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Folic Acid Analogs. Modifications in the Benzene-Ring Region. 3. Neohomofolic and Neobishomofolic Acids. An Improved Synthesis of Folic Acid and Its Analogs†

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An improved synthesis of folic acid was developed and applied to the synthesis of neohomofolic (**13**) and neobishomofolic (**14**) acids which were desired for testing as potential anticancer agents. The procedure consists of hydrolysis of the products of sodium borohydride reduction of the anils derived from the reaction of 2-acetamido-6-formylpteridin-4(3*H*)-one (**5**) with diethyl *N*-(*p*-aminophenylacetyl)glutamate (**7**) and diethyl *N*-(*p*-aminohydrocinnamoyl)glutamate (**8**). Analogs **13** and **14** were tested on single-dose (day 1 only) and daily (qd 1–9) schedules against leukemia L1210 in mice and were inactive. Analogs **13** and **14** were not cytotoxic to cells in culture and were not inhibitory toward *Streptococcus faecium* ATCC 8043 or pigeon liver dihydrofolate reductase.

Previous efforts in these laboratories to synthesize folic acid analogs[1,2] with an altered ability to function as one-carbon transfer agents have been directed toward molecules in which the electron density at position 10 (N^{10}) is reduced. The manner in which an increase or decrease in the electron density at N^{10} in reduced folic acid analogs may potentially affect their participation in folate metabolism has been discussed previously.[1] For example, an increased electron density at N^{10} may stabilize the one-carbon transfer agents formed and render more difficult the transfer of the one-carbon unit from the tetrahydrofolate type cofactor to the substrate. We now wish to report the synthesis of *N*-[*p*-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-phenylacetyl]glutamic acid (**13**, neohomofolic acid) and *N*-[*p*-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino]hydrocinnamoyl]glutamic acid (**14**, neobishomofolic acid) in which the carbonylglutamate moiety is removed from the benzene ring by one and two methylene units, respectively. Because the nonbonding electrons at N^{10} are effectively insulated from the electron-withdrawing effect of the carbonyl group, these compounds are examples of analogs in which the electron density at N^{10} is increased relative to folic acid. The added methylene units also elongate the analogs and may serve to alter their enzyme-binding abilities. It was anticipated that either or both of these effects might potentially affect folate metabolism at sites other than, or in addition to, the dihydrofolate reductase stage. Additionally, we are herein reporting an improved synthesis of folic acid and its application to the preparation of neohomofolic and neobishomofolic acids. This work is part of a program whose goal is to obtain potentially useful antineoplastic agents.

Chemistry. Neohomofolic acid (**13**) and neobishomofolic acid (**14**) were synthesized by hydrolysis of the products of $NaBH_4$ reduction of the anils **10** and **11** derived from the reactions of the pteridine aldehyde **5**[3] with **7** or **8**. The objective of preliminary efforts in the development of these procedural conditions was the model synthesis of folic acid (**12**), and we are also reporting its preparation *via* anil **9**. The synthetic procedure involved the reaction of the substituted anilines **6–8** with 1 equiv of **5** dissolved at its saturation concentration in DMSO. The anils **9–11** precipitated from this solution in high yields, and $NaBH_4$ in DMF was then used to effect reduction of the anil double bond. A ratio of 1.25 mol of $NaBH_4$ per mole of anil appeared to afford optimum yields in the model synthesis of **12** and minimized the extent of reduction of the pyrazine ring to a negligible level. Anaerobic alkaline hydrolysis simultaneously removed the glutamate ethyl esters and the N^2-acetyl blocking group and afforded the target compounds **12–14**. Purification was accomplished by utilizing DEAE-cellulose ion-exchange chromatography (Scheme I).

This improved synthesis is a modification of a procedure utilized originally by us in the synthesis[1] of 2′-azafolic acid. Subsequently, other workers reported related procedures for the preparation of pteroic acid and similar pteridines. In a synthesis of pteroic acid, Plante[4] utilized dimethyl-

