tion, and the mixture was refluxed 4 hr. The usual work-up gave 0.04 g of **3b**.

Circular Dichroism Measurements. (−)-3a·HCl. The CD measurements were obtained at a concentration of 9.21×10^{-3} *M* at 25°: $[\theta]_{360}$ ±0, $[\theta]_{349}$ −124, $[\theta]_{332}$ −157, $[\theta]_{318}$ −122, $[\theta]_{316}$ −94.

(+)-3a·HCl. The CD measurements were obtained at a concentration of 9.44×10^{-3} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{349}$ +132, $[\theta]_{332}$ +150, $[\theta]_{317}$ +115, $[\theta]_{314}$ +104.

(+)-3b·HCl. The CD measurements were obtained at a concentration of 8.96×10^{-3} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{350}$ +39, $[\theta]_{334}$ +41, $[\theta]_{320}$ +26.

(−)-3b·HCl. The CD measurements were obtained at a concentration of 9.04×10^{-3} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{350}$ −41, $[\theta]_{334}$ −40, $[\theta]_{320}$ −19.

(−)-4a·HCl. The CD measurements were obtained at a concentration of 1.84×10^{-2} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{321}$ +293, $[\theta]_{313}$ −1059, $[\theta]_{299}$ −1631.

(+)-4a·HCl. The CD measurements were obtained at a concentration of 1.80×10^{-2} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{321}$ −292, $[\theta]_{313}$ +1152, $[\theta]_{299}$ +1753.

(+)-4b·HCl. The CD measurements were obtained at a concentration of 1.59×10^{-2} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{319}$ −897, $[\theta]_{308}$ −120, $[\theta]_{297}$ +561.

(−)-4b·HCl. The CD measurements were obtained at a concentration of 1.59×10^{-2} *M* in MeOH at 25°: $[\theta]_{360}$ ±0, $[\theta]_{319}$ +929, $[\theta]_{308}$ +125, $[\theta]_{197}$ −498.

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Folic Acid Analogs. Modifications in the Benzene-Ring Region. 4. 3′-Ethyl- and 3′-Isopropylfolic Acids†

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3′-Ethylfolic acid (**12a**) and 3′-isopropylfolic acid (**12b**) were synthesized as part of a program to obtain folic acid analogs, the reduced forms of which may have an altered ability to function as one-carbon transfer agents. The condensation of 2-acetamido-6-formylpteridin-4(3*H*)-one (**10**) with diethyl *N*-(4-amino-3-ethylbenzoyl)-L-glutamate (**9a**) and with diethyl *N*-(4-amino-3-isopropylbenzoyl)-L-glutamate (**9b**) afforded anils **11a** and **11b** which were reduced with sodium borohydride. Anaerobic alkaline hydrolysis of the reduction products gave the desired folic acid analogs **12a** and **12b**. Analogs **12a** and **12b** were inactive when tested against leukemia L1210 in mice and were not cytotoxic to KB cells in culture. Although **12a** and **12b** were noninhibitory toward pigeon liver dihydrofolate reductase, the dihydrofolate reductase enzyme was apparently stimulated by **12b** at higher concentrations. When tested *vs. Streptococcus faecium*, **12a** was an effective inhibitor while **12b** was growth-supporting. The differences in the behavior of **12a** and **12b** in the dihydrofolate reductase and *S. faecium* systems were rationalized as possibly being attributable to a combination of relative steric requirements and hydrophobic bonding.

Some of the folic acid analogs previously synthesized in this laboratory have been designed so that the electron density at N^{10} is decreased[1,2] or increased[3] relative to folic acid, and a mechanistic interpretation of the means by which these changes may potentially affect the participation of the reduced forms of the analogs in folate metabolism has been postulated.[1] We now wish to report the synthesis of *N*-[4-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-3-ethylbenzoyl]-L-glutamic acid (3′-ethylfolic acid, **12a**) and *N*-[4-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-3-isopropylbenzoyl]-L-glutamic acid (3′-isopropylfolic acid, **12b**) in which additional changes are effected relative to folic acid. Analogs **12a** and **12b** have a slightly increased electron density at N^{10} because of the positive *R* effect[4] of the ethyl and isopropyl groups attached to the benzene ring. The alkyl groups may provide steric hindrance to reactions at N^{10} during the formation of one-carbon transfer agents by the reduced forms of the analogs. Also, the relative bulk of the alkyl groups and the increased lipophilic character

