynthesis of nucleic acids.[1] They exist in the cell as tetrahydrofolyl poly-γ-glutamate conjugates, which are the preferred substrates (cofactors) for most folate-requiring enzymes.[2,3] Conversion to polyglutamates, which is catalyzed by folylpolyglutamate synthetase (FPGS), contributes significantly to the intracellular retention of tetrahydrofolate cofactors.[2] A variety of structural analogues of folic acid, exemplified by methotrexate, is capable of undergoing intracellular polyglutamylation, which may play an important role in the antitumor activity of these antifolates.[4] Since FPGS is essential for cellular viability,[5,6] it represents a potential target for chemotherapy.[7,8]



Folic acid (PteGlu)

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†Current address: Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, 600 South 43rd Street, Philadelphia, PA 19104.

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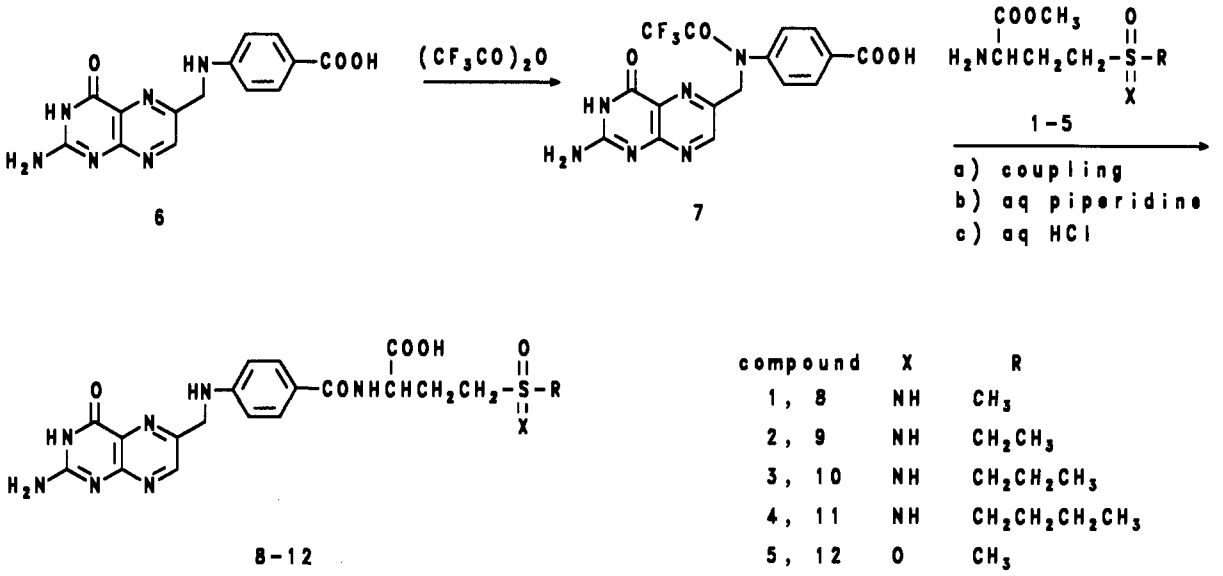
Scheme I



compound	X	R
1, 8	NH	CH_3
2, 9	NH	CH_2CH_3
3, 10	NH	$CH_2CH_2CH_3$
4, 11	NH	$CH_2CH_2CH_2CH_3$
5, 12	O	CH_3

We designed folate analogues, in which the glutamate moiety is replaced by *S*-alkylhomocysteine sulfoximines, as potential inhibitors of this enzyme.[9] Our strategy was based on the similarity between the catalytic mechanisms of FPGS[2,10] and γ-glutamylcysteine synthetase,[11] and on the susceptibility of γ-glutamylcysteine synthetase to inactivation by *S*-alkylhomocysteine sulfoximines.[11] These compounds were shown to become phosphorylated at the active site[11] to form enzyme-generated transition state analogue inhibitors.[12] In a recent report[9b] we discussed the rationale for the mechanism-based design in detail.

In this paper we describe the synthesis and characterization of a homologous series of pteroyl-*S*-alkylhomocysteine sulfoximines and pteroyl-*S*-methylhomocysteine sulfone, novel analogues of folic acid which cannot be polyglutamylated. We employed conventional synthetic strategies; however, formation of undesired products during condensation of pteroic acid with the amino acid analogues due to the reactivity of the sulfoximine group was of major concern. In order to establish the identity of the products, NMR and mass spectral studies were conducted, which proved that the desired structures were obtained. The analogues were also evaluated for substrate, cofactor, and cell growth inhibitory activity. Since the ability of folate-dependent enzymes to utilize tetrahydrofolate analogues in which the γ-COOH group is replaced by other functional groups has not been studied extensively, the results of the present work contribute to our understanding of how structural variations affect folate cofactor activity.