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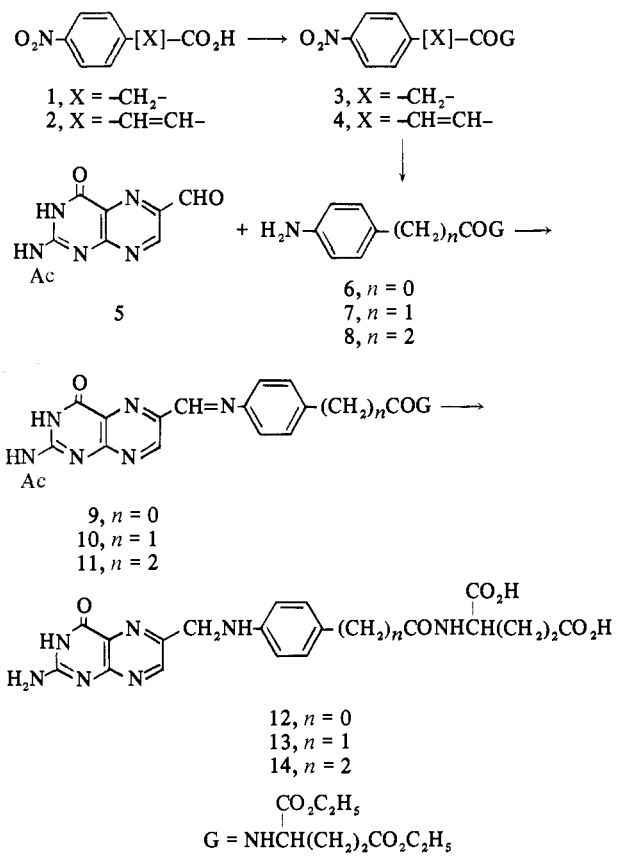
Scheme I



amineborane in acetic acid for reduction of the anil derived from **5** and ethyl *p*-aminobenzoate. Viscontini and Bieri[5] used an excess of $NaBH_4$ in aqueous ethanol for reduction of the anils derived from the reaction of 2-amino-6-formylpteridin-4(3*H*)-one with aniline, *p*-toluidine, and methyl *p*-aminobenzoate. Much earlier, the anil derived from **5** and *N*-(*p*-aminobenzoyl)glutamic acid had been isolated and converted to folic acid[6] of unspecified purity by catalytic hydrogenation and by formic acid and its Et_3N^+ salt; folic acid has also been prepared[3] by the reductive condensation of **5** and *N*-(*p*-aminobenzoyl)glutamic acid in the presence of an excess of *p*-toluenethiol, presumably *via* an anil intermediate. An advantage of the procedure herein reported is that relatively clean products are obtained which already have the glutamate moiety attached, thereby avoiding loss of product during glutamate coupling.

Biological Evaluation. Compounds **7**, **13**, and **14** were administered on a single-dose schedule to mice within 24 hr of implantation ip with 10^5 L1210 cells. Compound **7**, at a dose of 400 mg/kg, produced no evidence of toxicity and no increase in life span. Analogs **13** and **14** were toxic at all doses above 150 and 100 mg/kg, respectively, and produced no significant increase ($T/C \geq 25\%$) in life span at these specified doses. Analogs **13** and **14** were also administered daily (qd 1-9) to mice implanted ip on day 1 with 10^5 L1210 cells; doses of 75 and 50 mg/kg (chosen because of degree of weight loss observed on single-dose schedule) of **13** and **14**, respectively, produced no significant increase in life span over control animals.

When tested at concentrations of 1, 10, and 100 μg/ml, compounds **13** and **14** exhibited no cytotoxicity *vs.* HEp-2 cells and KB cells, respectively, in culture.

Analogs **13** and **14** were tested for inhibition of pigeon liver dihydrofolate reductase; **13** was noninhibitory at 1×10^{-4} *M*, and **14** was approximately 30% inhibitory at 1×10^{-4} *M*. In comparison, methotrexate was 50% inhibitory at 1×10^{-8} *M*.

Analogs **13** and **14** were found to effect <50% inhibition of *Streptococcus faecium* ATCC 8043 at a concentration of 1×10^{-5} *M*. In comparison, methotrexate was 50% inhibitory at 7×10^{-10} *M*.