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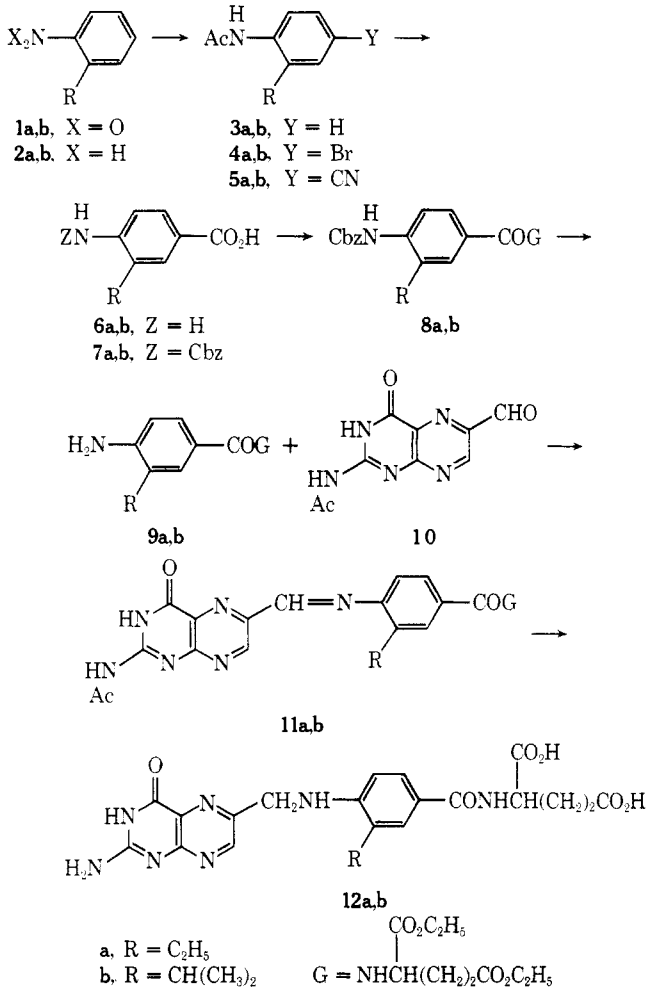
imparted to the analogs by their presence may serve to alter their enzyme-binding abilities. It was anticipated that any or all of these effects might potentially affect folate metabolism at sites other than, or in addition to, the dihydrofolate reductase stage. This work is part of a program devoted to the synthesis of potential antineoplastic agents.

Chemistry. The synthetic route to intermediates **9a** and **9b,** required for the reactions with **10,** is shown in Scheme I. Commercially available 2-nitroethylbenzene (**1a**) was converted to **4a** *via* **2a** and **3a,** according to the

Scheme I



procedures previously described by Altau, *et al.*[5] Fractional distillation of the nitration products of cumene afforded **1b,**‡ which was similarly converted[5] to **4b** *via* **2b** and **3b.** Acid hydrolysis of the nitriles, **5a** and **5b,** obtained from **4a** and **4b** by the method of Friedman and Schechter,[7] yielded 4-amino-3-ethylbenzoic acid (**6a**) and 4-amino-3-isopropylbenzoic acid (**6b**). The reaction of **6a** with carbobenzoxy chloride by the general Schotten–Baumann procedure[8] gave a poor yield (24%) of the protected amino acid, **7a.** The reaction of **6b** with carbobenzoxy chloride in an aqueous dioxane solution containing Na_2CO_3 afforded **7b** in 80% yield. The coupling of **7a** and diethyl glutamate with dicyclohexylcarbodiimide and the hydrogenolysis of **8a** were accomplished by standard techniques without difficulty. The reaction of **7b,** diethyl glutamate, and dicyclohexylcarbodiimide afforded a mixture of **8b** and the *N*-acylurea produced by rearrangement[9] of the product of the reaction of **7b** with dicyclohexylcarbodiimide. Attempted chromatographic isolation of **8b** from this mixture was unsuccessful, and the partially purified **8b** was subjected to hydrogenolysis. The crude product of hydrogenolysis was subjected to column chromatography, and **9b** was obtained as a mixture with *N*-cyclohexyl-(4-amino-3-isopropyl)benzamide. Pmr data indicated that the mixture contained approximately 85 mol % of **9b,** and this material was used without further purification.

