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Folate Analogues Altered in the C^9–N^{10} Bridge Region. 10-Oxafolic Acid and 10-Oxaaminopterin[1a]

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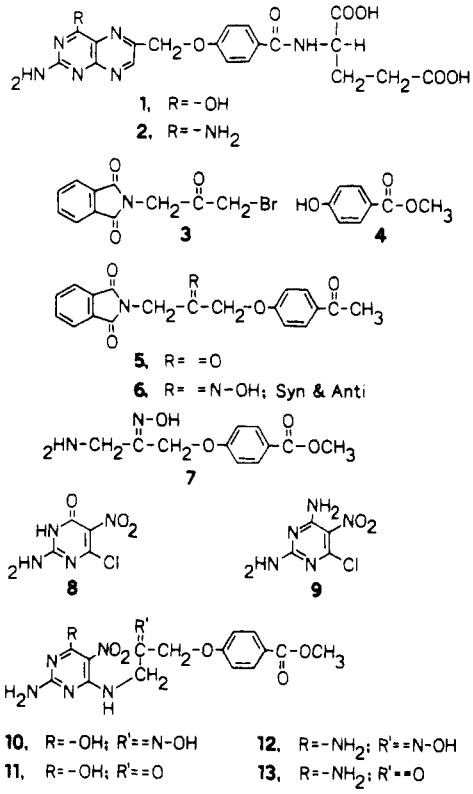
The unambiguous synthesis of two folate analogues, in which the 10-amino group of folic acid was replaced with oxygen, is described. The synthetic sequence employed commercially available methyl *p*-hydroxybenzoate and *N*-(2,3-epoxypropyl)phthalimide as starting materials. The use of cesium bicarbonate as a coreactant in the nucleophilic displacement reaction between bromo ketone **3** and the nucleophile **4** was found to be unique in character. The aminoacetonyl oxime **7** obtained by the hydrazinolysis of **6** was used as a common intermediate for the synthesis of both compounds. The generality of the use of the TFA–HCl mixture to deprotect the carbonyl group of both **10** and **12** and reductions involving sodium hydrosulfite in aqueous DMF were further substantiated by conversions of **11** and **13** to **14** and **15** quickly and efficiently without employing catalytic hydrogenations. Subsequent cyclizations, oxidations, and hydrolysis of these reduction products to the pteroate analogues **17** and **19** were carried out efficiently as described for the synthesis of the sulfur analogues. Activation of the carboxyl group of **19** by way of the mixed anhydride **22** and subsequent coupling to glutamic acid was carried out using the solid-phase coupling procedure. However, compound **17** required trifluoroacetylation to **20** prior to the coupling reaction due to solubility problems. Both 10-oxafolic acid (**1**) and 10-oxaaminopterin (**2**) showed potent antifolate activity when tested against two folate-requiring organisms. Compound **2** was a very powerful inhibitor of DCM-resistant *Lactobacillus casei* dihydrofolate reductase. The activity was comparable to that of methotrexate while the 4-hydroxy analogue did not show inhibition. 7,8-Dihydro-10-oxafolic acid failed to show any substrate activity to this enzyme and did not inhibit the enzymatic reaction when used with an equimolar concentration of the natural substrate.

The use of folate analogues as chemotherapeutic agents for the treatment of neoplastic diseases is well known.[1b-4] A concept of in vivo lethal synthesis[5,6] has led to the search for new folate analogues which are substrates of the enzyme dihydrofolate reductase (E.C. 1.5.1.3) and the reduction products thus formed capable of showing antifolate activity either by interference with folate uptake[7] or by inhibiting enzymes that are involved in DNA biosynthesis.[8] As part of a continuing program aimed at developing compounds which may meet these requirements, we have reported the design and synthesis of several folate analogues which are altered in the C^9–N^{10} bridge region.[9-13] In our continuing search for new and better substrates to this enzyme it appeared interesting to investigate the mode of interaction of the 10-oxa analogues toward dihydrofolate reductase and also their antifolate activities. This paper describes the chemical synthesis and characterization of the 10-oxa analogues of folic acid and aminopterin[14] and their preliminary antifolate activities.