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 A-60A 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 standard capacity Mannex (Mann Research Laboratories), and it was prepared for column chromatographic use (phosphate form) as described previously.[1] Solutions of NaCl used in elution were maintained at pH 7.0 by the use of 0.005 *M* potassium phosphate buffer and were 0.2 *M* in mercaptoethanol; all column washing solutions also contained mercaptoethanol as an antioxidant.[1] Washing of 12-14 by centrifugation was done with dilute HCl: 3-4 drops of 1 *N* HCl per 50 ml of H_2O. Analytical samples reported as hydrated 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 also used for column chromatography. Solvent systems used were A, BuOH-AcOH-H_2O (5:2:3); B, BuOH-AcOH-H_2O (4:1:5, upper layer); C, 5% aqueous Na_2HPO_4; D, 5% NH_4OH-3% NH_4Cl; E, 0.5 *M* NaCl, 0.2 *M* in mercaptoethanol, in 0.005 *M* potassium phosphate buffer at pH 7.0; F, $CHCl_3$; G, $CHCl_3$-EtOAc (95:5); H, $CHCl_3$-MeOH (98:2); I, $CHCl_3$-MeOH (97:3).

Diethyl *N*-(*p*-Nitrophenylacetyl)glutamate (3). *p*-Nitrophenylacetic acid (1, 9.06 g, 0.05 mol) was dissolved in 200 ml of pyridine and allowed to react with 10.32 g (0.05 mol) of DCI and 11.99 g (0.05 mol) of diethyl glutamate·HCl. After 5 days, the mixture was chilled, and the precipitate was removed by filtration and washed with $CHCl_3$. The filtrate was evaporated, and the residue was triturated with $CHCl_3$. The $CHCl_3$ filtrate was concentrated and applied to a chromatographic column (SGH), and the column was eluted with solvent F. Combination of tlc-homogeneous (SGH, solvent G) fractions afforded **2**: 10.1 g; 55% yield; mp 73-74°; pmr (DMSO-d_6) δ 3.67 (s, 2 H, $ArCH_2$); other pmr data were as expected.[1] *Anal.* ($C_{17}H_{22}N_2O_7$) C, H, N.

Diethyl *N*-(*p*-Nitrocinnamoyl)glutamate (4). *p*-Nitrocinnamic acid (2, 9.65 g, 0.05 mol) was dissolved in 1000 ml of pyridine and allowed to react with 10.32 g (0.05 mol) of DCI and 11.99 g (0.05 mol) of diethyl glutamate·HCl. After 2 days, the mixture was filtered, and the solid obtained was washed with $CHCl_3$. The filtrate was evaporated, and the residue was triturated with $CHCl_3$. More solid was removed, and the filtrate was washed with 1 *N* HCl, aqueous $NaHCO_3$, and H_2O. After drying, the $CHCl_3$ solution was evaporated, and the residue was recrystallized twice from C_6H_6: 10.29 g; 54% yield; mp 121°; pmr ($CDCl_3$) δ 6.64 (d, 1 H, C=CH), 7.70 (d, 1 H, C=CH). *Anal.* ($C_{18}H_{22}N_2O_7$) C, H, N.

Diethyl *N*-(*p*-Aminophenylacetyl)glutamate (7). Compound 3 (5.61 g, 0.015 mol) was hydrogenated at atmospheric pressure over Raney nickel in EtOH. After removal of the catalyst by filtration, evaporation of the filtrate afforded a solid. It was purified by column chromatography (SGH, solvent I): 4.09 g; 80% yield; mp 92°; pmr (DMSO-d_6) δ 3.18 (s, 2 H, $ArCH_2$). *Anal.* ($C_{17}H_{24}N_2O_5$) C, H, N.

Diethyl *N*-(*p*-Aminohydrocinnamoyl)glutamate (8). Compound 4 (5.67 g, 0.015 mol) was hydrogenated as described for 3. The crude solid was purified by column chromatography (SGH, solvent H): 2.4 g; 46% yield; mp 71°; pmr data were as expected. *Anal.* ($C_{18}H_{26}N_2O_5$) C, H, N.

***N*-[*p*-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]glutamic Acid (12).** Aldehyde **5**[3] (885 mg, 3.8 mmol) was dissolved in 18 ml of anhydrous DMSO, and 1.23 g (3.8 mmol) of **6**[7] was added; the flask was closed, and the contents were stirred at room temperature for 3 days. The precipitate was isolated by filtration and washed three times with 10 ml of Me_2CO and twice with Et_2O. The addition of 500 ml of anhydrous Et_2O to the filtrate yielded more yellow solid, and it was isolated and

washed similarly; total solid (9), 1.75 g, 86% crude yield. The anil was obtained analytically from one such reaction: pmr (DMSO-d_6) δ 8.80 (s, 1 H, CH=N), 9.53 (s, 1 H, C_7-H). *Anal.* ($C_{25}H_{27}N_7O_7$) C, H, N.

