Folate Analogues Altered in the C^9–N^{10} Bridge Region: N^{10}-Tosylisohomofolic Acid and N^{10}-Tosylisohomoaminopterin

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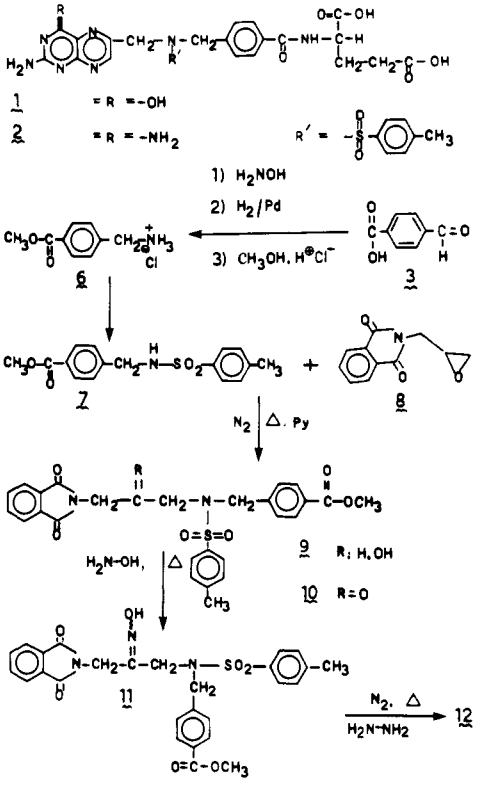
Synthetic methodology has been developed for the unambiguous construction of the isohomofolate framework. Fusion of 2,3-epoxypropylphthalimide (**8**) and *N*-tosyl-*p*-carbomethoxybenzylamine (**7**) with pyridine as a catalyst gave the essential intermediate **9** which was transformed to the aminoacetonyl oxime **12** by standard procedures. The dihydropteroate analogues **21** and **22** were quantitatively oxidized to the pteroate analogues **23** and **24** at elevated temperatures in DMF under aerobic conditions. This method has general applicability as evidenced by the quantitative conversion of both 4-oxo- and 2,4-diamino-4-deoxy-7,8-dihydro-10-thio- and -10-oxapteroates to the corresponding pteroates under these conditions. The free acids **25** and **26** were subjected to the solid-phase coupling procedures for their eventual elaboration to the folate analogues **1** and **2**. None of these 4-aminopteroate analogues inhibited dihydrofolate reductase, indicating that the introduction of bulky substituents at the bridge region could greatly alter their binding characteristics for this enzyme. However, compound **2** inhibited dihydrofolate reductase by 50% at 6.2×10^{-5} M, which was also reflected in its antifolate activity against *Lactobacillus casei*. Compounds **1** and **2** competed efficiently for the transport of 5-methyltetrahydrofolate in *L. casei* and did not inhibit thymidylate synthetase significantly. An improved procedure for the preparation of α-benzyl *tert*-butyloxycarbonyl-L-glutamate–resin for the solid-phase coupling reaction is also described.

As part of a continuing program[1-4] aimed at developing synthetic substrates to the enzyme dihydrofolate reductase (DHFR; E.C. 1.5.1.3) which in their enzymatically reduced tetrahydro form would either interfere with tetrahydrofolate utilization[5] or inhibit thymidylate synthetase (TS; E.C. 2.1.1.6),[6-8] we had occasion to explore the synthesis and biological properties of a number of isohomofolate analogues.[9,10] We were interested in delineating the effect of the introduction of bulky substituents at the 10-amino group, such as *p*-toluenesulfonyl, methanesulfonyl, trifluoroacetyl, and trifluoromethanesulfonyl, on their interaction with DHFR and TS in the isohomofolate series. This was done mainly to test the "bulk tolerance areas" of the enzymes beyond their active site in accordance with Baker's theory of drug design.[11,12] Slavik and co-workers have reported earlier that the parent compounds were competitive inhibitors of DHFR.[9] This paper details the synthesis and evaluation of the antifolate properties of N^{10}-tosylisohomofolic acid and N^{10}-tosylisohomoaminopterin.

Chemistry. At the outset, synthetic methodology for the construction of the isohomofolate framework with built-in substituents at the 10 position was required. The partial construction of the C^6 side chain of the pteridine nucleus was initially attempted by joining a three-carbon fragment to an appropriately substituted α-amino-*p*-toluic acid derivative.[13,14] This three-carbon fragment, which could give rise to an α-aminoacetonyl oxime by subsequent synthetic manipulations, was obtained by a fusion reaction.[15] Commercially available *p*-carboxybenzaldehyde (**3**) was converted to the corresponding oxime **4**, which on hydrogenation gave α-amino-*p*-toluic acid (**5**). Treatment of **5** with MeOH and HCl under reflux conditions yielded the amine hydrochloride **6**. Conversion of **6** to the tosylate **7** was accomplished using standard procedures. Fusion of **7** with 2,3-epoxypropylphthalimide (**8**) was conducted at 140 °C, using pyridine as a catalyst, to obtain the desired intermediate **9**. Jones oxidation of **9** in acetone gave the tosyl ketone **10** in excellent yield.