Chemistry

All folate analogues were prepared by coupling N^{10}-(trifluoroacetyl)pteroic acid with the sulfoximine- or sulfone-containing amino acid esters followed by hydrolysis as outlined in Scheme I. The unesterified amino acid analogues were either synthesized according to literature methods[11b] or were obtained commercially, when available. These compounds exhibited a characteristic behavior on ^1H NMR that later proved very useful in the identification of the corresponding esters and the folate analogues. A pronounced downfield shift of the protons on the carbons adjacent to the sulfoximine group was seen when the NMR samples were acidified with DCl. The change in chemical shift was highly consistent, averaging 0.73 ± 0.01 ppm for the methylene and 0.63 ppm for the methyl protons. Protonation of the imino group is known to occur in acid,[13]

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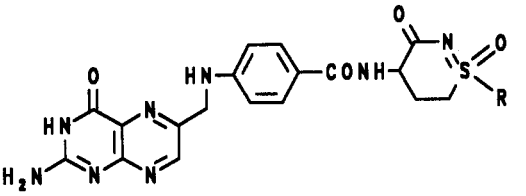
which increases the positive charge on sulfur, accounting for the observed magnitude of the shift. This is consistent with the much smaller change in chemical shift (0.14 ppm) for the methylene protons adjacent to the sulfone which is not protonated in acid.

The sulfoximine-containing amino acid methyl ester hydrochlorides (1–4) were synthesized by reaction of the free amino acids with an excess of 2,2-dimethoxypropane in the presence of an acid catalyst.[14] An alternative preparation of the *S*-methyl analogue (1) via Fischer–Speier esterification was reported,[11] however no analytical data were given. Due to the relatively poor solubilities of DL-methyl- and DL-ethyl-(*SR*)-sulfoximine in 2,2-dimethoxypropane, methanol was added to the reaction mixtures. After extraction and lyophilization, the products were obtained as hygroscopic glasses. These were shown to be essentially homogenous by TLC, although traces of unreacted starting material were usually present. Attempted purification of the sulfoximine-containing amino acid methyl esters by recrystallization, preparative TLC (cellulose), or column chromatography (silica gel, ion exchange, and gel filtration) were unsuccessful, therefore, they were used without further purification. All of the homologues produced NMR spectra that were in accord with the expected structures, i.e., the methyl group of the esters appeared as a sharp singlet in the range 3.83 to 3.92 ppm. However, the methylene protons on the carbons adjacent to the sulfoximine appeared farther downfield (3.97–4.20 ppm) than anticipated. The chemical shifts were similar to those obtained for the free amino acids in DCl (4.00–4.22 ppm). These results indicate that the amino acid esters were obtained as dihydrochloride salts (i.e., both the α-amino and the sulfoximine groups were protonated). In contrast, DL-methionine sulfone methyl ester[15] (**5**) was obtained as the crystalline monohydrochloride salt.

The final step in the synthesis of the folate analogues involved coupling of the appropriate amino acid esters with a pteroic acid derivative (Scheme I). Pteroic acid (**6**) of very high purity was synthesized in nearly quantitative yields by a modification of the preparative scale method of Martinelli et al.,[16] whereby folic acid is enzymatically hydrolyzed with carboxypeptidase G (EC 3.4.12.10; *N*-pteroyl-L-glutamate L-glutamate hydrolase). Each batch of pteroic acid was treated with the enzyme twice to remove trace amounts of folic acid that remained after the first hydrolysis. The pteroic acid was then converted to its N^{10}-trifluoroacetyl derivative **7** to enhance its solubility properties.[17] This was converted to a mixed anhydride by reaction with 3 equiv of isobutyl chloroformate.[18] Addition of 4 equiv of the appropriate amino acid methyl ester, followed by basic hydrolysis and acidification, provided the crude products, contaminated with pteroic acid. The compounds were purified by column chromatography on ion-exchange cellulose. The *S*-1-butyl analogue **11** was also synthesized via a carbodiimide-mediated coupling reaction using a modification of the method of Gangjee et al.[19] Comparable yields of **11** were obtained using either method.

The overall yields of the coupled products were relatively low.[20] The presence of the electron-withdrawing N^{10}-trifluoroacetyl group may reduce the nucleophilicity of the para-carboxylate, resulting in incomplete formation of the mixed anhydride. In addition, Levenson and Meyer[24] previously observed that sulfoximine derivatives of aspartic acid methyl esters are prone to cyclization via intramolecular nucleophilic attack by the sulfoximine nitrogen on the ester carbonyl. Although we could find no evidence for formation of cyclized products such as **13** in any of the



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coupling reactions of the corresponding glutamate derivatives, it is conceivable that it may have occurred, reducing the yields of the desired compounds. Considerable efforts were expended to further optimize the yields by altering the reaction conditions (i.e., time, temperature, reagent concentrations, etc.), however, all these attempts were unsuccessful. Other coupling methods were also investigated. Rosowsky et al.[25,26] have reported very good yields in the synthesis of a number of methotrexate analogues

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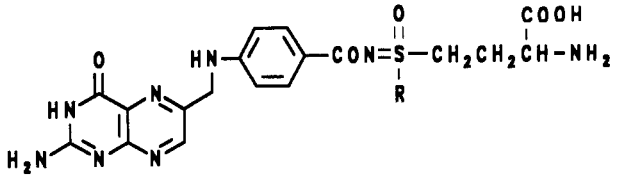
using diethyl phosphorocyanidate (DEPC). However, our attempts to use this reagent with the folate derivatives were unsuccessful. Reaction of DEPC with 7 under mild conditions led to rapid loss of the N^{10}-trifluoroacetyl group, and coupling with the amino acid esters did not occur. The mechanism of this unexpected deacylation reaction remains to be elucidated.