More than 20 years ago Fairburn[15,16] and co-workers reported the synthesis of 10-oxafolic acid (**1**) and 10-oxaaminopterin (**2**) (Chart I). In this connection, these authors reported the characterization of their respective pteroate analogues as well. All four compounds were evaluated for their antifolate activity by the method of Wooley[17] and all of these showed varying degrees of antifolate activity when tested against *Streptococcus faecalis* R. Significant among these results is the fact that none of the analogues showed any response when a 10:1 ratio of the respective analogue to folic acid was employed. At a ratio of 100:1, 10-oxaaminopterin (**2**) showed very little antifolate response. Furthermore, the 4-amino-4-deoxy pteroate analogue was remarkably more responsive to this organism than the respective folate analogue. The validity

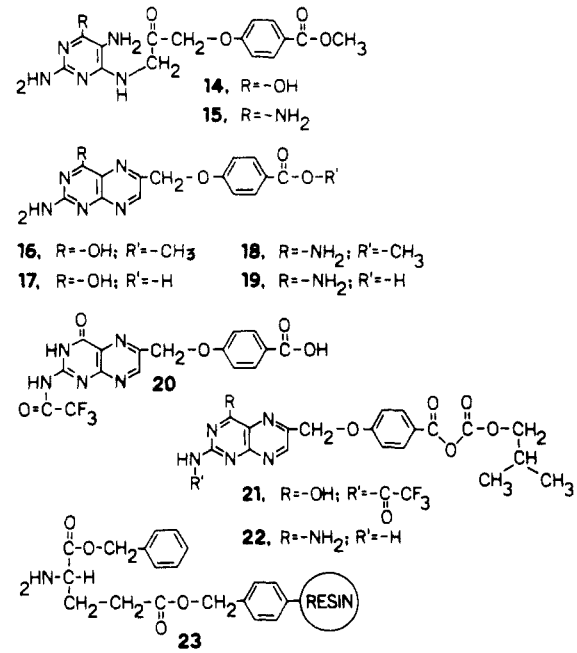
Chart I



of these results appeared to be rather questionable on the basis of our modern understanding about structure–activity relationships that exist among the antifol series,[6,18] according to which a folate analogue is expected to be more responsive than the corresponding pteroate analogue for this organism. The synthetic scheme employed by Fairburn made use of the classical approach of the condensation of a 4,5-diaminopyrimidine with a 1,2-dicarbonyl compound, a procedure that usually results in the formation of the 6- and 7-substituted pteridines. Although the uv spectrum and elemental analysis were reported for **1** no purity criteria or analytical evidence was given to compound **2** in the original paper. Therefore, an unambigious preparation of these potential antifols was of interest.

Chemistry. The synthetic methodology we have employed for the construction of the 10-oxafolate framework parallels the one utilized for the synthesis of 10-thiofolic acid and 10-thioaminopterin with certain modifications.[12,13] The quantitative conversion of *N*-(2,3-epoxypropyl)phthalimide to bromo ketone **3** has been described by us previously.[12] The displacement reaction between bromo ketone **3** and *p*-hydroxymethyl benzoate (**4**) did not proceed as expected, under a varying set of conditions which had been successfully used earlier with either thiols or amines as nucleophiles. In the presence of potassium *tert*-butoxide, sodium bicarbonate, or pyridine, the reaction proceeded in an unexpected manner giving rise to mixtures of water-soluble products whose structures were not investigated. However, the use of cesium bicarbonate as a coreactant facilitated the reaction to proceed in the desired direction with the formation of intermediate **5** in good yield. The keto group of **5** was then protected as the oxime by reaction with hydroxylamine, and the resulting product **6** was isolated as a mixture of the expected syn and anti isomers in a 3:2 ratio as evidenced by NMR and TLC criteria. No attempt was made to purify or characterize the individual isomers since the eventual regeneration of

Chart II



this carbonyl function was contemplated at a later stage of the synthetic sequence. Compound **6** was then subjected to the widely used hydrazinolysis[12,19] to generate the amino ester **7**, which proceeded in good yield over a period of 72 h in ethanol. The structure of **7** was established beyond doubt by high-resolution mass spectrometry and NMR.