Protection of the carbonyl group of **10** was accomplished by oximation,[16] which gave a mixture of the syn and anti isomers of **11** (TLC, NMR). This synthetic step was followed by the standard hydrazinolysis[2-4] of **11** to generate the aminoacetonyl oxime **12**. These reactions are summarized in Scheme I. Nitration of 2-amino-6-chloro-4-pyrimidinol and 2,4-diamino-6-chloropyrimidine to their respective 5-nitro derivatives **13** and **14**[17] was carried out using fuming HNO_3 and concentrated H_2SO_4 according to earlier procedures. Reaction of **12** with the nitrochloropyrimidines **13** and **14** in refluxing EtOH under N_2 with 1 equiv of *N*-methylmorpholine as a proton acceptor gave the pyrimidine derivatives **15** and **16** in good yield. Deprotection of the carbonyl function of both these compounds was desired at this stage and was easily accomplished by the use of a 1:1 mixture of 1 N HCl and trifluoroacetic acid (TFA) at 60 °C for 20 min. The ketones **17** and **18** thus obtained were subjected to dithionite reduction to the corresponding 5-amino compounds **19** and

Scheme I



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20. The conditions for this quick and efficient reduction have been previously reported from this laboratory.[2-4] The reduction products 19 and 20, although stable below 0 °C in the absence of air for several days, are very unstable in air above 0 °C. Treatment of the reduction products with a pyridine–pyridine hydrochloride solution in absolute EtOH at pH 5 under N_2, followed by refluxing the solution for 1 h, resulted in the cyclization of these compounds to the more stable dihydropteroate analogues 21 and 22. These compounds were resistant to the previously reported one-step cyclization–oxidation procedure developed for the 10-thio- and 10-oxapteroate analogues.[2-4]

Oxidation of the dihydro derivatives 21 and 22 to the corresponding analogues 23 and 24 was initially accomplished by the use of alkaline $KMnO_4$ in DMF containing EtOH. Although this procedure gave satisfactory results, it was observed that both compounds were neatly and quantitatively oxidized when their dilute solutions in DMF were heated slowly to 120 °C while stirring in air over a period of 1 h. At this point it was reasonable to assume that the function of DMF was to bring the substance in solution where a simple air oxidation occurs. On subsequent experimentation it was found that the oxidation will not proceed to any appreciable extent, even after stirring a dilute solution of 22 in DMF under aerobic conditions for several days, at room temperature. Elevated temperatures in the range of 100–130 °C are required for this conversion. In order to check the general applicability of this simple oxidation, four additional 7,8-dihydropteroate analogues were tested. These were methyl 7,8-dihydro-10-thiopteroate, methyl 7,8-dihydro-10-oxapteroate, methyl 2,4-diamino-4-deoxy-7,8-dihydro-10-thiopteroate, and methyl 2,4-diamino-4-deoxy-7,8-dihydro-10-oxapteroate.[2-4] The methyl esters of the respective pteroic acids were isolated by evaporation of the solvent. Hydrolysis of compounds 23 and 24 with 0.1 N NaOH in acetonitrile and subsequent workup and purification by ion-exchange chromatography gave the free acids 25 and 26 in good yield based on 15 and 16 (Scheme II).

The problem of attaching the L-glutamate moiety to the pteroate analogues remained. We have reported previously that under carefully controlled conditions protection of the amino groups on the pteridine ring is unnecessary for the selective activation of the carboxyl group with isobutyl chloroformate.[14] Later we extended this procedure for the synthesis of [^{14}C]methotrexate.[18] Similar treatment of pteroate analogues 25 and 26 with 1 equiv of isobutyl chloroformate and 1.25 equiv of *N*-methylmorpholine in a 1:1 Me_2SO–THF solvent mixture at 0 °C for 15 min and its subsequent coupling with α-benzyl L-glutamate–resin at room temperature for 18 h resulted in the formation of the resin-bound products. Detachment of the final products from the resin with simultaneous deprotection of the α-carboxyl group was accomplished by hydrolysis of the resin-bound products with a 1:1 mixture of 2 N NaOH and dioxane under an atmosphere of N_2. These procedures are detailed in our previous reports.[19,20]

A new and efficient method of esterification of α-benzyl *tert*-butyloxycarbonyl-L-glutamate to the Merrifield chloromethyl resin was employed for the solid-phase coupling procedure. In the original procedure,[21] triethylamine and absolute EtOH were employed for the esterification. The use of the cesium salt of α-benzyl *tert*-butyloxycarbonylglutamate[22] in DMF resulted in higher esterification values and gave a product devoid of contamination with quarternary ammonium sites on the resin. Details of this procedure are presented in the Experimental Section.