A total of 2.82 g (5.3 mmol) of anil 9 was dissolved in 262 ml of anhydrous DMF and 250 mg (6.6 mmol) of $NaBH_4$ was added. The contents of the closed flask were stirred at room temperature; after 96 hr, the DMF was distilled off *in vacuo* using a warm water bath. The residue was dissolved in 1300 ml of deaerated 0.1 *N* NaOH and allowed to stir for 24 hr under an N_2 atmosphere. The solution was filtered, acidified (HCl) to pH 3.5, and refrigerated. The solid was isolated and washed twice with dilute HCl by centrifugation; after drying *in vacuo* (P_2O_5), the tan solid weighed 1.35 g.

The solid was dissolved in 1500 ml of dilute NH_4OH, and the solution was made 0.2 *M* in mercaptoethanol. This solution (pH 6.5) was applied to an 85-g DEAE-cellulose column (4.8 × 50 cm), and the column was then washed with 2000 ml of aqueous 0.2 *M* mercaptoethanol. The column was eluted with 2000 ml of 0.2 *M* NaCl and then with 0.3 *M* NaCl until the product had been completely eluted; the uv absorbance of the eluate was continuously monitored. The product appeared as a distinctly yellow-colored band on the column, and the eluted fractions in which this band was contained were more intensely uv-absorbing than any other fractions. Colorless, uv-absorbing impurities were eluted in advance of the product band, and immobile, intensely colored impurities remained at the top of the column. Acidification (HCl) to pH 3.5 of the combined product fractions yielded a solid which was isolated and washed twice with dilute HCl by centrifugation. The wet solid was redissolved in 1200 ml of dilute NH_4OH containing mercaptoethanol, and the solution was acidified to pH 3.5. The yellow solid was isolated and washed four times with dilute HCl by centrifugation; the wet solid was dried (P_2O_5) *in vacuo*, and the product was pulverized before final drying (P_2O_5) for 24 hr at 0.35 mm and room temperature: 969 mg; 48% yield from **5**; uv_{max} (0.1 *N* HCl) 246 nm (ϵ 13,200), 296 (19,400); uv_{max} (pH 7) 281 nm (ϵ 27,400), 346 (7120); uv_{max} (0.1 *N* NaOH) 256 nm (ϵ 24,300), 283 (23,900), 364 (8460); pmr data were as expected. *Anal.* ($C_{19}H_{19}N_7O_6 \cdot H_2O$) C, H, N.

***N*-[*p*-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]phenylacetyl]glutamic Acid (13, Neohomofolic Acid).** Aldehyde **5** (1.53 g, 6.5 mmol) was dissolved in 30 ml of anhydrous DMSO, and 2.2 g (6.5 mmol) of **7** was added; the reaction was conducted and the product (**10**) was isolated as described for **9**: 3.6 g; 100% crude yield. The anil was obtained analytically by recrystallization of a small amount from Me_2CO–Et_2O: pmr (DMSO-d_6) δ 8.77 (s, 1 H, CH=N), 9.50 (s, 1 H, C_7-H); uv_{max} (DMF) 292 nm (ϵ 18,600), 388 (24,300). *Anal.* ($C_{26}H_{29}N_7O_7$) C, H, N.

This anil was dissolved in 328 ml of anhydrous DMF containing 312 mg (8.2 mmol) of $NaBH_4$. The reduction, hydrolysis (in 820 ml of 0.2 *N* NaOH), and isolation were conducted as described for the conversion of **9** to **12**, and 1.53 g of crude **13** was obtained.

The solid was dissolved in 1700 ml of dilute NH_4OH, and the solution was made 0.2 *M* in mercaptoethanol. This solution (final pH 6.6) was applied to a 90-g DEAE-cellulose column (4.8 × 55 cm), and the column was then washed with 2000 ml of aqueous 0.2 *M* mercaptoethanol. The column was eluted with 0.2 *M* NaCl, and the elution pattern and product detection were as described in the purification of **12**. The product was isolated, reprecipitated, and dried as described for **12**. The final drying yielded a yellow solid: 893 mg; 29% yield from **5**; uv_{max} (0.1 *N* HCl) 232 nm (ϵ 15,400), 245 (13,900), 282 (4130), 321 (8260); uv_{max} (pH 7) 242 nm (ϵ 22,700), 272 (17,600), 346 (7100); uv_{max} (0.1 *N* NaOH) 254 nm (ϵ 33,900), 275 (sh), 365 (8730); pmr data were as expected. *Anal.* ($C_{20}H_{21}N_7O_6 \cdot H_2O$) C, H, N.