The desired analogs, **12a** and **12b,** were obtained from the reactions of **10** with **9a** and **9b** in yields of 28 and 25%, respectively, according to a previously described[3] method of synthesis of folic acid analogs. Purification was accomplished by utilizing DEAE-cellulose ion-exchange chromatography. The anils **11a** and **11b** were not isolated but were assumed to be intermediates in the reaction.

Biological Evaluation. Analogs **12a** and **12b** were administered on a single-dose schedule to mice within 24 hr of implantation ip with 10^5 L1210 cells. Analog **12a,** at doses of 200, 100, and 50 mg/kg, produced no significant increases ($T/C \geq 25\%$) in life span over control animals; the degree of weight loss observed in animals treated with **12a** in this test indicated that 200 mg/kg approached the maximum tolerated dose. Analog **12b** exhibited chronic toxicity at 400 mg/kg; at 200 mg/kg, **12b** was nontoxic and produced no significant increase in life span over control animals. Analog **12a** was also administered daily (qd 1–9) at a dose of 100 mg/kg to mice implanted ip on day 1 with 10^5 L1210 cells, and no significant increase in life span over control animals was observed.

When tested at 1, 10, and 100 μg/ml, compounds **12a** and **12b** exhibited no cytotoxicity *vs.* KB cells in culture.

When analogs **12a** and **12b** were tested[10] at pH 7.4 for inhibition of pigeon liver dihydrofolate reductase at a concentration of 1×10^{-5} *M*, neither analog was inhibitory. At a concentration of 1×10^{-4} *M*, **12a** was again noninhibitory while **12b** caused an apparent doubling in the rate of reduction of dihydrofolic acid. This phenomenon may have been due either to stimulation of the normal enzymatic reaction by **12b** or to the substrate activity of **12b,** since the standard test method[10] which was used indirectly measures the dihydrofolate reductase-mediated conversion of dihydrofolic acid to tetrahydrofolic acid by directly measuring the disappearance (decreasing optical density at 340 nm) of TPNH as it is oxidized to TPN.[11] Since folic acid itself is not a substrate for dihydrofolate reductase at pH 7.4,[11] and since **12b** did not exhibit substrate activity (at a concentration of 6×10^{-6} *M*), stimulation of the normal enzymatic reaction by **12b** becomes the more plausible explanation. The differentiation by dihydrofolate reductase between **12a** and **12b** may be due to a combination of steric requirements and hydrophobic bonding.[11] Even though the possibility exists that **12a** may be accommodated by the enzyme in such a way that hydrophobic bonding may occur, the absence of enzyme inhibition or other effects indicates that the 3′-ethyl function of **12a** is not participating to any detectable extent in such hydrophobic bonding. It is further possible that the enzyme may accommodate **12b** at the normal folic acid binding site or within the neighboring hydrophobic site[11] in such a way as to allow hydrophobic bonding by the 3′-isopropyl function. Yet increased binding of **12b** within this region would presumably lead to enzyme inhibition and not to the stimulation of the enzyme, which was actually observed. It may then be postulated that the 3′-isopropyl function of **12b** is not accommodated at or within the normal binding site because of steric requirements, and that **12b** may, therefore, be participating in hydrophobic bonding elsewhere on the enzyme in such a way as to induce stimulation of the normal enzymatic reaction.

‡ Intermediate **1b** was obtained by fractional distillation of the products of nitration of cumene: bp 110.5–112.5° (7.6 mm); lit.[6] bp 115–117° (12 mm).