Table I. Inhibition of *L. casei* Dihydrofolate Reductase by Derivatives of Isohomopteroates

Compd	Concn, M	% inhibn
22	1×10^{-4}	0
26	1×10^{-4}	17
2	6.2×10^{-5}	50
23	1×10^{-4}	23
25	1×10^{-4}	25
1	1×10^{-4}	32

Table II. Inhibition of *L. casei* Thymidylate Synthetase by Derivatives of Isohomopteroates

Compd	Concn, M	% inhibn
22	1×10^{-4}	0
26	1×10^{-4}	0
2	1×10^{-4}	20
23	6×10^{-5}	16
25	6×10^{-5}	15
1	6×10^{-5}	12

Table III. Inhibition of the Uptake of 5-[^{14}C]Methyltetrahydrofolate by Derivatives of Isohomopteroates in Suspensions of *L. casei*

Compd	Concn, M	% inhibn
22	1×10^{-4}	32
26	1.6×10^{-6}	50
2	4×10^{-7}	50
23	1.5×10^{-5}	50
25		
1	4×10^{-7}	50

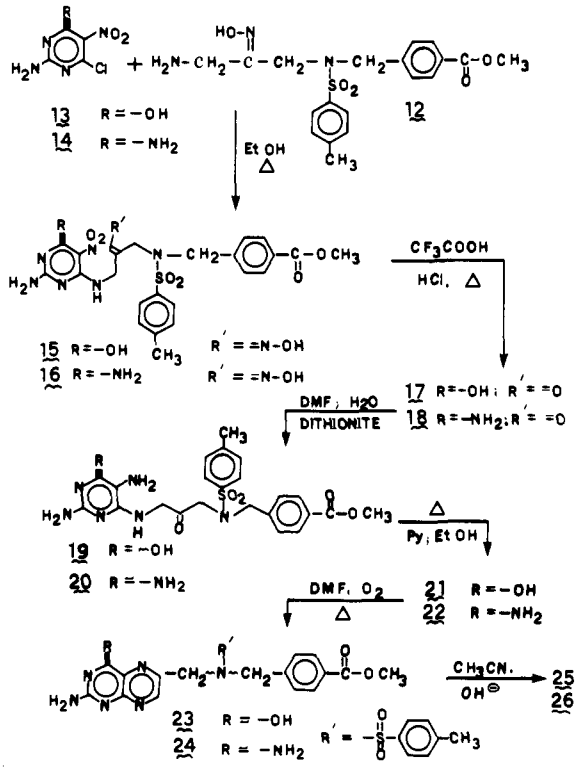
Both compounds 1 and 2 and their respective pteroate analogues 25 and 26 were purified by ion-exchange chromatography on DEAE-cellulose and exhibited NMR resonances expected of their structures. Although all four compounds showed similar UV maxima in 0.1 N NaOH, the UV spectra of the 4-amino analogues differed considerably in 0.1 N HCl from their respective 4-hydroxy derivatives.

Biological Evaluation and Discussion. Both compounds 1 and 2 were evaluated for their antifolate activities by the use of *Lactobacillus casei* (ATCC 7469), *Streptococcus faecium* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081). Compound 2 inhibited the growth of *L. casei* by 50% at 0.2 μg/mL and that of *S. faecium* at a concentration of 3.8 μg/mL. The methotrexate-resistant strains of both *L. casei* and *S. faecium* were insensitive to compound 2 up to a concentration of 4 μg/mL. Compound 2 was also inactive against *P. cerevisiae*. The 4-hydroxy analogue 1 was inactive against all the above organisms.

Selected compounds were also tested for their in vitro inhibition of *L. casei* DHFR and TS. These results are summarized in Tables I and II. Only compound 2 showed any appreciable inhibition of DHFR. None of the analogues tested was a good inhibitor of TS.

The failure of these analogues to exhibit powerful antifolate activity against microorganisms could presumably be explained in terms of their inability to be transported across the bacterial wall because of the presence of a bulky substituent at the 10 position. Competitive transport experiments using [^{14}C]methyltetrahydrofolate and the analogues revealed that compounds 1 and 2 are capable of interacting with the transport receptor sites used by 5-methyltetrahydrofolate. Therefore, it is reasonable to assume that the weak antifolate response exhibited by compound 2 and the complete lack of activity of 1 is due to their relative inhibitory characteristics to DHFR rather

Scheme II



than transport. Results are shown in Table III.