2-Amino-6-chloro-4-hydroxy-5-nitropyrimidine (**8**) was prepared by the 5-nitration of the corresponding pyrimidine by established procedures. Condensation of amino ester **7**, with the nitropyrimidine **8**, proceeded well in refluxing ethanol. The use of a molar equivalent of *N*-methylmorpholine as a proton acceptor in the reaction medium considerably improved the yield of intermediate **10**. Similarly, 6-chloro-2,4-diamino-5-nitropyrimidine, prepared by the 5-nitration of 2,4-diamino-6-chloropyrimidine according to the procedure of Pfleiderer,[20] was condensed with the amino ester **7**, as described for **10**, to give the pyrimidine intermediate **12**. Both compounds **10** and **12** were now deprotected by treatment with 1 N HCl in trifluoroacetic acid to regenerate their original carbonyl moiety.

The next key step in the synthetic procedure was the reduction of the nitro groups of **11** and **13** to the corresponding amino functions. These reactions were accomplished easily and neatly by using a procedure described earlier by us[12,13] which involves the use of sodium dithionite in aqueous DMF solution. The progress of this reduction was monitored spectrophotometrically at 345 nm. This absorption maximum due to the 5-nitro group shifts to lower wavelength as the reduction proceeds to completion. The reduction products, **14** and **15**, thus obtained (Chart II) were then subjected to a cyclization procedure which involved treatment with a pyridine–pyridine hydrochloride buffer system (pH 5) in N_2 under reflux conditions for 2 h. The fully cyclized 7,8-dihydropteridine derivatives (uv spectra) thus obtained were allowed to stir at room temperature under aerobic conditions for several hours to obtain the fully oxidized pteroic acid analogues, **16** and **18**. However, the spontaneous oxidation described in this procedure was somewhat slower for compound **15** compared to **14**. Subsequently, it was discovered that after the initial cyclization step, the 4-amino-4-deoxy analogue could be converted directly to **19**,

bypassing intermediate **18** by direct hydrolysis with a 1:1 mixture of 2 N NaOH and Me_2SO at room temperature, a procedure which also resulted in the oxidation. In the case of the 4-hydroxy compound **16**, hydrolysis with 0.75 N NaOH in a nitrogen atmosphere gave the pteroic acid analogue **17** in ~50% yield after workup and purification. Similar treatment of **18** resulted in the deamination of the 4-amino group of the pteridine structure to give compound **17**.

The remaining steps to complete the synthesis of **1** and **2** were the preparation of the respective L-glutamate conjugates of **17** and **19**. A procedure frequently used by us[10-13] to accomplish this involves a typical solid-phase coupling of L-glutamic acid with the activated carboxyl group of the pteroate analogue under carefully controlled conditions. However, the applicability of this scheme is limited severely by the solubility of the pteroic acid analogue. Although the 4-amino-4-deoxy analogue **19** was soluble in a 1:1 Me_2SO–THF mixture and presented no problems, the 4-hydroxy analogue **17** was found to be insoluble in all organic solvents commonly employed in solid-phase reaction procedures. Consequently, it became necessary to derivatize **17** to a soluble compound. This was accomplished by reaction of **17** at room temperature with excess trifluoroacetic anhydride for 72 h to obtain a trifluoroacetyl derivative **20**, which was soluble in Me_2SO. The precise structural elucidation of this intermediate appeared to be unwarranted, because on treatment of this material with 0.05 N NaOH at room temperature for 1 h, it was quantitatively converted to compound **17**. Since similar treatment of pteroic acid results in the formation of N^2,N^{10}-ditrifluoroacetylpteroic acid,[21] the trifluoroacetyl derivative must have the structure **20** as written. The clean conversion of **20** to **17** rules out trifluoroacetylation at position 7[22] of the pteridine nucleus.