Elemental analyses indicated that all of the folate analogues exhibited varying degrees of solvation with water or organic solvents. Fractional solvation of other folates has been reported in the literature.[18,19,25,26] All products were homogeneous on TLC. As expected, the UV spectra were virtually identical to those previously reported for folic acid under acidic, neutral, and basic conditions.[27] The IR spectra were also consistent with the assigned structures; however, the sulfoximine N–H stretch, which normally appears at about 3230 cm^{-1},[13] was obscured by the strong O–H and N–H stretching bands. Using negative ion FAB/MS in glycerol matrix, the experimentally determined masses for the $(M - H)^-$ ions were within ±150 ppm of theory. An ion corresponding to $(M - 2H + glycerol)^-$ was detected in the FAB mass spectrum of the *S*-1-propyl analogue **10**. This ion was within 20 ppm of its theoretical mass. These observations are important since cyclization[24] of the sulfoximine group (i.e., **13**) could occur (see above) and would have led to loss of water with 18 mass units.

Signal assignments on 270 MHz ^1H NMR spectra could be made for all of the protons. As was noted with the sulfoximine-containing amino acids, the methylene and methyl protons adjacent to the sulfoximine group exhibited a marked downfield shift (0.87 to 0.98 ppm) upon acidification of the NMR samples. This indicates that the sulfur atom is positively charged due to protonation of the imino group,[13] and suggests that the sulfoximine group is intact in the folate analogues. In comparison, essentially no shift was noted in the corresponding protons in pteroyl-*S*-methylhomocysteine sulfone, which is also in accord with the results obtained for methionine sulfone. An exchangeable peak arising from the amide proton was present at 8.28–8.36 ppm in the ^1H NMR of all the folate analogues. These results are important since they indicate that the coupling reaction occurred with the α-amino group and not the sulfoximine nitrogen of the amino acids. We were thus able to eliminate another alternative structure, i.e., **14**, for the final products. Although only the desired



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products could be isolated from the reaction mixtures and identified, side reactions leading to **13** to **14** may have contributed to the relatively low yields obtained (see above). This could also explain why the yield of **12**, with an unreactive sulfone group, was substantially higher than that of any of the sulfoximine products.

Biological Results and Discussion

Since reduction of folates to the tetrahydrofolate level is a prerequisite for conversion to cofactor derivatives, we examined whether **11**, as a representative member of the series with the largest *S*-alkyl group, can serve as a substrate for dihydrofolate reductase (DHFR). As shown in Figure 1, the bovine liver enzyme can reduce racemic **11** as readily as folic acid, but only to a maximum extent of 50% conversion, due to the specificity of DHFR for the L-configuration. Since sulfoximines are chiral, this result shows that the enzyme does not discriminate between the (*S*) and (*R*) isomers of **11**, present in equal amounts.

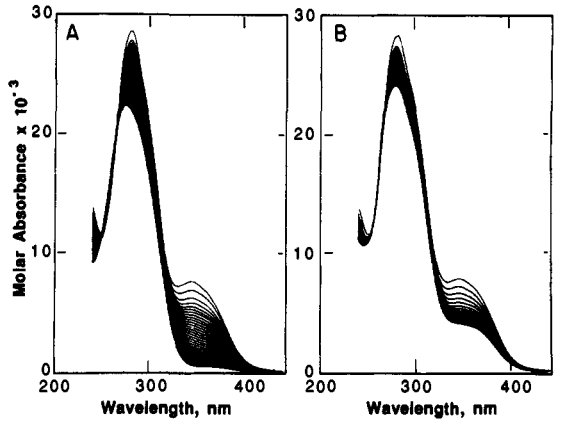

Figure 1. Reduction of 50 μM folate (A) and **11** (B) by 0.2 unit of bovine liver DHFR. NADPH (0.1 mM) was maintained in the reduced form by glucose 6-phosphate (G-6-P, 2 mM) and G-6-P dehydrogenase (Sigma, 10 units). The reduction of folate and **11** at pH 6.5 and 25 °C was monitored by repetitive (5-min) scanning. Pteroyl compounds were omitted from the reference cuvettes.

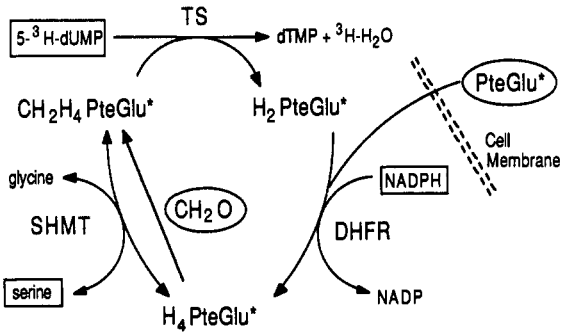

Figure 2. Outline of the TS cycle and the permeabilized cell assay. PteGlu* is pteroylglutamate (folate) or a γ-substituted analogue (**8–12**); H_2PteGlu*, 7,8-dihydro-PteGlu*; H_4PteGlu*, 5,6,7,8-tetrahydro-PteGlu*; CH_2H_4PteGlu*, 5,10-methylene-H_4PteGlu*. In the experimental system, the TS cycle is composed of TS, DHFR, and serine hydroxymethyl transferase (SHMT) and their respective substrates, H_4-pteroyl cofactors, H_2PteGlu* and products, with the exception of those *encircled*. The complete cycle is initiated by adding those in a *box* and PteGlu* to permeabilized cells (see Experimental Section). In the absence of serine, CH_2O can form CH_2H_4PteGlu* nonenzymatically, allowing the measurement of TS in the absence of the SHMT reaction. Activity of the TS cycle is measured by the amount of cofactor-dependent release of tritium into water from the substrate, [5-^{3}H]dUMP, catalyzed by TS (see Table I).