Analog **12a** effected 50% inhibition of *Streptococcus faecium* ATCC 8043 at a concentration of 2.6×10^{-8} *M*; in a simultaneous test, methotrexate was 37 times more effective ($I_{50} = 7 \times 10^{-10}$ *M*) than **12a**. In a previous test,[2] methotrexate ($I_{50} = 3.5 \times 10^{-9}$ *M*) had been 43 times more effective *vs. S. faecium* than tetrahydrohomofolate ($I_{50} = 1.5 \times 10^{-7}$ *M*). Thus, **12a** and tetrahydrohomofolate appear to be essentially equivalent as inhibitors of *S. faecium*. 3'-Methylfolic acid, a compound very similar in structure to **12a**, has also been reported[12] previously to be an inhibitor of *S. faecium*. In contrast to 3'-methylfolic acid and **12a**, analog **12b** appeared to be growth-supporting at concentrations above 1×10^{-7} *M* when tested *vs. S. faecium*. The concentration of folic acid present in each of the tests reported herein was 2.5×10^{-10} *M*, the concentration required to give one-half maximum growth under standard conditions.[13] Unlike the dihydrofolate reductase enzyme alone, the *S. faecium* microorganism has the ability to reduce folic acid to dihydro- and tetrahydrofolic acids and may, therefore, have the potential for converting 3'-methylfolic acid, **12a**, and **12b** to the corresponding dihydro and tetrahydro forms. Although this reduction is normally essential for growth-supporting activity, such activity for **12b** may possibly be attributed to the demonstrated ability of **12b** (unreduced form) to stimulate the dihydrofolate reductase enzyme. While it is not possible to state, from the data presently available, in which stage of oxidation (or reduction) 3'-methylfolic acid and **12a** are inhibiting *S. faecium*, it is, however, essential that 3'-methylfolic acid and **12a** undergo reduction by *S. faecium* to their tetrahydro forms if the reason for their inhibition of *S. faecium* is to be attributed to the increased electron density[1] at N^{10}. However, other analogs[3] of folic acid having increased electron density at N^{10} were not found to be inhibitory toward *S. faecium*. While this does not eliminate the possibility that increased electron density at N^{10} contributes to the inhibition of *S. faecium*, it does suggest that the inhibition of *S. faecium* by 3'-methylfolic acid and **12a**, perhaps in one of their reduced forms, may be due to hydrophobic bonding by the 3'-methyl and 3'-ethyl functions.

While folic acid analogs **12a** and **12b** possessed no antileukemic activity and were not cytotoxic, the differences in their behavior toward dihydrofolate reductase and toward *S. faecium* are significant. These differences, as described and rationalized above, are most likely attributable to a combination of relative steric requirements and hydrophobic bonding.

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of theoretical values. Melting points were determined with a Kofler Heizbank (gradiently heated bar) apparatus. Pmr data were determined with a Varian XL-100 spectrometer and are given in parts per million downfield from Me_4Si. Uv spectra were determined with a Cary Model 17 recording spectrometer and wavelengths are given in nanometers. Linde Type 4A molecular sieves were used to dry solvents designated anhydrous. Na_2SO_4 was used to dry solutions in organic solvents, and solvent evaporations were done *in vacuo*. The DEAE-cellulose used was regular capacity Mannex (Schwarz/Mann Bioresearch, Inc.), and it was prepared for column chromatographic use (phosphate form) as described previously.[1] Solutions of NaCl used for elution were maintained at pH 7.0 by the use of 0.005 *M* potassium phosphate buffer and were 0.2 *M* in 2-mercaptoethanol (antioxidant). Washing of **12a** and **12b** by centrifugation was done with dilute HCl (0.004 *N* HCl). Hydrated analytical samples were handled in a dry bag.

Tlc was run on MN 300 cellulose-coated plates (Cell-A) obtained from Analtech, Inc., Bakerflex DEAE-cellulose sheets (Cell-DEAE), and Brinkmann's silica gel H (SGH); SGH was used for column chromatography unless indicated otherwise.

4-Bromo-2-isopropylacetanilide (4b). 2-Isopropylacetanilide [**3b**, 24.0 g, 0.135 mol, mp 73° (lit.[6] 70°), prepared from 2-nitrocumene‡ according to the procedures of Altau, *et al.*[5]] was brominated according to Altau, *et al.*,[5] and the product was recrystallized from C_6H_6: 23.4 g (68% yield); mp 139°. *Anal.* ($C_{11}H_{14}NOBr$) C, H, N.

4-Acetamido-3-ethylbenzonitrile (5a). 4-Bromo-2-ethylacetanilide[5] (**4a**, 24.2 g, 0.1 mol) was heated for 12 hr at reflux in 65 ml of DMF containing 1 ml of H_2O and 10.0 g (0.11 mol) of CuCN.[7] The reaction solution was poured into 150 ml of a warm aqueous solution of NaCN (50 g), and, after vigorous shaking, the mixture was extracted with EtOAc. The EtOAc-soluble residue was recrystallized from EtOH: 13.8 g (73% yield); mp 181°. *Anal.* ($C_{11}H_{12}N_2O$) H, N; C: calcd, 70.18; found, 69.68.