It has been suggested by Baker[23] that the enzyme DHFR contains three binding regions. The active site recognizes the 2,4-diamino functionality of the pteridine moiety and the hydrophobic region recognizes the C^9–N^{10} bridge and the phenyl ring. This hydrophobic region was thought to be rather nonspecific[24] and able to tolerate structural changes involving the presence of substituents such as methyl, ethyl, formyl, and nitroso groups[25] at C^9 or N^{10} or the replacement of the heteroatom with a methylene group.[20] Plante and Friedkin[25] have observed that elongation of this bridge region by two or more carbon units and the introduction of halogens at both the ortho positions in the phenyl ring of the folate, homofolate, and bis(homofolate) skeleton actually enhance the binding of these compounds to DHFR. We have recently suggested[3] that changes in the bridge region could alter the binding characteristics of 2,4-diamino analogues of folic acid to DHFR. It is interesting to note that the diaminopteroate analogues **22** and **26** failed to show inhibition while the glutamate conjugate **2** exhibits significant inhibition of this enzyme, indicating that the third polar region[23] of the enzyme is also important in binding of these analogues. From these studies, it is apparent that the introduction of bulky substituents at the N^{10} position of isohomofolate or pteroate derivatives could result in a decrease in efficacy of their binding to DHFR although the 2,4-diaminopteridine functionality is present.

Experimental Section

Melting points are uncorrected and were determined on a Fisher-Johns apparatus. NMR spectra were run in CF_3COOH or $CDCl_3$ on a 90-MHz Perkin-Elmer R-32 spectrometer with Me_4Si as internal lock signal. Field strengths of the various proton resonances are expressed in parts per million and coupling constants in 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 on 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 of 1 L each of 0–0.5 M NaCl in 0.005 M phosphate buffer, pH 7, was used to elute the column. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, Tenn. 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. Yields represent the actual amount of pure compound isolated, assuming 100% reaction.

***p*-Carbomethoxybenzylamine Hydrochloride (6).** Using all oven-dried apparatus, 9.3 g (56.36 mmol) of α-amino-*p*-toluic acid (**5**) was suspended in 200 mL of MeOH. Gaseous HCl was bubbled through the solution until **5** had dissolved and the solution was refluxed for 6 h. The MeOH was evaporated, ether was added, and the white precipitate was filtered, giving **6** in quantitative yield: mp 225 °C. Anal. ($C_9H_{12}NO_2Cl$) C, H, Cl.

***N*-*p*-Tosyl-4-carbomethoxybenzylamine (7).** The reaction was carried out by suspending 2.1 g (10.5 mmol) of **6** in 30 mL of lutidine under N_2, followed by addition of 3.0 g (15.7 mmol) of *p*-toluenesulfonyl chloride over a 10-min period. The yellow solution was heated, with stirring, in an oil bath at 80 °C for 1 h and then added to 600 mL of ice and water. The white precipitate which formed was filtered, washed with distilled water, and dried in vacuo over P_2O_5: yield 3.21 g (98.7%); mp 152 °C (MeOH); NMR ($CDCl_3$) 7.90 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 7.74 (d, J = 9 Hz, 2 protons, $H_{3',5'}$), 7.26 (c, 4 tosyl protons), 4.18 (d, J = 7 Hz, 2 protons, methylene), 3.89 (s, 3 protons, carbomethoxy), 2.41 ppm (s, 3 protons, tosyl methyl). Anal. ($C_{16}H_{17}NO_4S$) C, H, N.

***N*-*p*-Tosyl-*N*-(2-hydroxypropylphthalimido)-4-carbomethoxybenzylamine (9).** In a round-bottomed flask, 7.143 g (22.3 mmol) of **7** and 4.53 g (22.3 mmol) of **8** were combined and heated slowly in an oil bath under N_2 until both had melted. This was followed by the addition of 0.5 mL of pyridine and the solution was kept stirring under N_2 at 140–145 °C for an additional 2 h. The cooled melt was triturated with MeOH and recrystallized from MeOH: mp 148–150 °C; yield from two crops, 8.9 g (76.7%); NMR ($CDCl_3$) 7.66 (c, 8 protons, 4 phthalimide, 4 phenyl), 7.24 (c, 4 tosyl protons), 5.38 (d, 2 protons, J = 5 Hz, methylene), 3.82 (s, 3 protons, carbomethoxy), 3.56 (t, J = 5 Hz, 2 protons, phthaloyl methylene), 3.18 (d, 2 protons, J = 4 Hz, methylene), 2.40 ppm (s, 3 protons, tosyl methyl). Anal. ($C_{27}H_{26}N_2O_7S$) C, H, N.

***N*-*p*-Tosyl-*N*-(2-oxopropylphthalimido)-4-carbomethoxybenzylamine (10).** A solution of 1 g (1.92 mmol) of **9** in 100 mL of acetone was chilled in an ice bath. Jones reagent (10 mL) was added dropwise while stirring at 0 °C, and the solution was stirred at room temperature for 10 min. The acetone was removed by flash evaporation at 40 °C. The solution was extracted twice with ethyl acetate, the ethyl acetate layer washed with distilled water until clear, and the organic layer evaporated. The resulting gum was dissolved in $CHCl_3$–MeOH, the volume was reduced by boiling, and the white crystals were collected: mp 98–104 °C; yield 837 mg (83.8%); NMR ($CDCl_3$) 8.05, 7.8 (d, 4 protons, phenyl), 7.85 (c, 4 protons, phthalimide), 3.95 (s, 3 protons, methoxy), and 2.45 ppm (s, 3 protons, tosyl methyl). Anal. ($C_{27}H_{24}N_2O_7S \cdot 0.75H_2O$) C, H, N.

