Folate Analogues Altered in the C^9–N^{10} Bridge Region: 11-Thiohomofolic Acid

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The synthesis of 11-thiohomofolic acid (**2**) has been accomplished by an unambiguous procedure. Reaction of 1-chloro-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone (**10**) with hydroxylamine under carefully controlled conditions gave the corresponding oxime **33**. Conversion of this oxime to 1-phthalimido-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone oxime (**4**) was carried out by its reaction with potassium phthalimide using crown 18 ether as a catalyst. Hydrazinolysis of compound **4** gave 1-amino-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone oxime (**5**), which was used for the construction of the title compound **2** by modification of the Boon and Leigh procedure. An alternate synthesis utilizing 1-hydroxy-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone (**11**) and 4-hydroxy-2,5,6-triaminopyrimidine has also been carried out. Compound **2** did not exhibit any antifolate activity against *Lactobacillus casei* or *Streptococcus faecium*. The dithionite reduction product, 7,8-dihydro-11-thiohomofolic acid, was able to function as a substrate of *L. casei* dihydrofolate reductase. The catalytic reduction product of **2**, consisting of a mixture of diastereomers, exhibited powerful antifolate activity against both these organisms.

Several years ago, DeGraw and co-workers[1] synthesized an analogue of folic acid in which an extra methylene group was inserted between the C^9 and the N^{10} position of the vitamin. This compound, homofolic acid (**1**), showed some very interesting biological properties and was the subject of extensive investigation during the past decade. For example, 7,8-dihydrohomofolic acid was shown to be a substrate of *L. casei* dihydrofolate reductase, and its tetrahydro form was shown to be a powerful inhibitor of thymidylate synthetase.[2] Dihydrohomofolate substituted for folate as a growth factor for *S. faecium*, while the tetrahydro derivative antagonized its growth. This inhibition of growth was later attributed to the *d*,L-diastereomer[3] of tetrahydrohomofolic acid. The enzymatic reduction product of 7,8-dihydrohomofolic acid, i.e., *l*,L-tetrahydrohomofolic acid, was capable of acting as a pseudocofactor of thymidylate synthesis in *S. faecium* and *L. casei*.[4] Mishra[5] and co-workers have shown that dihydrohomofolate was active against an antifolate-resistant leukemia $L1210/FR_8$, which contains high levels of dihydrofolate reductase, and that the tetrahydro derivative prolonged the life span of MTX-resistant leukemic mice.[6]

The lack of antifolate activity of 7,8-dihydro and *l*,L-tetrahydro derivatives of homofolic acid in certain microorganisms could be explained in terms of their ability to be metabolized to N^5,N^{11}-methylenetetrahydrohomofolate and its subsequent participation as a pseudocofactor in thymidylate biosynthesis.[3,4] Therefore, we were interested in designing a potential substrate of dihydrofolate reductase which, in its enzymatically reduced tetrahydro form, is unable to carry cyclic 1 carbon fragments in the bridge region. This molecular modification would effectively prevent the molecule from participating as a pseudocofactor in thymidylate synthesis. Therefore, the enzymatically reduced tetrahydro derivative of this homofolate analogue was envisioned to be potentially capable of interfering with tetrahydrofolate utilization. Substitution of a sulfur atom for the 11-amino group of homofolic acid appeared to meet these requirements. As part of a continuing program aimed at developing analogues of folic acid which are alterd in the C^9–N^{10} bridge region,[7-10] this paper details the synthesis and preliminary biological evaluation of 11-thiohomofolic acid.

Chemistry. The partial side chain **5** could be obtained from **4** by hydrazinolysis.[7,8] Therefore, initial attempts were directed toward the synthesis of **3** and **4**. The preparation of *p*-(carbomethoxy)thiophenol from *p*-aminobenzoic acid was accomplished using earlier procedures.[11,12] We proposed to prepare **3** by the reaction of potassium phthalimide with chloromethyl ketone **10**, which in turn could be prepared by the Michael addition of *p*-(carbomethoxy)thiophenol and hydroxymethyl vinyl ketone (**15**) and the subsequent treatment of the product with thionyl chloride. To optimize conditions for this reaction, the Michael addition of this thiophenol with acrolein and methyl vinyl ketone was examined as models which resulted in the formation of compounds **19** and **21**. Conversions of 1,4-butynediol (**14**) to **15**, **16**, and **17** were carried out using $HgSO_4$ as a catalyst and using the appropriate reaction conditions.[13] Michael addition of *p*-(carbomethoxy)thiophenol to these substituted vinyl ketones gave the hydroxymethyl, chloromethyl, and acetoxymethyl ketones **11**, **10**, and **12**, respectively. The chloromethyl ketone **10** can also be prepared from **20** by converting it successively to the acid chloride, diazo ketone, and chloromethyl ketone by the Arndt–Eistert procedure.[14] Thus, treatment of *p*-(carbomethoxy)thiophenol with β-propiolactone or iodopropionic acid resulted in the formation of **20**, which was converted to the acid chloride **23b** by treatment with thionyl chloride. This acid chloride was treated with diazomethane, and the diazo ketone thus formed was reacted with gaseous HCl to produce **10** in good yield. Compounds **9**, **11**, and **12** were also prepared as potential starting materials for **10**, which in turn could be converted to **3** by reaction with potassium phthalimide.