It was of interest to test for cofactor activity of the reduced derivatives as tetrahydropteroylglutamate analogues. In the thymidylate synthase (TS) cycle (Figure 2) of permeabilized L1210 murine leukemia cells, all analogues served as cofactors for the two sequential reactions catalyzed by serine hydroxymethyl transferase (SHMT) and TS. The results are summarized in Table I. In these experiments the analogues were used at 2 times the concentration of folate (pteroyl-L-glutamate) because only the L-forms of the DL-mixtures are reduced by DHFR (see above). The data indicate that in the presence or absence of extracellular bovine liver DHFR all derivatives were

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Table I. Cofactor Activities in the Thymidylate Synthase Cycle of Permeabilized L1210 Cells

		tritium release,[a] %			
		+bovine DHFR[b]		no bovine DHFR	
compd	concn, mM	serine	CH_2O	serine	CH_2O
8	0.4	9.5	8.3	3.9	5.0
9	0.4	8.3	6.8	4.2	4.9
10	0.4	9.2	7.3	4.2	4.3
11	0.4	7.0	5.8	4.0	4.4
12	0.4	7.8	6.7	3.6	4.6
PteGlu	0.2	24.1	21.8	19.1	20.6

[a] Release of tritium from 1 μM [5-^{3}H]dUMP into water expressed as percent of total radioactivity, corrected by the background as described in the Experimental Section. Data represent averages of two experiments run in duplicates. [b] For each tube, 0.016 unit of enzyme was added to 0.15 mL of cell suspension.

Table II. Inhibition of the Growth of L1210 Leukemia Cells in Culture[a]

compd	IC_{50},[b] mM	% inhibn at 0.5 mM
8	0.19	76.4
9	0.22	80.2
10	0.30	75.2
11	0.27	75.2
12	0.39	78.6
PteGlu	>10.0[c]	*d*

[a] Values represent averages of the results of two experiments run in duplicates. [b] Concentration required for 50% growth inhibition. [c] 33.6% inhibition. [d] No significant inhibition.

active as cofactors of SHMT and TS as analogues of tetrahydrofolate and 5,10-methylenetetrahydrofolate, respectively, but were only about 20–40% as effective as folic acid (PteGlu) in supporting the TS cycle. When the potent TS inhibitor CB3717[28] (10 μM) was added, all reactions were inhibited about 99% (data not shown), indicating that the release of tritium in the presence of folate or its analogues was due to their respective cofactor activities. As expected, inhibition of SHMT (in the absence of CH_2O) or DHFR could also interrupt the cycle (see Figure 1).

The IC_{50} values (Table II) of the inhibition of the growth of L1210 murine leukemia cells in culture by the analogues were similar [$(2.7 \pm 0.78) \times 10^{-4}$ M], reflecting a low degree, but significant cytotoxicity, which is of interest for nonpolyglutamylatable folic acid derivatives with cofactor activities. The parent folic acid (PteGlu) was at least 2 orders of magnitude less potent as a cell growth inhibitor than the analogues. As reported previously,[10] none of the folate analogues (or their reduced tetrahydro derivatives) showed any substrate or inhibitory activity using partially purified mammalian FPGS. Thus, interaction with FPGS cannot account for the growth inhibition observed. It is not known which of the isomers of the reduced or unreduced forms are inhibitory and how easily these compounds can penetrate the cell membrane. Since they are presented to the cell as unreduced folate analogues, they must be poor substrates for the reduced folate/methotrexate carrier transport system, but may bind to the folate receptor. It is expected that once internalized, the L-isomers of the sulfoximine derivatives (8–11) are reduced by DHFR to *l*, L, *S* and *l*, L, *R* analogues of tetrahydrofolate (see Figure 1) and may interfere as cofactor analogues with one-carbon metabolism. Alternatively, the unreduced D, *S* and D, *R* isomers may inhibit cell growth. If only one isomer of each sulfoximine analogue is responsible for the cytotoxity, the associated potencies should be higher than those observed. Since the IC_{50} values of sulfone **12** and the corresponding sulfoximine **8** differ only by a factor of 2 (Table II) and the difference between IC_{50} values for the *S*-methyl (**8**) and *S*-butyl (**11**) sulfoximines is insignificant, it may be concluded that the lack of the γ-COOH group is more important for growth inhibitory activity than the nature of the γ-substituent. Further work is required to determine the site(s) of action and the mechanism of cytotoxicity of the various derivatives.