4-Acetamido-3-isopropylbenzonitrile (5b). Compound **4b** (23.0 g, 0.09 mol) was allowed to react with CuCN (8.9 g, 0.099 mol) in DMF (50 ml) as described for **5a**. The EtOAc-soluble residue was recrystallized from C_6H_6: 15.5 g (85% yield); mp 141°. *Anal.* ($C_{12}H_{14}N_2O$) C, H, N.

4-Amino-3-ethylbenzoic Acid (6a). The benzonitrile **5a** (13.0 g, 0.069 mol) was heated at reflux for 6 hr in 100 ml of concentrated HCl. After cooling, the reaction was made alkaline with 50% NaOH and filtered. Acidification (HCl) of the filtrate to pH 4.5 afforded a crystalline solid which was recrystallized from H_2O (charcoal): off-white crystals; 7.2 g (63% yield); mp 140°. *Anal.* ($C_9H_{11}NO_2$) C, H, N.

4-Amino-3-isopropylbenzoic Acid (6b). The benzonitrile **5b** (14.8 g, 0.073 mol) was hydrolyzed and recrystallized as described for **6a**: 10.08 g (77% yield); mp 152°; pmr (CF_3CO_2D) δ 8.39 (d, 1 H, C_2-H), 8.17 (doublet of doublets, 1 H, C_6-H), 7.62 (d, 1 H, C_5-H), 3.24 (septuplet, 1 H, isopropyl CH), 1.44 (d, 6 H, isopropyl CH_3's). *Anal.* ($C_{10}H_{13}NO_2$) C, H, N.

4-Benzyloxycarbonylamino-3-ethylbenzoic Acid (7a). Carbobenzoxy chloride (8.0 g, 0.045 mol) and 3 *N* NaOH (45 ml) were added alternately and in portions[8] to a solution of **6a** (7.0 g, 0.041 mol) in 15 ml of 3 *N* NaOH maintained at 0–5°. The solid that separated during the 6-hr reaction period was isolated by filtration and washed with 2 *N* HCl and with H_2O. The combined filtrates were acidified to pH 4.5, and a second solid was isolated and washed with H_2O. The combined solids were recrystallized from HOAc: white needles; 3.03 g (24% yield); mp 229°. *Anal.* ($C_{17}H_{17}NO_4$) C, H, N.

4-Benzyloxycarbonylamino-3-isopropylbenzoic Acid (7b). Carbobenzoxy chloride (9.75 g, 0.057 mol) was added to a solution of **6b** (10.3 g, 0.057 mol) in 820 ml of dioxane and 570 ml of H_2O containing 6.1 g (0.057 mol) of Na_2CO_3. The reaction mixture was stirred and maintained at 5–10° for 7 hr during which time additional carbobenzoxy chloride was added (9.75 g after 1 hr and 9.75 g more after 3.5 hr). The reaction mixture was acidified to pH 4.5 and, after standing overnight, afforded a red oil (7.6 g) and a clear supernatant solution. Removal of the dioxane by partial evaporation of the decanted supernatant afforded a pink solid (10.4 g, mp 178–180°).

A $CHCl_3$ solution of the red oil was washed with aqueous Na_2CO_3, and acidification of the Na_2CO_3 washings afforded a solid (1.8 g, mp 184°). Evaporation of the $CHCl_3$ layer yielded a liquid which was found to contain the benzyl alcohol ester (*m/e* 403, M^+) of **7b** and carbobenzoxy chloride (*m/e* 170, M^+). The carbobenzoxy chloride was esterified by allowing the liquid to stand in EtOH, and the product of this treatment was hydrolyzed in 760 ml of dioxane–0.2 *N* NaOH (1:1) for 24 hr. Acidification of the hydrolysate and evaporation of the dioxane yielded a solid (3.9 g, mp 184°).

The combined solids (16.1 g) were recrystallized from C_6H_6: 14.4 g (80% yield); mp 185°. *Anal.* ($C_{18}H_{19}NO_4$) C, H, N.

Diethyl *N*-(4-Benzyloxycarbonylamino-3-ethylbenzoyl)-L-glutamate (8a). A solution of **7a** (2.86 g, 9.6 mmol), DCI (1.98 g, 9.6 mmol), and diethyl L-glutamate hydrochloride (2.30 g, 9.6 mmol) in 100 ml of anhydrous pyridine was allowed to react for 3 days. The solid formed was isolated by filtration and washed with $CHCl_3$; more solid was isolated by $CHCl_3$ trituration of the total filtrate residue. The $CHCl_3$ filtrate was washed with 1 *N* HCl, aqueous $NaHCO_3$, and H_2O and dried before evaporation, and the residue was purified by column chromatography [SGH, 100 g; $CHCl_3$–MeOH (97:3)]: 2.30 g (50% yield); mp 122–123°. *Anal.* ($C_{26}H_{32}N_2O_7$) C, H, N.