A more direct approach to the synthesis of **5** or **6** consisted of converting **10** to the corresponding keto azide **13**, protection of the carbonyl group of **13** as the oxime or ketal, and subsequent reduction of the azide to a primary amine. Although these reactions appeared straightforward, treatment of **10** with sodium azide in aqueous acetone resulted in the formation of a major product whose structure was established as **24** by mass spectrometry[15] and NMR. Therefore, attempts were made to prepare **3** from **10** by reaction with potassium phthalimide using acetonitrile as a solvent and crown 18 ether as a catalyst. The molecular ion of this reaction product had an m/e value of 383[24] but had the rearranged structure **25** rather than the expected **3**.[15] Since the occurrence of this rearrangement[15] was discovered only after experiencing the consistent failures to ring close the dithionite reduction product of either compound **31** or **32** to a pteridine, all the pyrimidine intermediates derived from **27** and **28** are also described in this paper.

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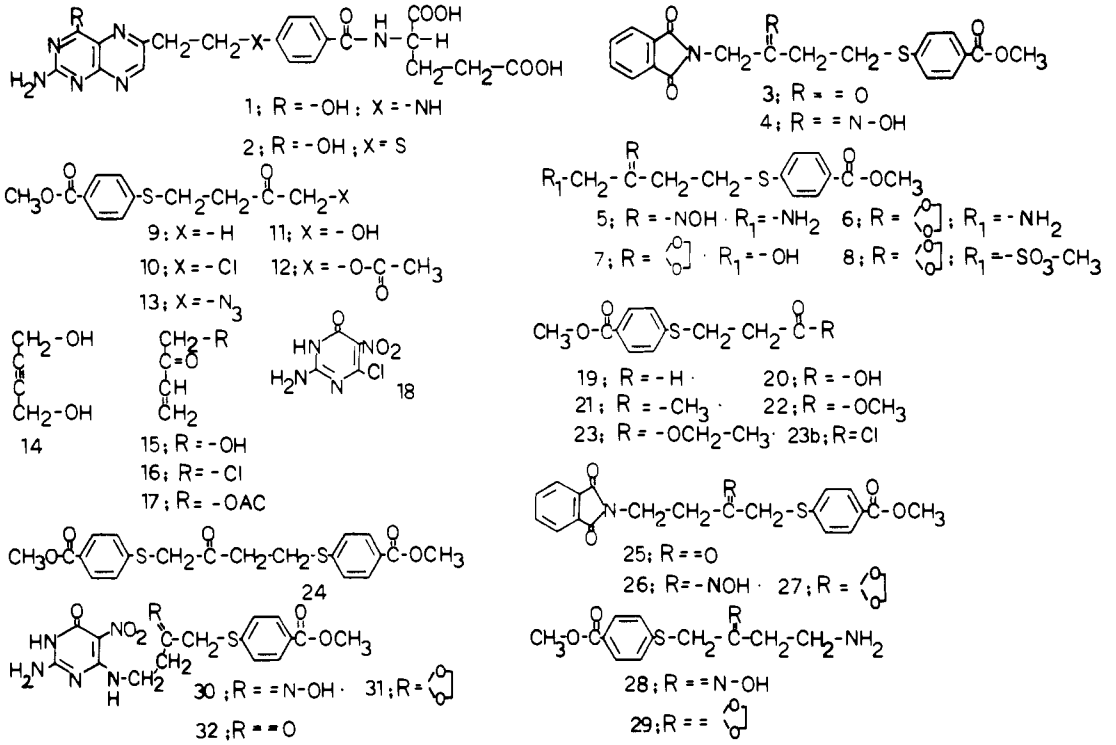
Chart I