Experimental Section

Melting points were determined in open-end capillary tubes on a Mel-Temp apparatus and are uncorrected. Infrared spectra were run on a Perkin-Elmer Model 197 spectrophotometer or a Nicolet Model 7199 Fourier Transform Interferometer. ^{1}H NMR spectra were obtained on Varian T-60A or Jeol FX-270 spectrometers. Chemical shifts are reported in ppm downfield from Me_4Si (DMSO-d_6) or DSS (D_2O). Splitting patterns are abbreviated as: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), or br (broad). UV spectra were determined on a Cary Model 118C spectrophotometer equipped with a repetitive scanning accessory. Fast atom bombardment mass spectra (FAB/MS) were obtained on a Kratos MS80 instrument equipped with a DS55 data system. Samples were analyzed in the negative ion mode in a glycerol matrix. Radioactivity was quantitated in a Packard liquid scintillation counter with a 40–45% counting efficiency for tritium. TLC was performed on Merck F_{254} cellulose plates (0.1 mm thickness) containing a fluorescent indicator. The plates were developed with 1-BuOH–HOAc–H_2O (system A) or 3% NH_4Cl (system B). Spots were visualized with ninhydrin spray reagent (2% solution in acetone) or under UV light (254 or 360 nm). Elemental analyses were obtained from Atlantic Microlabs, Inc., Atlanta, GA. Ion-exchange chromatography was performed at 4 °C using Whatman DE53 DEAE-cellulose. All folate analogues were protected from light whenever possible and were stored at −10 °C under N_2. The solvents DMF and DMSO were distilled from BaO and were stored over 4A molecular sieves. Isobutyl chloroformate was fractionally distilled into a receiver containing $CaCO_3$ prior to use. For the coupling reactions, Et_3N was distilled sequentially from phenyl isocyanate and KOH and was then stored over KOH pellets.

Materials. DL-Buthionine (*SR*)-sulfoximine was purchased from Chemical Dynamics, South Plainfield, NJ. DL-Methionine sulfone and DL-methionine (*SR*)-sulfoximine, bovine liver dihydrofolate reductase (8 units/mg), *dl*-L-tetrahydrofolate, and NADPH were products of the Sigma Chemical Co., St. Louis, MO. DL-Ethionine (*SR*)-sulfoximine, DL-prothionine, and DL-prothionine (*SR*)-sulfoximine were prepared according to published procedures.[11,12] N^{10}-(Trifluoroacetyl)pteroic acid (**7**) was synthesized by trifluoroacetylation of pteroic acid.[17] Diethyl phosphorocyanidate (DEPC) was prepared as described[25] from triethyl phosphate and cyanogen bromide. Carboxypeptidase G was obtained from the New England Enzyme Center, Boston, MA.

Methyl DL-2-Amino-4-[*S*-methyl-(*SR*)-sulfonimidoyl]butanoate Dihydrochloride (1). To a suspension of DL-methionine (*SR*)-sulfoximine (3.6 g, 0.02 mol) in 300 mL 2,2-dimethoxypropane was added 25 mL of concentrated HCl. To aid in dissolution, the reaction mixture was supplemented with 60 mL MeOH and was then stirred at room temperature for 48 h. The resulting dark solution was concentrated to a brown oil under reduced pressure. This residue was dissolved in 100 mL of H_2O and was then extracted with CH_2Cl_2 (3 × 100 mL) and ether (1 × 100 mL). Lyophilization of the aqueous layer provided 4.26 g (80.0%) of **1** as a white, highly hygroscopic glass: TLC (system A) R_f 0.47; ^{1}H NMR (D_2O) δ 2.63 (m, 2 H, CH_2), 3.73 (s, 3 H, *S*-CH_3), 3.92 (s, 3 H, CO_2CH_3), 4.20 (m, 2 H, *S*-CH_2), 4.40 (t, 1 H, CH).

Methyl DL-2-Amino-4-[*S*-ethyl-(*SR*)-sulfonimidoyl]butanoate Dihydrochloride (2). The *S*-ethyl analogue was prepared in a fashion similar to that for **1** using DL-ethionine (*SR*)-sulfoximine in place of DL-methionine (*SR*)-sulfoximine. After lyophilization, 2.62 g (72.2%) of product was obtained as a white,

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hygroscopic glass: TLC (system A) R_f 0.53; ^{1}H NMR (D_2O) δ 1.55 (t, 3 H, *S*-ethyl CH_3), 2.60 (m, 2 H, CH_2), 3.83 (s, 3 H, CO_2CH_3), 3.97 (m, 4 H, CH_2SCH_2), 4.33 (t, 1 H, CH).

Methyl DL-2-Amino-4-[*S*-1-propyl-(*SR*)-sulfonimidoyl]-butanoate Dihydrochloride (3). This compound was prepared as described above using DL-prothionine (*SR*)-sulfoximine (no methanol was added). The product (3.05 g, 86.1%) was obtained as a colorless, hygroscopic glass: TLC (system A) R_f 0.54; ^{1}H NMR (D_2O) δ 1.17 (t, 3 H, *S*-propyl CH_3), 2.03 (m, 2 H, *S*-propyl CH_2), 2.67 (m, 2 H, CH_2), 3.90 (s, 3 H, CO_2CH_3), 3.96 (m, 4 H, CH_2SCH_2), 4.40 (t, 1 H, CH).

Methyl DL-2-Amino-4-[*S*-1-butyl-(*SR*)-sulfonimidoyl]butanoate Dihydrochloride (4). The 1-butyl analogue was prepared from DL-buthionine (*SR*)-sulfoximine in a manner similar to that for the 1-propyl compound. The product (1.42 g, 68.1%) was obtained as an off-white, hygroscopic glass: TLC (system A) R_f 0.73; ^{1}H NMR (D_2O) δ 0.97 (distorted t, 3 H, *S*-butyl CH_3), 1.77 (m, 4 H, *S*-butyl CH_2CH_2), 2.63 (m, 2 H, CH_2), 3.92 (s, 3 H, CO_2CH_3), 3.96 (m, 4 H, CH_2SCH_2), 4.43 (t, 1 H, CH).