Diethyl *N*-(4-Amino-3-ethylbenzoyl)-L-glutamate (9a). H_2 was bubbled continuously for 6 hr into a suspension of Pd (from 806 mg of $PdCl_2$) in EtOH containing 2.2 g (4.5 mmol) of **8a**. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was suspended in aqueous $NaHCO_3$ and extract-

ed with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried, and evaporated: crystals; 1.45 g (91% yield); mp 109°; pmr (CF_3CO_2D) δ 8.02 (d, 1 H, C_2-H), 7.96 (doublet of doublets, 1 H, C_6-H), 7.64 (d, 1 H, C_5-H); the remainder of the pmr spectrum was as expected. *Anal.* ($C_{18}H_{26}N_2O_5$) C, H, N.

Diethyl *N*-(4-Amino-3-isopropylbenzoyl)-L-glutamate (9b). A solution of **7b** (14.4 g, 46 mmol), DCI (9.47 g, 46 mmol), and diethyl L-glutamate hydrochloride (11.05 g, 46 mmol) in 600 ml of anhydrous pyridine was allowed to stir for 6 days, at which time tlc [SGH, $CHCl_3$–MeOH (97:3)] indicated that only a trace of **7b** remained unreacted. The reaction mixture was treated as described for **8a**, and mass spectral analysis of the residue obtained by evaporation of the $CHCl_3$ filtrate indicated the presence of the desired **8b** (*m/e* 364, M^+) and 1-(4-benzyloxycarbonylamino-3-isopropylbenzoyl)-1,3-dicyclohexylurea (*m/e* 519, M^+). Attempted purification of **8b** by column chromatography was unsuccessful [900 g of Davison silica gel from Applied Science Laboratories, 140–200 mesh, $CHCl_3$–C_6H_6 (85:15)].

The best column fractions (16.45 g) in 300 ml of EtOH were added to prereduced (H_2) $PdCl_2$ (7.1 g, 40 mmol) in 300 ml of EtOH; H_2 was bubbled continuously for 5 hr into this suspension. Removal of the catalyst by filtration and evaporation of the filtrate afforded a residue which was dissolved in $CHCl_3$ and washed with aqueous $NaHCO_3$ and H_2O. The $CHCl_3$ residue was subjected to column chromatography [SGH, 600 g, $CHCl_3$–MeOH (99:1)], and the best fractions were combined (4.3 g, 26% yield from **7b**). Mass spectral data indicated the presence of **9b** (*m/e* 364, M^+) and *N*-cyclohexyl-(4-amino-3-isopropyl)benzamide (*m/e* 260, M^+): pmr (CF_3CO_2D) δ 8.08 (d, C_2-H), 7.88 (doublet of doublets, C_6-H), 7.62 (d, C_5-H), 5.01 (m, NCH of glutamate), 4.38 (six lines, CH_2O of esters), 3.61 (m, NCH of cyclohexyl), 3.24 (septuplet, CH of isopropyl), 2.58 (m, CH_2CH_2 of glutamate), 1.90 [m, $(CH_2)_5$ of cyclohexyl], 1.28–1.50 (six lines, CH_3 of esters, isopropyl); relative integral areas correspond to a mixture of **9b**–cyclohexylamide of approximately 85:15. This material was used without further purification for the preparation of **12b**.

***N*-[4-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-3-ethylbenzoyl]-L-glutamic Acid (3′-Ethylfolic Acid, 12a).** A solution of **9a** (1.005 g, 2.87 mmol) and **10** (670 mg, 2.87 mmol) in 14 ml of anhydrous DMSO was stirred at room temperature for 4 days; the reaction mixture was added to anhydrous DMF (130 ml) containing 137 mg (3.59 mmol) of $NaBH_4$. After 96 hr, the DMF was evaporated, and the residue was hydrolyzed at room temperature in an N_2 atmosphere in 360 ml of 0.2 *N* NaOH for 16 hr.