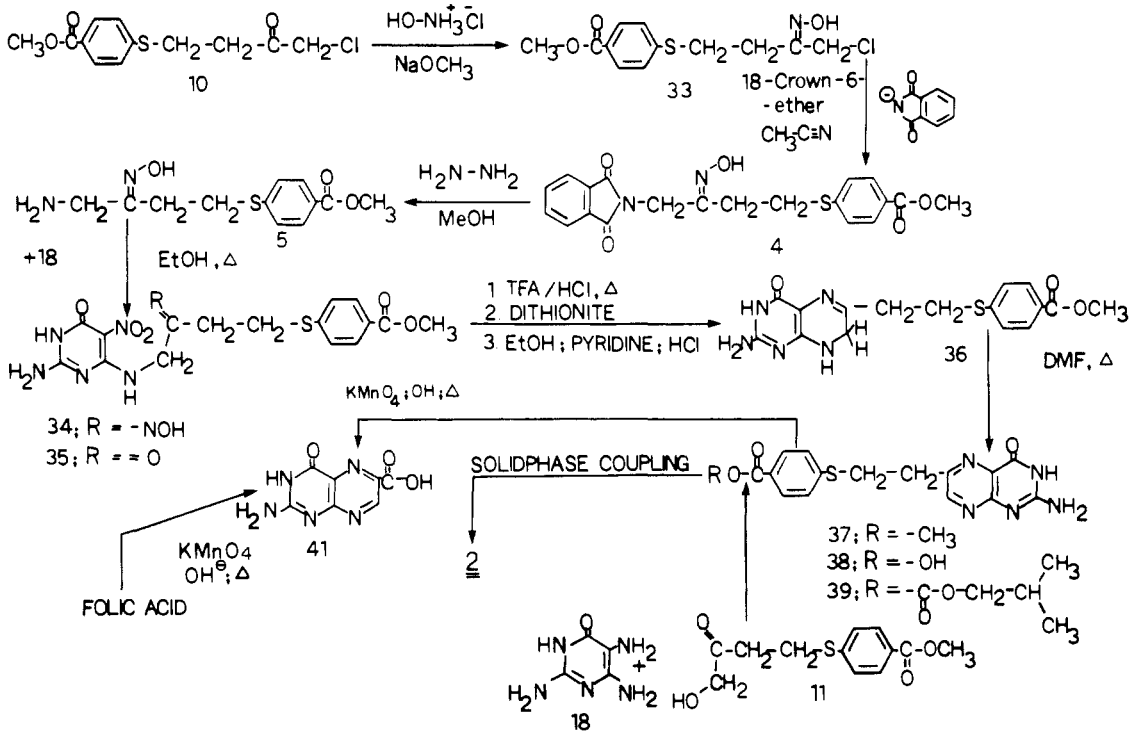
An alternate procedure to prepare **6** from the hydroxymethyl ketone **11** was attempted by protecting the keto group of this compound in the form of an ethylene ketal. This gummy ketal **7** was then reacted with methanesulfonyl chloride in pyridine, which gave a crystalline mesylate **8**. All attempts to convert the mesylate **8** to an azido ketal by treatment with sodium azide failed due to the high stability of **8** toward nucleophiles. Elevated temperatures and phase-transfer catalysts were employed for these trial reactions. The crown ether reaction product **25** was converted to the ketal **27** by standard procedures. Hydrazinolysis of **27** gave **29**, which on treatment with the nitrochloropyrimidine **18** gave **31**. In a similar manner, **25** was converted to the oxime **26**, which on hydrazinolysis gave the amine **28**. Reaction of **28** with **18** gave **30**. Treatment of either **30** or **31** with TFA and HCl gave the same ketone **32**. Dithionite reduction of **32** gave the corresponding amino compound, which when subjected to synthetic manipulations[7,8,12] failed to yield a pteridine ring system (Chart I).

The desired nonrearranged **4** was made by the following procedure. The carbonyl group of **10** was protected as the oxime **33**, by its reaction with hydroxylamine hydrochloride under carefully controlled conditions. This protective measure effectively prevented enolate formation of **10** under the stipulated reaction conditions, with potassium phthalimide giving the nonrearranged compound **4**. Deprotection of **4** with TFA/HCl gave the butanone derivative **3**, which was isomeric with **25**. Hydrazinolysis of **4** in methanol gave the desired primary amine **5**. Treatment of **5** with 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine (**18**) in ethanol using 1 equiv of *N*-methylmorpholine as a proton acceptor gave the intermediate **34**. The carbonyl group of **34** was deprotected using TFA/HCl as described previously.[7,8] This deprotection was accompanied by a blue shift of the λ_{max} of **34** from 348 to 303 nm. The nitro ketone thus obtained was quickly and efficiently reduced with sodium dithionite in aqueous DMF at 55 °C during a period of 15 min. The reduction product, after drying under vacuum, was cyclized to the dihydrohomopteroate analogue **36** using the Py/HCl mixture in MeOH. Aerobic oxidation of **36** to the methyl homopteroate analogue **37** was accomplished at elevated temperatures[8] in DMF. The methyl ester thus obtained was hydrolyzed according to the procedure of Mautner and co-workers[16] to yield 11-thiohomopteroic acid. This compound was purified by ion-exchange chromatography on DEAE-cellulose (Scheme I).