Methyl DL-2-amino-4-(methylsulfonyl)butanoate hydrochloride (5) was prepared as described above from DL-methionine sulfone and 2,2-dimethoxypropane. Recrystallization of the crude product from MeOH–THF afforded 1.92 g (69.2%) of **5** as a white crystalline solid: mp 171–174 °C (lit.[15] mp 164 °C); TLC (system A) R_f 0.46; ^{1}H NMR (D_2O) δ 2.57 (m, 2 H, CH_2), 3.17 (s, 3 H, *S*-CH_3), 3.53 (distorted t, 2 H, *S*-CH_2), 3.87 (s, 3 H, CO_2CH_3), 4.40 (t, 1 H, CH). Anal. ($C_6H_{14}ClNO_4S$) C, H, Cl, N, S.

4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoic Acid (6, Pteroic Acid). Folic acid (dihydrate, 6.0 g, 12.5 mmol) was dissolved in 500 mL of 0.1 M Tris buffer with vigorous stirring at room temperature. To the deep orange solution were added carboxypeptidase G (50 units) and $ZnCl_2$ (10 mg, 0.07 mmol). The pH was carefully adjusted to 7.2 with concentrated HCl and the reaction was then stirred at room temperature. After 48 h the mixture was diluted with 500 mL of H_2O and acidified (pH 2.5) with concentrated HCl. The crude product was collected by centrifugation (4000*g*) and washed with H_2O (3 × 1000 mL). TLC (system B) indicated that some folic acid still remained. Therefore, the crude product was dissolved in Tris buffer and reincubated with carboxypeptidase G as described above. After an additional 72 h the reaction was complete, and the product was isolated by acidification and centrifugation. The product was dried in vacuo (40 °C/P_2O_5) to provide 3.95 g (99.7%) of **6** as a pale yellow powder: mp >300 °C; TLC (system B) R_f 0.52; ^{1}H NMR (0.1 M NaOD) δ 4.7 (s, 2 H, C_9-H), 6.8 (d, 2 H, phenyl), 7.7 (d, 2 H, phenyl), 8.55 (s, 1 H, C_7-H). Anal. ($C_{14}H_{12}N_6O_3 \cdot 0.5H_2O$) C, H, N.

***N*-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-DL-2-amino-4-[*S*-1-butyl-(*SR*)-sulfonimidoyl]butanoic Acid (11, Pteroyl-*S*-1-butylhomocysteine Sulfoximine). Method A. Mixed Anhydride Method.** To a solution of **7** (446 mg, 1 mmol) and Et_3N (0.3 g, 3 mmol) in dry DMSO (8 mL) was added *i*-BuOCOCl (0.41 g, 3 mmol). The solution was stirred at room temperature for 45 min and then Et_3N (0.8 g, 8 mmol) followed by **4** (1.25 g, 4 mmol) was added. The mixture was stirred at 30–35 °C for 24 h and was then poured into 100 mL of 0.1 M aqueous piperidine. After stirring at room temperature for 1 h, the dark orange solution was acidified (pH 3) with concentrated HCl. The resulting gelatinous brown material was collected by centrifugation (4000*g*) and was washed with H_2O. The gel was then dissolved in 200 mL of 0.1 M triethylammonium bicarbonate (TEAB) buffer (pH 8) with stirring at 4 °C and was subsequently applied to a 2.5 × 26 cm DE53 (HCO_3^-) column. The column was washed with 0.1 M TEAB buffer and then eluted with a 0.1 to 0.43 M linear gradient of the same buffer until the desired material (monitored by UV at 254 nm) was eluted. The appropriate fractions (as determined by TLC) were combined and evaporated to dryness in vacuo to yield a yellow residue. This was dissolved in 50 mL of H_2O and the pH was adjusted to 3 with 10% HCl. Centrifugation (3000*g*) followed by lyophilization provided 64.4 mg (11.5%) of **11** as a yellow-orange powder: mp >300 °C; TLC (system B) R_f 0.12; ^{1}H NMR (DMSO-d_6) δ 0.86 (distorted t, 3 H, *S*-butyl CH_3), 1.37 (m, 2 H, *S*-butyl CH_2), 1.58 (m, 2 H, *S*-butyl CH_2), 2.22 (m, 2 H, CH_2), 2.98 (m, 4 H, CH_2SCH_2), 4.48 (br s, 3 H, overlap of C_9-H and CH), 6.65 (d, 2 H, $C_{3',5'}$-H), 7.02 (m, NH_2), 7.66 (pseudo t, 2 H, $C_{2',6'}$-H), 8.36 (m, CONH), 8.65 (s, 1 H, C_7-H); ^{1}H NMR (DMSO-d_6 + DCl) δ 3.96 (m, 4 H, CH_2SCH_2), peaks at 7.02 and 8.36 absent, all other peaks unchanged; FAB/MS (M – H)$^-$ theor. 515.18, found 515.19; (M – 2H + Na^+)$^-$ theor. 537.16, found 537.17. Anal. ($C_{22}H_{28}N_8O_5S \cdot 2.5H_2O$) C, H, N, S.