Both compounds **19** and **20** were solubilized in a Me_2SO–THF mixture and were converted to their respective mixed anhydrides **22** and **21** by reaction with molar equivalents of isobutyl chloroformate at 0 °C in the presence of *N*-methylmorpholine. *tert*-Boc-L-glutamic acid α-benzyl ester was coupled to the Merrifield chloromethyl resin through the γ-carboxyl group and the resin-bound glutamate derivative was deprotected to **23** as usual by using standard procedures.[12,21] Reaction of **21** and **23** for 18 h at room temperature resulted in the expected coupling, and the resin-bound folate analogue was cleaved from the resin and simultaneously deprotected at the α-carboxyl group and 2-amino group by treatment with an NaOH–dioxane mixture in a nitrogen atmosphere. Similarly, reaction between **22** and **23** and subsequent treatment as described above gave 10-oxaaminopterin in good yield after chromatographic purification (Chart II). The uv spectra of compounds **1** and **2** were very similar in 0.1 N NaOH while they differed considerably in 0.1 N HCl. 10-Oxafolic acid (**1**) showed relevant NMR signals in D_2O–NaOD using SDSS as an internal standard at δ 8.65 (s, 1 proton), 7.8 (d, 2 protons, $H_{2',6'}$, J = 9 Hz), 7.0 (d, 2 protons, $H_{3',5'}$, J = 9 Hz), 5.18 (br s, 2 protons, 9, $-CH_2-$), and between 2–2.5 ppm (c, 4 protons of glutamic acid). 10-Oxaaminopterin (**2**) showed signals due to the C_7 proton at δ 8.78 (s, 1 proton), 7.85 (d, 2 protons, $H_{2',6'}$, J = 9 Hz), 7.03 (d, 2 protons, $H_{3',5'}$, J = 9 Hz), 5.2 (br s, 2 protons, 9, $-CH_2-$), and between 2.2 and 2.7 ppm (c, 4 protons of glutamic acid). Spectral characteristics are in complete agreement with the required structures.

Biological Results and Discussion. As expected, 10-oxafolic acid per se or as its 7,8-dihydro derivative was not an effective inhibitor of dihydrofolate reductase from DCM-resistant *Lactobacillus casei*[23] DHFR at a concentration of 1.3×10^{-4} M. On the other hand, 10-oxaaminopterin (**2**) ($I_{50} = 4.5 \times 10^{-9}$ M) showed very powerful inhibition of this enzyme which is comparable to that of methotrexate (MTX $I_{50} \simeq 4.5 \times 10^{-9}$ M). 7,8-Dihydro-10-oxafolic acid failed to show substrate activity when substituted for the natural substrate 7,8-dihydrofolic acid in the assay medium.

It is noteworthy that while 7,8-dihydrofolic acid, 7,8-dihydrohomofolic acid, and 7,8-dihydro-10-thiofolic acid show substrate activity to this enzyme, the 4-amino analogues aminopterin, 10-thioaminopterin, and 10-oxaaminopterin are examples of the most potent inhibitors. A comparison of these observations with isofolic acid,[10] isoaminopterin,[11] and 10-deazaaminopterin[24] reveals that the presence of a 4-amino function in folate analogues, altered in the C^9–N^{10} bridge region, is the prime requisite to elicit antifolate activity and is rather insensitive to minor structural changes in this region. The corresponding 4-hydroxy analogues, on the other hand, are very susceptible to subtle changes in this region in exhibiting substrate activity. The successive changes from nitrogen to sulfur to oxygen result in the progressive loss of substrate activity, although all of these heteroatoms are capable of providing a lone pair of electrons. Since sulfur is a better nucleophile than either nitrogen or oxygen, it appears that introduction of substituents which are less electronegative than either sulfur or oxygen might elicit better substrate activity in this series. Therefore, the excellent substrate activity shown by 7,8-dihydrohomofolic acid and the relatively weak (~28% of the natural substrate) activity shown by 7,8-dihydro-10-thiofolic acid can easily be understood.

Both 10-oxafolic acid (**1**) and 10-oxaaminopterin (**2**) were tested for their ability to inhibit the growth of *Streptococcus faecium* (ATCC 8043) and showed 50% inhibition of growth as monitored turbidimetrically at 650 nm at a concentration of 5×10^{-8} and 5×10^{-9} g/ml, respectively. The Difco folic acid assay media for the specific organism contained 5×10^{-10} g of folic acid per milliliter. In the case of *L. casei* (ATCC 7469) the concentrations of **1** and **2** required for 50% inhibition of growth were 5×10^{-8} and 5×10^{-11}, respectively, with a folate concentration of 5×10^{-11} g/ml in the assay medium. It is noteworthy that these results do not agree with those reported by Fairborn and co-workers, and this is perhaps a reflection of the heterogeneity of the material used by them.