***N*-*p*-Tosyl-*N*-(2-oxopropylphthalimido)-4-carbomethoxybenzylamine Oxime (11).** **10** (10 mmol, 5.20 g) and 700 mg (10 mmol) of hydroxylamine hydrochloride were dissolved in 65 mL of a 1:1 (v:v) mixture of pyridine–absolute EtOH and refluxed for 1 h. The pyridine–EtOH was evaporated, and water and ethyl acetate were added. After separating the layers, the ethyl acetate layer was washed three times with distilled water and evaporated. The resulting gum, on treatment with MeOH, gave white crystals: mp 153–155 °C; yield 3.83 g (71.7%). Anal. ($C_{27}H_{25}N_3O_7S$) C, H, N.

***N*-(3-Aminoacetonyl)-*N*-*p*-tosyl-4-carbomethoxybenzylamine Oxime (12).** A solution of 3.83 g (7.17 mmol) of 11 was made in absolute EtOH by refluxing under N_2. The mixture was cooled to room temperature, 229 mg (7.17 mmol) of hydrazine was added, and the solution was stirred under N_2 for 72 h. The solution, containing a thick white precipitate, was then refluxed for 1 h under N_2 and cooled to 40 °C, and 7.17 mL (1 molar equiv) of 1 N HCl was added. This solution was evaporated to dryness, and, after adding 50 mL of water, the pH was adjusted to 2 with HCl. The white precipitate which formed was collected and discarded. The filtrate was adjusted to pH 8 with NH_4OH

and chilled, and the white precipitate thus obtained was collected by filtration and vacuum dried over P_2O_5: mp 149–150 °C; yield 2.14 g (73.8%); NMR ($CDCl_3$–CF_3COOH) showed two sets of splitting of two methylene protons due to the presence of the syn and anti isomers. Other relevant signals were at 3.97 (s, 2 protons, benzylmethylene), 3.64 (s, 3 protons, carbomethoxy), 2.15 ppm (s, 3 protons, tosyl methyl), and no trace of the phthalimide protons. Recrystallization from $CHCl_3$ gave the analytical sample. Anal. ($C_{19}H_{23}N_3O_5S \cdot 0.25H_2O$) C, H, S.

***N*-*p*-Tosyl-*N*-[3-(2-amino-4-hydroxy-5-nitro-6-pyrimidinyl)aminoacetonyl]-4-carbomethoxybenzylamine Oxime (15).** Compound **12**, 2.15 g (5.28 mmol), was dissolved in 150 mL of 95% EtOH by refluxing under N_2. A solution of **13** (5.28 mmol) in 95% EtOH was added to the amino ester solution with a dropping funnel. After 15 min 0.59 mL (5.28 mmol) of *N*-methylmorpholine was added and refluxing was continued for an additional 2 h. After cooling to 0 °C, the precipitate which formed was filtered and washed successively with cold water, EtOH, and ether: yield 1.88 g (65.5%); UV (0.1 N NaOH) λ_{max} 347 and 231 nm. Anal. ($C_{23}H_{25}N_7O_8S$) C, H, N, S.

Preparation of 16. In a similar manner, 1.7 g of **12** was treated with 794 mg (4.2 mmol) of the chloropyrimidine **14** to give 1.33 g (57%) of **16**: mp 210 °C; UV λ_{max} (0.1 N NaOH) 342 and 230 nm. Anal. ($C_{23}H_{26}N_8O_7S$) C, H, N.

***N*-*p*-Tosyl-*N*-[3-(2-amino-4-hydroxy-5-nitro-6-pyrimidinyl)aminoacetonyl]-4-carbomethoxybenzylamine (17). a. Preparation of 17.** **15** (559 mg, 1.0 mmol) was dissolved in 10 mL of TFA and heated in a 55 °C water bath. After 15 min, 10 mL of 1 N HCl was added dropwise with occasional stirring. The solution was kept at 55 °C for another 15 min, then evaporated, and triturated after adding ice. The precipitate thus formed was filtered, washed with distilled water, and dried over P_2O_5: yield 523 mg; mp 140–145 °C; UV λ_{max} (0.1 N NaOH) 330 and 232 nm. Anal. ($C_{23}H_{24}N_6O_8S \cdot 1.5H_2O$) C, H, N.

b. Preparation of 18. **16** (700 mg, 1.25 mmol) was treated in a similar manner with 10 mL each of TFA and 1 N HCl, yielding 625 mg of **18**: mp 125 °C; UV λ_{max} (0.1 N NaOH) 330 and 225 nm. Anal. ($C_{23}H_{25}N_7O_7S \cdot HCl$) C, H, N, S.