Method B. Carbodiimide Method. To a solution of **7** (485.0 mg, 1 mmol) in dry DMF (20 mL) at 0 °C was added 1-hydroxybenzotriazole hydrate (154 mg, 1 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (192 mg, 1 mmol) and 4-(dimethylamino)pyridine (123 mg, 1 mmol). The reaction solution was stirred at 0 °C for 60 min and then at room temperature for 60 min. To this was added a solution of **4** (311 mg, 1.0 mmol) in dry DMF (7 mL), followed immediately by Et_3N (0.1 g, 2 mmol). After being stirred at room temperature for 45 h, the reaction mixture was poured into 100 mL of 0.1 M aqueous piperidine and was then stirred at room temperature for 1 h. The crude product was purified by ion-exchange chromatography as described above (method A). After drying (60 °C/P_2O_5), **11** was obtained as 49.6 mg (9.3%) of a yellow solid: mp >180 °C slow dec; TLC (system B) R_f 0.11. Anal. ($C_{22}H_{28}N_8O_5S \cdot H_2O$) C, H, N, S.

***N*-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-DL-2-amino-4-[*S*-methyl-(*SR*)-sulfonimidoyl]butanoic acid (8, pteroyl-*S*-methylhomocysteine sulfoximine)** was prepared from **7** and **1** essentially as described under method A. After trituration with EtOH and drying in vacuo (60 °C/P_2O_5), 204 mg (17.9%) of **8** was obtained as a yellow powder: mp >180 °C slow dec; TLC (system B) R_f 0.11; ^{1}H NMR (DMSO-d_6) δ 1.18 (t, CH_3 of solvated ethanol), 2.24 (m, 2 H, CH_2), 2.90 (s, 3 H, *S*-CH_3), 3.05 (s, 2 H, *S*-CH_2), 4.49 (br s, 3 H, overlap of C_9-H and CH), 6.66 (d, 2 H, $C_{3',5'}$-H), 6.97 (br s, NH_2), 7.67 (distorted d, 2 H, $C_{2',6'}$-H), 8.29 (distorted d, CONH), 8.65 (s, 1 H, C_7-H); ^{1}H NMR (DMSO-d_6 + DCl) δ 2.37 (m, 2 H, CH_2), 3.77 (s, 3 H, *S*-CH_3), 4.01 (m, 2 H, *S*-CH_2), 4.53 (m, 1 H, CH), 4.77 (s, 2 H, C_9-H), 6.84 (distorted d, 2 H, $C_{3',5'}$-H), 7.72 (dd, 2 H, $C_{2',6'}$-H), 8.84 (s, 1 H, C_7-H); FAB/MS (M – H)$^-$ theor. 473.14, found 473.20. Anal. ($C_{19}H_{22}N_8O_5S \cdot 0.4CH_3CH_2OH \cdot 0.8H_2O$) C, H, N, S.

***N*-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-DL-2-amino-4-[*S*-ethyl-(*SR*)-sulfonimidoyl]butanoic acid (9, pteroyl-*S*-ethylhomocysteine sulfoximine)** was prepared by mixed anhydride-mediated coupling (method A) of **7** and **2**. The product **9** was obtained as 81.8 mg (7.7%) of a yellow solid following trituration with EtOH and drying in vacuo for 24 h: mp >200 °C; TLC (system B) R_f 0.15; ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3 H, *S*-ethyl CH_3), 2.22 (m, 2 H, CH_2), 2.96 (m, 4 H, CH_2SCH_2), 4.48 (br s, 3 H, overlap of C_9-H and CH), 6.66 (d, 2 H, $C_{3',5'}$-H), 6.95 (br s, NH_2), 7.67 (d, 2 H, $C_{2',6'}$-H), 8.33 (m, CONH), 8.65 (s, 1 H, C_7-H); ^{1}H NMR (DMSO-d_6 + DCl) δ 1.38 (distorted t, 3 H, *S*-ethyl CH_3), 2.35 (m, 2 H, CH_2), 3.90 (m, 4 H, CH_2SCH_2), 4.60 (s partly obscured by HDO, overlap of C_9-H and CH), 6.69 (d, 2 H, $C_{3',5'}$-H), 7.70 (d, 2 H, $C_{2',6'}$-H), 8.78 (s, 1 H, C_7-H); FAB/MS (M – H)$^-$ theor. 487.15, found 487.24. Anal. ($C_{20}H_{24}N_8O_5S \cdot 0.5CH_3CH_2OH \cdot H_2O$) C, H, N, S.

***N*-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-DL-2-amino-4-[*S*-1-propyl-(*SR*)-sulfonimidoyl]butanoic acid (10, pteroyl-*S*-1-propylhomocysteine sulfoximine)** was prepared from **7** and **3** via method A. Trituration with EtOH followed by drying in vacuo (60 °C/P_2O_5) provided 233 mg (18.9%) of **10** as a yellow-orange powder: mp >180 °C; TLC (system B) R_f 0.17; ^{1}H NMR (DMSO-d_6) δ 0.95 (distorted t, 3 H, *S*-propyl CH_3), 1.18 (t, CH_3 of solvated ethanol), 1.80 (m, 2 H, *S*-propyl CH_2), 2.30 (m, 2 H, CH_2), 3.20 (m, partly obscured by HDO peak, CH_2SCH_2), 4.49 (br s, 3 H, C_9-H and CH), 6.65 (d, 2 H, $C_{3',5'}$-H), 6.91 (br s, NH_2), 7.63 (dd, 2 H, $C_{2',6'}$-H), 8.29 (m, CONH), 8.65 (s, 1 H, C_7-H); ^{1}H NMR (DMSO-d_6 + DCl) δ 1.07 (m, 3 H, *S*-propyl CH_3), 1.80 (m, 2 H, *S*-propyl CH_2), 2.40 (m, 2 H, CH_2), 3.98 (m, 4 H, CH_2SCH_2), 4.52 (m, 1 H, CH), 4.68 (s, 2 H, C_9-H), 6.83 (d, 2 H, $C_{3',5'}$-H), 7.76 (dd, 2 H, $C_{2',6'}$-H), 8.86 (s, 1 H, C_7-H); FAB/MS (M – 2H + glycerol)$^-$ theor. 592.21, found 592.22. Anal. ($C_{21}H_{26}N_8O_5S \cdot 0.2CH_3CH_2OH \cdot 2H_2O$) C, N, S; H: calcd, 5.75; found, 5.30.