Experimental Section

Melting points are uncorrected and were determined on a Fisher-Johns apparatus. NMR spectra were run in $CDCl_3$ on a 90-MHz Perkin-Elmer R-32 spectrometer with Me_4Si or TSP as internal lock signal unless otherwise specified. Field strengths of the various proton resonances are expressed in parts per million and coupling constants as hertz. Peak multiplicity is depicted as usual: s, singlet; d, doublet; t, triplet; q, quartet; br, broadened singlet or unresolved multiplet; and c, complex signal whose center is given. Uv spectra were determined in a Beckman Model 25 spectrophotometer. Chromatography was carried out on DEAE cellulose in the chloride form with 1.2 × 22 cm packing unless otherwise specified. A linear NaCl gradient in 0.005 M phosphate buffer, pH 7, 1 l. each from 0 to 0.5 M NaCl, was used to elute the column. Mass spectra were run at Research Triangle Institute in N.C. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, Tenn.[25] Yields represent the actual amount of pure compound isolated, assuming 100% reaction.

Displacement Reaction between 3 and 4. Preparation of 5. Methyl *p*-hydroxybenzoate (**4**), 1 mmol (152 mg), was dissolved in 100 ml of 95% EtOH and distilled H_2O (50 ml) was added. To this solution $CsHCO_3$, 1 mmol (194 mg), was added and stirred to get a clear solution, which was evaporated to dryness in a flash

evaporator in vacuo. A 1:1 mixture of EtOH and benzene (50 ml) was added and the evaporation repeated two times. The gummy product thus obtained was dissolved in DMF (100 ml) and transferred to another flask fitted with a N_2 inlet and reflux condenser. The bromo ketone **3**, 1 mmol (284 mg), was then added in a N_2 atmosphere and the reaction mixture heated under stirring to 160° (outside bath temperature) for 2 h. After this period, the heat was turned off and the solution was stirred at room temperature overnight in N_2. The solvent was removed in vacuo and 80 g of ice was added. The precipitate thus obtained was filtered, washed, and recrystallized from MeOH: mp 160°; yield 290 mg. Anal. ($C_{19}H_{15}NO_6$) C, H, N, O.

Preparation of Oxime 6. A solution of 10 mmol (3.53 g) of **5** in a 1:1 mixture of pyridine and EtOH (60 ml) was refluxed with 10 mmol (695 mg) of hydroxylamine hydrochloride in a nitrogen atmosphere for 2 h and then evaporated in a flash evaporator to get a slightly yellow gum. To this, H_2O (20 ml) was added followed by extraction with two 50-ml portions of ethyl acetate. The combined EtoAc layers were washed three times with H_2O (20 ml each), and the organic layer was dried over Na_2SO_4. Evaporation of ethyl acetate solution gave crude oxime **6** as a white solid which was recrystallized from methanol: mp 170–175°; yield 2.2 g; TLC (silica gel, chloroform) showed two spots in a ratio of 3:2 corresponding to the syn and anti isomers. Anal. ($C_{19}H_{16}N_2O_6$) C, H, N, O.

Hydrazinolysis of 6. Preparation of 7. A solution of 13.6 mmol (5 g) of **6** in 350 ml of absolute EtOH was treated with an equimolar amount of hydrazine (435 mg) at room temperature and in an atmosphere of N_2. The reaction mixture was allowed to stir at this temperature for 72 h in N_2. After this period the solution was refluxed for 1 h and cooled to ~50°, and 13.6 ml of 1 N HCl was added and stirred at room temperature for 1 h. It was then cooled to 0° and filtered and the precipitate was discarded. The filtrate thus obtained was evaporated to dryness and the residue treated with 100 ml of ice-cold H_2O. The pH was then adjusted to 3 with 1 N HCl. After filtration, NH_4OH was added to the clear filtrate until the pH was 10. On refrigeration, a thick white precipitate was formed which was filtered, washed free of NH_4OH, and recrystallized from MeOH: mp 135–140°; yield 2.5 g; NMR (Me_2SO-d_6) 8.0 (d, J = 9 Hz), 7.15 (d, J = 9 Hz, 4 aromatic protons), 5.05 (s, 2 protons, anisoylmethylene), 3.9 (s, 3 protons, carbomethoxy), and 3.4 ppm (s, 2 protons, methylamino); calcd mass for $C_{11}H_{14}N_2O_4$, 238.0953; found, 238.0956.