***N*-*p*-Tosyl-*N*-[3-(2,5-diamino-4-hydroxy-6-pyrimidinyl)aminoacetonyl]-4-carbomethoxybenzylamine (19).** A solution of 1.67 g (2.84 mmol) of **17** in 60 mL of DMF was stirred and heated to 50 °C, at which point 10 g of sodium dithionite (57.69 mmol) was added. Distilled water was added a few milliliters at a time for 15 min, keeping the solution at 50–55 °C, until solution was effected and the color lightened. Distilled water was slowly added for 5 min more until a white precipitate started to appear. The solution was then diluted to 1.4 L with ice and water and filtered. The brown precipitate thus obtained was collected and dried over P_2O_5 under vacuum: yield 1.00 g of **19** (63%); UV λ_{max} (0.1 N NaOH) 343, 280, and 240 nm. Reduction of **18** to **20** was done in a similar manner with similar yield: UV λ_{max} (0.1 N NaOH) 335, 285, and 225 nm.

Methyl 7,8-Dihydro-N^{10}-tosylisohomopteroate (21). A solution of 2.8 g (5.5 mmol) of **19**, in 200 mL of a 1:1 (v/v) mixture of pyridine–absolute EtOH adjusted to pH 5 with HCl, was refluxed under N_2 for 1 h. The solvent was evaporated, ice was added, and a brown precipitate of **21** was collected: yield 2.2 g (81.4%); UV λ_{max} (0.1 N NaOH) 343 and 272 nm [*A* (272/343) = 1.45]. Compound **20** was treated in a similar manner to give **22** in 85% yield with UV λ_{max} (0.1 N NaOH) at 332 and 270 nm, which is typical of a 6-substituted 7,8-dihydropteridine.

Methyl N^{10}-Tosylisohomopteroate (23). Compound **21**, 500 mg (1.02 mmol), was dissolved in 500 mL of DMF and slowly heated to 120 °C while stirring in air. This temperature was maintained for 2 h; then the solution was stirred at room temperature for 5 h. The DMF was evaporated to approximately 5 mL, ice was added, and the orange precipitate which formed was filtered, washed with cold water, and dried under vacuum over P_2O_5: yield 490 mg; UV λ_{max} (0.1 N NaOH) 362 and 247 nm; NMR (CF_3COOH) 8.4 (s, 1 proton, C_7), 7–7.8 (c, 8 protons, aromatic), 4.2, 4.3 (br, 4 protons, methylenes), 3.65 (3 protons, methoxy), 2.15 ppm (3 protons, tosyl methyl). Oxidation of **22** to **24** also proceeded with quantitative yield: UV λ_{max} (0.1 N HCl) 338, 282, and 237 nm.

N^{10}-Tosylisohomopteroic Acid (25). **23** (1 mmol, 482 mg) was dissolved in 36 mL of CH_3CN; 9 mL of 1 N NaOH and 21 mL of distilled water were added and the mixture was stoppered and stirred overnight at room temperature. The CH_3CN was removed by vacuum evaporation and the solution adjusted to pH 7 with HCl, diluted, and chromatographed. The effluent containing the product was reduced to a small volume by evaporation and titrated to pH 4 with HOAc. Compound **25** precipitated as a yellow solid which was collected and washed with dilute HOAc and dried over P_2O_5 under vacuum: yield 320 mg; UV λ_{max} (0.1 N NaOH) 363 nm (ϵ 6504), 255 (23 292), and 240 (29 976); UV λ_{max} (0.1 N HCl) 325 nm (ϵ 6144) and 237 (31 404); mp >300 °C; NMR (CF_3COOH) 8.95 (s, 1 proton, C_7), 8.13 (d, J = 9 Hz, 2 protons, $H_{3',5'}$), 7.95 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 7.53 (c, 4 tosyl protons), 4.79 (s, 2 protons, C_9), 4.69 (s, 2 protons, C_{11}), 2.62 ppm (s, three protons, tosyl methyl). Anal. ($C_{22}H_{20}N_6O_5S$) C, H, N, O.

4-Amino-4-deoxy-N^{10}-tosylisohomopteroic Acid (26). The hydrolysis was carried out in a similar manner to that of **23** by dissolving 721 mg (1.5 mmol) of **24** in 48 mL of CH_3CN, followed by addition of 12 mL of 1 N NaOH and 28 mL of distilled water. The solution was stirred at room temperature for 19 h. The CH_3CN was removed by vacuum evaporation; the pH was adjusted to 7 with HCl and diluted to 1100 mL with distilled water. Chromatography and isolation as above yielded 450 mg of **26**: UV λ_{max} (0.1 N NaOH) 372 nm (ϵ 5265), 257 (18 981), and 236 (27 621); UV λ_{max} (0.1 N HCl) 339 nm (ϵ 7340), 279 (4993), and 240 (34 229); NMR (CF_3COOH) 8.80 (s, 1 proton, C_7), 7.91 (c, 4 tosyl protons), 4.75 (s, 2 protons, C_9), 4.56 (s, 2 protons, C_{11}), 2.52 ppm (s, 3 protons, tosyl methyl). Anal. ($C_{22}H_{21}N_7O_4S$) C, H, N, S.