***N*-[*p*-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]hydrocinnamoyl]glutamic Acid (14, Neobishomofolic Acid).** Aldehyde **5** (1.30 g, 5.5 mmol) was dissolved in 26 ml of anhydrous DMSO, and 1.95 g (5.5 mmol) of **8** was added. The reaction was conducted as described for **9**, but the product (**11**) was not isolated. This anil had been isolated in 88% yield in a previous reaction; washing with Me_2CO afforded an analytical sample: uv_{max} (DMF) 296 nm (ϵ 15,400), 373 (24,400). *Anal.* ($C_{27}H_{31}N_7O_7 \cdot 0.3DMSO$) C, H, N.

The thick suspension of the anil product in DMSO was transferred to a solution of 264 mg (6.9 mmol) of $NaBH_4$ in anhydrous DMF (total reduction solution volume = 277 ml). The reduction, hydrolysis (695 ml of 0.2 *N* NaOH), and isolation were conducted as described for the conversion of **9** to **12** and 2.0 g of crude **14** was obtained.

Crude **14** was dissolved in 1500 ml of dilute NH_4OH made 0.1 *M* in mercaptoethanol (final pH 7.0), and this solution was applied to a 24-g DEAE Sephadex column‡ (chloride form). The column was eluted with a linear gradient of NH_4Cl (0.01–1.0 *M* NH_4Cl, total volume of 2000 ml), then with 1000 ml of 1.0 *M* NH_4Cl, and finally with 500 ml of 2.0 *M* NH_4Cl (all NH_4Cl solutions were 0.1 *M* in mercaptoethanol and were of pH 7.0). The fractions were monitored by uv absorbance; acidification (HCl) of the desired collected fractions yielded an orange solid which was isolated in the usual manner by centrifugation.

This solid (615 mg) was dissolved in 1000 ml of dilute NH_4OH, and the solution was made 0.2 *M* in mercaptoethanol. This solution (final pH 6.7) was applied to a 30-g DEAE-cellulose column (2.8 × 51 cm), and the column was washed with 1000 ml of aqueous 0.2 *M* mercaptoethanol. The column was eluted with a linear gradient of NaCl (0.0–0.7 *M*; total volume, 4000 ml); the eluate was monitored continuously by uv absorbance, and the elution pattern was as described for **12**. The yellow product band was eluted between 0.32 and 0.38 *M* NaCl. The product was isolated, reprecipitated, and dried as described for **12**. The final drying yielded a yellow solid: 380 mg; 14% yield;‡ uv_{max} (0.1 *N* HCl) 232 nm (ϵ 14,600), 246 (13,200), 281 (4100), 321 (8110); uv_{max} (pH 7) 240 nm (ϵ 22,600), 273 (14,100), 346 (6840); uv_{max} (0.1 *N* NaOH) 254 nm (ϵ 32,400), 278 (sh), 364 (8200); pmr data were as expected. *Anal.* ($C_{21}H_{23}N_7O_6 \cdot 0.5H_2O$) C, H, N.

Tlc Analysis of 12, 13, and 14. Analysis by tlc of **12** and **13** on Cell-A (solvents C and D) and Cell-DEAE (solvent E) indicated that each contained a major uv-absorbing component and a minor blue-fluorescent (long uv) impurity. The impurity in each was determined to be 2-amino-6-formylpteridin-4(3*H*)-one by tlc comparison with an authentic sample; side-by-side tlc comparison with standards indicated the level of this impurity to be <1% in **12** (Cell-DEAE, solvent E) and approximately 4% in **13** (Cell-A, solvent C). The level of occurrence of this impurity in **14** was very likely similar to that in **13**, but it had R_f's identical with those of **14** in each of the solvent systems tested: Cell-A (solvents A, B, C, and D) and Cell-DEAE (solvent E). Neobishomofolic acid (**14**) also contained a trace component presumed to be the corresponding neobishomopteroic acid because of its R_f values and its identical green fluorescence on tlc.

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‡The use of a DEAE-Sephadex column did not improve the quality of the crude analog and apparently lowered the final yield. Other analogs have since been obtained (E. C. Roberts and Y. F. Shealy, unpublished results) in yields comparable to that of **13** by the general procedure described for the synthesis and purification of **12** and **13**.