Reaction of Amino Ester 7 with Chloropyrimidine (8). (a) Preparation of 10. In a three-neck, round-bottomed flask equipped with stirrer, N_2 inlet, and reflux condenser, 4.2 mmol (1.0 g) of amino ester **7** and 75 ml of 95% EtOH were added. The mixture was allowed to reflux in N_2 for 10 min until a clear solution was obtained. During this period, a solution of 4.2 mmol (794 mg) of **8** in 95% EtOH was made and added through a dropping funnel into the flask containing **7**. After 15 min, 4.2 mmol (0.47 ml) of *N*-methylmorpholine was added and the refluxing continued for 2 h. The reaction mixture was cooled to 0 °C and the fine precipitate **10** thus formed was filtered and washed successively with ice-cold water, EtOH, and $(Et)_2O$: mp 222° dec; λ max (0.1 N NaOH) 348 and 253 nm. Recrystallization of this crude product from DMF–$(Et)_2O$ gave the analytical sample: yield 1.5 g. Anal. ($C_{15}H_{16}N_6O_7$) C, H, N, O.

(b) Preparation of 12. The procedures described above for the preparation of **10** were utilized for obtaining **11**, the only difference being the substitution of chloropyrimidine (**9**) for **8**: yield of **12** 1.39 g; mp 232–234°; λ max (0.1 N NaOH) 345 and 237 nm. Anal. ($C_{15}H_{17}N_7O_6$) C, H, N, O.

Deprotection of the Carbonyl Groups of 10 and 12. Preparation of 11 and 13. In a typical experiment, 1.39 g (3.55 mmol) of **10** was dissolved in 50 ml of trifluoroacetic acid by slowly heating to 50° in an Erlenmeyer flask. The stirred solution was then treated with 5-ml portions of 1 N HCl while the temperature was slowly raised to 60° during a period of 15 min until the addition of 50 ml of 1 N HCl was complete. The reaction mixture was stirred at this temperature for an additional 5 min. It was then evaporated to dryness in vacuo and treated with 25 g of crushed ice. The precipitate **11** that was formed was filtered and then washed successively with water, MeOH, and $(Et)_2O$: mp >250°; λ max (0.1 N NaOH) 337 and 295 nm; yield 1.16 g. Anal. ($C_{15}H_{15}N_5O_7$·0.5 H_2O) C, H, N, O.

Preparation of 13. Compound **12**, 1.5 g (3.84 mmol), was treated in a similar manner with 60 ml each of trifluoroacetic acid and 1 N HCl. After workup as above, **13** was isolated as a brown solid: yield 1.3 g; λ max (0.1 N NaOH) 336 and 295 nm; mp >250°. Anal. ($C_{15}H_{16}N_6O_6$·HCl) C, H, N, O.

Sodium Hydrosulfite Reduction of 11 and 13. Preparation of 14 and 15. This reduction was carried out in aqueous DMF using sodium hydrosulfite as follows. Compound **11**, 1 g (2.59 mmol), was dissolved in 50 ml of hot DMF in an Erlenmeyer flask and the solution was cooled to 50°. Sodium hydrosulfite, 3 g (17.24 mmol), was then added to this solution and the mixture stirred at this temperature with the gradual addition of distilled water (3 ml/min). When ~50 ml of water was added by this manner, a clear, yellow solution was obtained which slowly began turning colorless with the formation of a light yellow precipitate. After 20 min the reaction mixture was diluted to 500 ml with distilled water and then cooled to 0°. The pH of this solution was adjusted to 4 by the addition of glacial acetic acid. The precipitate was filtered, washed with distilled water, and dried in vacuo over P_2O_5 to obtain 600 mg of **14**. The compound was found to be unstable and turned dark on exposure to air: λ max (0.1 N NaOH) 344 and 247 nm; A = 247/334 = 3.12. The reduction of compound **13** to **15** was accomplished in a similar manner. The yield of the final product remained the same in both instances. Due to the instability of these compounds, they were immediately subjected to the cyclization procedure described below.