N^{10}-Tosylisohomofolic Acid (1). In an oven-dried graduated cylinder, 262 mg (0.545 mmol) of **25** was dissolved in 25 mL of dry Me_2SO; 25 mL of dry tetrahydrofuran (THF) was added, the solution chilled to 0 °C in an ice bath, and 0.076 mL (0.681 mmol) of *N*-methylmorpholine added. After 15 min at 0 °C, 0.071 mL (0.545 mmol) of freshly distilled isobutyl chloroformate was added; the solution was mixed well and kept at 0 °C for an additional 15 min. Excess (2.1 mmol) resin-bound α-benzyl *tert*-butyloxycarbonyl-L-glutamate was placed in a dried reaction vessel and deprotected and neutralized as described previously by this laboratory.[19,21] The mixed anhydride was added to the reaction vessel and the vessel was rotated on a solid-phase apparatus[21] at room temperature for 5 h. The unreacted anhydride was removed by filtration and the resin was washed twice with Me_2SO–THF and twice with dioxane. The resin-bound folate analogue was suspended in 30 mL of a deaerated 1:1 2 N NaOH–dioxane mixture, shaken manually at room temperature for 1 h, and then incubated in a 55 °C water bath, with intermittent shaking, for 20 min. The product was filtered and the resin was washed twice with a 1:1 dioxane–water mixture and then three times with water. The filtrate and washings were diluted to 1 L, adjusted to pH 7, and purified by chromatography. The major UV absorbing peak was evaporated to a small volume and the product was precipitated by titrating to pH 4.0 with HOAc: UV λ_{max} (0.1 N NaOH) 367 nm (ϵ 7524) and 245 (27 556); UV λ_{max} (0.1 N HCl) 325 nm (ϵ 7500) and 240 (40 128); NMR (CF_3COOH) 8.9 (s, 1 proton, C_7), 7.85 (t, 4 protons, $H_{2',6'}$ and $H_{3',5'}$), 7.55 (t, 4 protons, tosyl), 4.75, 4.65 (s, 2 protons, C_9, C_{11}), 2.5 (s, 3 protons, tosyl methyl), and between 2 and 2.7 ppm (c, 4 protons, glutamate). Anal. ($C_{27}H_{27}N_7O_8S \cdot H_2O$) C, H, N, S.

N^{10}-Tosylisohomoaminopterin (2). Activation of the carboxyl group of **26** was accomplished as described for **25**, using 15 mL each of Me_2SO and THF for 207 mg of the pteroate. The rest of the procedure was identical with that for compound **1**, and compound **2** was isolated after chromatographic purification: yield 105 mg; UV λ_{max} (0.1 N NaOH) 374 nm (ϵ 5988) and 239 (31 279); UV λ_{max} (0.1 N HCl) 338 nm (ϵ 7782), 283 (5000), and 242 (35 233); NMR (CH_3COOH) relevant signals at 8.70 (s, 1 proton, C_7), 7.83 (c, 4 tosyl protons), 7.34 (d, J = 9 Hz, 2 protons, $H_{2',6'}$), 4.70 (s, 2 protons, C_9), 4.52 (s, 2 protons, C_{11}), 2.50 ppm (s, 3 protons, tosyl methyl). Anal. ($C_{27}H_{28}N_8O_7S \cdot 2H_2O$) C, H, N, S.

Improved Method for Esterification of Merrifield Chloromethyl Resin. Chloromethylated resin beads (Schwarz/Mann) containing 1.6 mequiv of Cl^- per gram of resin were used in this experiment. Typically, 11.975 g (35.62 mmol) of α-benzyl *tert*-butyloxycarbonyl-L-glutamate (Vega Fox) was dissolved in 100 mL of 95% EtOH and diluted to 200 mL with distilled water. To this solution 6.91 g (35.62 mmol) of cesium bicarbonate was slowly added with stirring. The clear solution

thus obtained was evaporated to dryness and the resulting gum was codistilled three times with benzene to remove water. The cesium salt thus obtained was dried under vacuum over P_2O_5. Dry DMF, 200 mL, was added to the dry cesium salt, followed by 21.3 g (34.08 mmol) of chloromethylated resin. The vessel was stoppered and the contents gently stirred at 80 °C in an oil bath for 48 h. The resin was filtered and washed successively with DMF, 1 N HOAc, dioxane, and EtOH and then dried over P_2O_5 under vacuum. A sample of this resin was hydrolyzed by refluxing with 6 N HCl in HOAc for 6 h and the liberated amino acid quantitated by the ninhydrin method. The resin thus prepared contained 1.2 mequiv of glutamic acid per gram. This yield could be improved by using a 20% excess of *tert*-butyloxycarbonylamino acid relative to the chloride substitution on the resin.