Acidification (HCl) to pH 3.5 afforded a yellow solid which was isolated and washed once with dilute HCl by centrifugation; freeze-drying of the wet solid afforded 779 mg of crude **12a**. A solution (final pH 6.8) of this material in 1500 ml of dilute NH_4OH containing 22 ml of 2-mercaptoethanol was applied to a DEAE-cellulose column (30 g, 2.8 × 50 cm, phosphate form), and the column was then washed with 500 ml of 0.2 *M* 2-mercaptoethanol. The column was eluted with a linear gradient of NaCl (0.0–0.7 *M* NaCl, 4000 ml total volume); input of 0.7 *M* NaCl from the reservoir was ended at the halfway point in the gradient, and elution was continued with 0.35 *M* NaCl. All fractions were monitored by uv absorbance, and those fractions collected between 200 and 610 ml of 0.35 *M* NaCl were pooled and acidified (HCl) to pH 3.5. The yellow solid was isolated and washed once with dilute HCl by centrifugation and then redissolved in 500 ml of dilute NaOH containing 5 ml of 2-mercaptoethanol. This solution was acidified (HCl) to pH 3.5 and refrigerated, and the solid was isolated and washed three times with dilute HCl by centrifugation before drying (P_2O_5) for 24 hr at 0.1 mm and room temperature: 378 mg; 28% yield; uv_{max} (0.1 *N* HCl) 245 nm (ϵ 13,900), 298 (21,300); uv_{max} (pH 7) 280 nm (ϵ 27,100), 297 (sh), 346 (7730); uv_{max} (0.1 *N* NaOH) 255 nm (ϵ 25,800), 286 (23,300), 365 (9270); pmr data were as expected. *Anal.* ($C_{21}H_{23}N_7O_6 \cdot 0.25H_2O$) C, H, N.

***N*-[4-[[2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-3-isopropylbenzoyl]-L-glutamic Acid (3′-Isopropylfolic Acid, 12b).** A solution of 698 mg of the column product containing **9b** (89% of **9b** by weight; 621 mg or 1.7 mmol of **9b**) and 466 mg (2 mmol) of **10** in 10 ml of anhydrous DMSO was allowed to stir at room temperature for 3 days; the reaction mixture was added to 90 ml of anhydrous DMF containing 95 mg (2.5 mmol) of $NaBH_4$. The DMF was evaporated after 96 hr, the residue was hydrolyzed in 500 ml of 0.1 *N* NaOH, and crude **12b** (322 mg) was obtained by acidification as described for **12a**. A solution (final pH 6.5) of this material in 500 ml of dilute NH_4OH containing 7.5 ml of 2-mercaptoethanol was applied to a DEAE-cellulose column (20 g, 2.8 × 33 cm, phosphate form), and the column was washed with 500 ml of 0.2 *M* 2-mercaptoethanol before elution with 0.2 *M* NaCl. All fractions were monitored by uv absorbance, and the most intensely uv-absorbing fractions corresponded to a yellow-colored zone on the column. These fractions were pooled, and the product was isolated and reprecipitated as described for **12a**. The product was pulverized before final drying (P_2O_5) for 24 hr at 0.25 mm and room temperature: 210 mg; 25% yield; uv_{max} (0.1 *N* HCl) 246 nm (ϵ 13,900), 299 (21,500); uv_{max} (pH 7) 280 nm (ϵ 26,100), 298 (sh), 346 (6950); uv_{max} (0.1 *N* NaOH) 255 nm (ϵ 25,600), 285 (22,600), 365 (8940); pmr data were as expected. *Anal.* ($C_{22}H_{25}N_7O_6 \cdot H_2O$) C, H, N.

Tlc Analysis of 12a and 12b. It was determined that analogs **12a** and **12b** contained a major uv-absorbing component and a minor uv-fluorescent impurity by tlc analysis on Cell-DEAE developed with a potassium phosphate (0.005 *M*) buffer solution at pH 7.0 containing NaCl (0.5 *M*) and 2-mercaptoethanol (0.2 *M*). The fluorescent impurity in **12a** and **12b** was determined to be 6-formylpterin by tlc comparison with an authentic sample; side-by-side tlc comparisons with standards containing known amounts of 6-formylpterin indicated that the content of this impurity was approximately 1% in **12a** (Cell-A developed with 5% aqueous Na_2HPO_4) and 3% in **12b** (Cell-DEAE developed with the potassium phosphate buffer solution described above).

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