Conversion of 14 to 17. Preparation of 10-Oxapteroic Acid. A solution of 50 ml of absolute EtOH and 50 ml of pyridine was adjusted to pH 5.0 by the addition of concentrated HCl. This solution was transferred to a 250-ml three-neck flask fitted with stirrer, N_2 inlet, and reflux condenser. After the solution was deaerated for 10 min by bubbling N_2, the hydrosulfite reduction product **14** was suspended in it under vigorous stirring in a N_2 atmosphere. The reaction mixture was then refluxed for 1 h and cooled to room temperature. The N_2 flow was then cut off and the reaction allowed to proceed for 72 h under aerobic conditions. On evaporation and trituration with water, a dark brown precipitate **16** was obtained which showed uv maxima in 0.1 N NaOH at 362 and 258 nm and at 323 and 253 nm in 0.1 N HCl. Spectral observations substantiate that the product is a fully oxidized pteridine. This material was treated with 50 ml of 0.75 N deaerated NaOH and refluxed in N_2 for 2 h. After cooling to room temperature and acidifying with glacial HOAc to pH 3.5, a bright yellow precipitate appeared which was filtered and washed with water. This precipitate was suspended in 500 ml of distilled water and treated with enough 0.1 N NaOH to form a clear solution. The pH was readjusted with 0.1 N HCl to 7.3 and the solution was chromatographed on a DEAE, Cl^- column by elution with a linear NaCl gradient at pH 7.0 from 0 to 0.5 M. A major uv-absorbing peak was eluted from this column. All the effluents representing this major peak were pooled and evaporated to a small volume. On acidification with glacial HOAc, pure 10-oxapteroic acid was precipitated as a yellow solid which was filtered and washed several times with distilled water and then dried over P_2O_5 in vacuo. The compound showed λ max (0.1 N NaOH) 366 nm (7512) and 258 (34 273); λ max (0.1 N HCl) 327 nm (16 120) and 251 (38 968); mp >300°; yield 440 mg; NMR (TFA) 9.25 (s, 1 proton, H_7), 8.25 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 7.31 (d, J = 9 Hz, 2 protons, $H_{3',5'}$), and 5.7 ppm (br s, 2 protons, 9, methylene). Anal. ($C_{14}H_{11}N_5O_4$) C, H, N, O.

Conversion of 15 to 19. Preparation of 4-Amino-4-deoxy-10-oxapteroic Acid (19). The initial cyclization and subsequent oxidation procedure, described above for the 4-hydroxy compound, was repeated to the stage of **18**. However, it was noticed that the conversion of **15** to **18** was rather slow under these conditions as evidenced by the uv spectral changes associated with this oxidation. Hydrolysis of an aliquot of the fully oxidized material, as described above with 0.75 N NaOH, gave **17** rather than **19**. Therefore, immediately after the initial cyclization procedure, using pyridine–pyridine hydrochloride buffer, the reaction mixture was evaporated to dryness. After treatment with ice, a product was filtered whose uv spectrum clearly showed a 7,8-dihydropteridine structure [λ max (0.1 N NaOH) 326, 287, and 242 nm; A (λ287/242) = 2]. This crude product was then treated with a 0.75 N solution of NaOH in Me_2SO at room

temperature for 5 h. During this period, the product showed the uv spectrum of a fully oxidized pteridine [λ max (0.1 N NaOH) 372 and 262 nm]. The solution was diluted to 0.01 N with respect to NaOH, adjusted to pH 7.3 by the addition of glacial HOAc, and chromatographed as described above on DEAE cellulose to get ~45% yield of the desired pteroic acid analogue: λ max (0.1 N HCl) 337 nm (9516) and 254 (26 364); λ max (0.1 N NaOH) 372 nm (6864) and 262 (31 434); NMR (D_2O with SDSS as internal standard) 8.7 (s, 1 proton, H_7), 7.9 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 6.95 (d, J = 9 Hz, 2 protons, $H_{3',5'}$), and 5.05 ppm (br s, 2 protons, H_9). Anal. ($C_{14}H_{12}N_6O_3$) C, H, N, O.

Trifluoroacetylation of 17. Preparation of 20. Finely powdered **17**, 200 mg (0.64 mmol), was suspended in a 100-ml round-bottomed flask, and 40 ml of trifluoroacetic anhydride was added to it. This mixture was stirred at room temperature and protected from moisture for 4 days. During this period, the bright yellow material changed to a white suspension. On evaporation in vacuo and treatment with ice-cold water, a cream-colored solid was obtained. This derivative was then dried in vacuo over P_2O_5 at 40° for several days to obtain 195 mg of **20**, which was soluble in a Me_2SO–THF mixture. The compound was unstable in air and slowly converted to the insoluble starting material.