Methods Used for Biological Testing. The uptake system for 5-methyltetrahydrofolate, slightly modified from that described,[26] consisted of 0.1 M potassium phosphate buffer, pH 6.5, 1% glucose, 1.0×10^9 cells (1.0 mg dry weight), and 0.4 mg (40000 cpm) of 5-[^{14}C]methyltetrahydrofolate (Amersham) in a volume of 1.0 mL. Incubations were for 1 min at 37 °C. Uptake was terminated by filtration through membrane filters and washing three times with 2.0 mL of ice-cold saline. The filters were dried and counted in vials with Liquifluor (New England Nuclear). Thymidylate synthetase,[27] dihydrofolate reductase,[28] and microbiological assays[29] were carried out as described.

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References and Notes

(1) M. G. Nair and C. M. Baugh, *J. Med. Chem.*, **17**, 223 (1974).
(2) M. G. Nair, P. T. Campbell, and C. M. Baugh, *J. Org. Chem.*, **40**, 1745 (1975).
(3) M. G. Nair and P. T. Campbell, *J. Med. Chem.*, **19**, 825 (1976).
(4) M. G. Nair and C. M. Baugh in "Chemistry and Biology of Pteridines", W. Pfleiderer, Ed., Walter de Gruyter, Berlin and N.Y., 1975, pp 503–513.
(5) D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin, and E. J. Crawford, *Nature (London)*, **189**, 39 (1961).
(6) M. Friedkin, E. J. Crawford, and L. T. Plante, *Ann. N.Y. Acad. Sci.*, **186**, 209 (1971).
(7) J. I. DeGraw, J. P. Marsh, Jr., E. M. Acton, O. P. Crews, C. W. Mosher, A. N. Fujiwara, and L. Goodman, *J. Org. Chem.*, **30**, 3404 (1965).
(8) L. Goodman, J. I. DeGraw, R. L. Kisliuk, M. Friedkin, E. J. Pastore, E. J. Crawford, L. T. Plante, A. Nahas, J. F. Morningstar, Jr., G. Kwok, L. Wilson, E. F. Donovan, and J. Ratzan, *J. Am. Chem. Soc.*, **86**, 308 (1964).
(9) K. Slavik, R. Cihar, J. Soucek, K. Pristoupilova, E. Hermanova, and V. Slavikova in "Advances in Antimicrobial and Antineoplastic Chemotherapy", Vol. II, University Park Press, Baltimore, London, and Tokyo, 1972, p 69.
(10) K. Slavik, R. Cihar, J. Soucek, E. Hermanova, K. Pristoupilova, and V. Slavikova, *Mol. Pharmacol.*, **8**, 740 (1972).
(11) B. R. Baker, "Design of Active Site Directed Irreversible Enzyme Inhibitors", Wiley, New York, N.Y., 1967.
(12) C. Silipo and C. Hansch, *J. Am. Chem. Soc.*, **97**, 6849 (1975).
(13) M. G. Nair and C. M. Baugh, *J. Org. Chem.*, **38**, 2185 (1973).
(14) M. G. Nair, L. P. Mercer, and C. M. Baugh, *J. Med. Chem.*, **17**, 1268 (1974).
(15) D. I. Weisblat, B. J. Magerlein, A. R. Hanze, D. R. Myers, and S. T. Rolfson, *J. Am. Chem. Soc.*, **75**, 3625 (1953).
(16) M. G. Nair, P. T. Campbell, E. Braverman, and C. M. Baugh, *Tetrahedron Lett.*, **31**, 2745 (1975).
(17) D. E. O'Brien, C. C. Cheng, and W. Pfleiderer, *J. Med. Chem.*, **9**, 573 (1966).
(18) M. G. Nair and C. M. Baugh, *J. Labelled Compd. Radiopharm.*, **13**, 147 (1977).
(19) M. G. Nair and C. M. Baugh, *Biochemistry*, **12**, 3973 (1973).
(20) J. I. Degraw, R. L. Kisliuk, Y. Gaumont, C. M. Baugh, and M. G. Nair, *J. Med. Chem.*, **17**, 552 (1974).
(21) C. L. Krumdieck and C. M. Baugh, *Biochemistry*, **8**, 1568 (1969).
(22) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(23) B. R. Baker, *Ann. N.Y. Acad. Sci.*, **186**, 214 (1971).
(24) W. B. Neely, *Ann. N.Y. Acad. Sci.*, **186**, 249 (1971).
(25) L. T. Plante, E. J. Crawford, and M. Friedkin, *J. Biol. Chem.*, **242**, 1466 (1967).
(26) F. Mandelbaum-Shavit, *Biochim. Biophys. Acta*, **428**, 674 (1976).
(27) A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **237**, 3794 (1962).
(28) M. Chaykovsky, A. Rosowsky, N. Papathanasopoulos, K. N. Chen, E. J. Modest, R. L. Kisliuk, and Y. Gaumont, *J. Med. Chem.*, **17**, 1212 (1974).
(29) R. L. Kisliuk, D. Strumpf, Y. Gaumont, R. P. Leary, and L. Plante, *J. Med. Chem.*, **20**, 1531 (1977).