***N*-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]-DL-2-amino-4-(methylsulfonyl)butanoic acid (12, pteroyl-*S*-methylhomocysteine sulfone)** was obtained as a yellow solid from **7** and **5** in 29.7% yield (138.1 mg) using method

A: mp >180 °C slow dec; TLC (system B) R_f 0.12; ^{1}H NMR (DMSO-d_6) δ 2.20 (m, 2 H, CH_2), 2.99 (s, 3 H, S-CH_3), 3.18 (m, 2 H, S-CH_2), 4.49 (br s, 3 H, C_9-H and CH), 6.66 (d, 2 H, $C_{3',5'}$-H), 7.05 (m, NH_2), 7.66 (d, 2 H, $C_{2',6'}$-H), 8.28 (distorted d, CONH), 8.65 (s, 1 H, C_7-H); ^{1}H NMR (DMSO-d_6 + DCl) δ 2.24 (m, 2 H, CH_2), 3.00 (s, 3 H, S-CH_3), 3.22 (m, 2 H, S-CH_2), 4.58 (m, 1 H, CH), 4.80 (s, 2 H, C_9-H), 6.64 (d, 2 H, $C_{3',5'}$-H), 7.56 (d, 2 H, $C_{2',6'}$-H), 8.84 (s, 1 H, C_7-H). Anal. ($C_{19}H_{21}N_7O_6S\cdot1.1H_2O$) C, H, N, S.

Determination of Cofactor Activity in Permeabilized Cells. Murine leukemia L1210 cells were transplanted, maintained, and harvested as described.[29] TS activity was measured in suspensions of permeabilized cells[30] [(2-5) × 10^7 cells/mL]. Partial permeabilization[31] using dextran sulfate (500,000 MW, Pharmacia) was carried out as described.[32] Cells were incubated with folate or analogs 8-12 at the indicated concentrations at 37 °C for 15 min in the presence of 0.2 mM NADPH, 1 mM serine (or 7.5 mM CH_2O), 200 mM 2-mercaptoethanol, 40 mM $MgCl_2$, 0.6 mM EDTA, 100 mM NaF and 80 mM Tris-acetate buffer, pH 7.4 [5-^{3}H]dUMP (1 μM) was added, and after an additional 30 min incubation, the reaction was terminated and the tritium released into water was measured as described.[29,32]

Inhibition of Tumor Cell Growth. Duplicate cultures of L1210 murine leukemia cells were exposed to increasing concentrations of test compounds in comparison with folic acid (PteGlu). After 48 h incubation at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, cell numbers were counted and concentrations corresponding to 50% inhibition of control growth (IC_{50} values) were determined graphically. Viability was ≥90% as determined by dye exclusion.

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Supplementary Material Available: Complete UV and IR data for compounds 8-12 and three tables of NMR chemical shift data for the free amino acids, amino acid esters 1-5, and folic acid analogues 8-12 before and after acidification of samples (5 pages). Ordering information is given on any current masthead page.

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Cholesterol Lowering Bile Acid Binding Agents: Novel Lipophilic Polyamines

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A series of novel lipophilic polyamines was synthesized by the sodium cyanoborohydride-mediated reductive amination of various ketones and aldehydes with the polyamine tris(2-aminoethyl)amine. Two of these compounds, *N,N*-bis[2-(cyclododecylamino)ethyl]-*N'*-benzyl-1,2-ethanediamine trihydrochloride (**36**·3HCl) and *N,N*-bis[2-(cyclododecylmethylamino)ethyl]-*N',N'*-dimethyl-1,2-ethanediamine (**23**), are 29 and 24 times more potent than colestipol hydrochloride, respectively, for lowering animal serum cholesterol levels.

It is estimated that one-third of all deaths in industrial societies result from coronary artery diseases. The most compelling evidence for the positive relationship between high cholesterol levels and the incidence of coronary artery diseases comes from the results of the Lipid Research Clinics Coronary Primary Prevention Trial.[1] Due mainly to this evidence, the nonabsorbable, bile acid binding resins Colestid granules and Questran resin are recommended as first line therapy when patients cannot control their cholesterol levels through diet.[2] Colestid in combination with niacin has been reported to reverse the atherosclerotic process.[3a,b] These resins bind to bile acids and cause a 3-10-fold increase in fecal bile acid excretion.[4] This interruption in enterohepatic circulation causes increases in bile acid synthesis, HMG-CoA reductase activity, and hepatic low density lipoprotein (LDL) receptor-mediated uptake of LDL. It is the latter response that effectively lowers serum cholesterol levels.[5] The search for more

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