After treating with 0.1 N NaOH at room temperature for 1 h and subsequent chromatography on DEAE cellulose, the reaction mixture showed the presence on only one uv absorbing peak which was identified as **17** by comparison with an authentic sample: NMR (0.1 N NaOD–D_2O using SDSS as internal standard) 8.7 (s, 1 proton, H_7), 7.91 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 7.08 (d, J = 9 Hz, 2 protons, $H_{3',5'}$), and 5.3 ppm (br s, 2 protons, H_9).

Preparation of 10-Oxafolic Acid (1). In an oven-dry graduated cylinder, 1 mmol (409 mg) of **20** was suspended in 30 ml of purified dry Me_2SO. The cylinder was closed and heated to 100°, dissolving all of the material. The solution was cooled to room temperature and 30 ml of dry THF was added. The solution was thoroughly mixed by shaking and then cooled in an ice bath for 15 min, followed by the addition of 1.25 mmol (0.16 ml) of freshly distilled *N*-methylmorpholine. It was then kept at this temperature for an additional 15 min. Freshly distilled isobutyl chloroformate (1 mmol, 0.1311 ml) (bp 128–129°) was added, mixed well, and kept in the ice bath for an additional 15 min.

The resin-bound *tert*-Boc-L-glutamic acid α-benzyl ester was deprotected and neutralized in a reaction vessel, as described previously from this laboratory. The active mixed anhydride **21** was then poured into the reaction vessel and allowed to react at room temperature for 18 h while the vessel was subjected to continuous rocking in a closed system. After the reaction, the unreacted **21** was removed by filtration and the resin washed twice with a Me_2SO–THF mixture and then twice with two 30-ml portions of dioxane. The combined filtrates were diluted to 1 l.; the pH was adjusted to 10 by the addition of 1 N NaOH and kept at room temperature for 4 h to remove the protective trifluoracetyl group and to hydrolyze the anhydride. This solution was applied on a DEAE cellulose column and, after application, washed with distilled water. The unreacted **21**, now as **17**, was eluted from the column with 10% NH_4OH, and the eluate evaporated to a small volume and quantitated spectrophotometrically.

The resin-bound glutamate conjugate derivative of **20** was then suspended in a deaerated 1:1 2 N NaOH–dioxane solution (30 ml) and shaken for 1 h at room temperature and then for 20 min at 50°. The α-benzyl ester group of glutamic acid and the trifluoroacetyl moiety at the 2-amino groups were hydrolyzed as well as the product being cleaved from the resin by this procedure. The product was separated from the resin by filtration and washed with H_2O. The filtrate and washings were combined and adjusted to pH 7.2 by 1 N HCl, diluted to 1 l., and purified by chromatography on DEAE cellulose. The uv absorbing effluent corresponding to the major peak was pooled and concentrated, and, after acidifying to pH 3 by glacial HOAc, gave the final product **1** in 80% yield based on reacted **21**: λ max (0.1 N NaOH) 365 nm (7433) and at 263 (38 167); λ max (0.1 N HCl) 322 nm (7300), 276 (12 610), and 252 (15 132). Anal. ($C_{19}H_{18}N_6O_7$) C, H, N, O.

Preparation of 10-Oxaaminopterin (2). The activation of the carboxyl group of **19** was accomplished as described above for **20**. Since **19** was soluble in Me_2SO, there was no need to derivatize this compound. The rest of the experimental procedures were the same as described for **1** and **2** was isolated after chromatographic purification in 85% yield based on reacted **19**: λ max (0.1 N NaOH) 371 nm (8158) and 262 (42 115); λ max (0.1 N HCl) 335 and 248 nm. Anal. ($C_{19}H_{19}N_7O_6$) C, H, N; O: calcd, 21.77; found, 22.19.

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References and Notes

(1) (a) 10-Oxafolic acid = *N*-[α-(2-amino-4-hydroxy-6-pteridinyl)-*p*-anisoyl]-L-glutamic acid; 10-oxaaminopterin = *N*-[α-(2,4-diamino-4-deoxy-6-pteridinyl)-*p*-anisoyl]-L-glutamic acid. Other abbreviations include DHFR, 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase (E.C. 1.5.1.3); SDSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate. (b) J. R. Bertino, *Ann. N.Y. Acad. Sci.*, **186**, 1–519 (1971).
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(25) Where analyses are indicated only by symbols of elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.