An alternate synthesis of **37** was also attempted using the hydroxymethyl ketone **11** and 4-hydroxy-2,5,6-triaminopyrimidine (**40**). When the original conditions[17] for the synthesis of 6-hydroxymethylpteridine were employed for this reaction, no pteridine was isolated presumably because of the insolubility of **11**. However, this reaction could be carried out in DMF or glacial acetic acid,[18] which resulted in the formation of a mixture of several pteridine fractions from which the desired compound could be isolated after hydrolysis of the pteridine mixture, followed by extensive ion-exchange chromatography in unacceptably low yield. The purified pteroic acid obtained by this procedure was shown to have the correct structure by comparing it with the authentic material obtained by use of the unambiguous procedure (UV, NMR, and cochromatography). This material was also oxidized by alkaline permanganate to pterin-6-carboxylic acid, which was also the oxidation product of folic acid under these conditions.[19] Application of this reaction to the preparation of the 4-amino analogue of **2** using **11** and 2,4,5,6-tetraminopyrimidine ended in failure, due to the formation of complex mixtures which were unsuitable for further investigations.

The final elaboration of **38** to **2** was initially carried out using the solid-phase coupling procedure. Although this procedure was preferred over the others for the synthesis of several folate analogues reported from this laboratory, clearly this was not the method of choice in the present instance. The yield of **2** was extremely poor by the solid-phase procedure. The carboxyl group of **38** was activated by the isobutyl chloroformate method under conditions which did not require the protection of the

Scheme I



pteridine ring,[7,11] and introduction of the glutamate moiety was accomplished using diethyl-L-glutamic acid. The diethyl ester of **2** thus obtained was hydrolyzed using 0.33 N NaOH in aqueous acetonitrile,[16] and the final product was purified by ion-exchange chromatography. The very poor yield of **2**, using the solid-phase procedure, was subsequently attributed to the severity of conditions which were employed for the cleavage of the product from the resin. When **38** was subjected to identical conditions of this cleavage, it was observed that 60% of the material was degraded to less polar products. The structures of these products were not investigated. Compound **2** exhibited UV and NMR spectral characteristics which were in excellent agreement with the desired structure.

Biological Evaluation and Discussion. The biological responses of some of these compounds were determined using two folate-requiring microorganisms, *Streptococcus faecium* (ATCC 8043) and *Lactobacillus casei* (ATCC 7469). These assays were carried out using previously reported procedures[8] in the appropriate assay medium for each organism. Compound **2** did not inhibit the growth of either of these organisms at 1.6 μg/mL. Neither the corresponding pteroic acid **38** nor its methyl ester **37** were found to be active by this assay procedure. Therefore, it became clear that none of these analogues with a 4-hydroxypteridine ring was capable of interfering with folate metabolism. It is interesting to note that both 10-thiofolic acid[11,16] and 10-oxafolic acid[7] showed excellent antifolate activity against the growth of these organisms. Next, the interaction of **2** with *L. casei* dihydrofolate reductase[20] (DHFR; EC 1.5.1.3) and thymidylate synthetase[21] (TS; EC 2.1.1.6) was investigated. Compound **2** was neither a substrate nor an inhibitor of DHFR, and it did not inhibit TS to any significant level.

Treatment of an aqueous solution of the potassium salt of **2** with excess sodium dithionite at room temperature and acidification of the solution with glacial HOAc gave 7,8-dihydro-11-thiohomofolic acid, which showed λ_{max} in 0.1 N NaOH at 280 and 320 nm which was typical of a 7,8-dihydropteridine. This compound served as a good substrate of *L. casei* DHFR. Catalytic reduction[22] of **2** gave the diastereomeric mixture of the tetrahydro derivative, which showed λ_{max} at 290 nm at pH 7.6, typical of a tetrahydropteridine. This diastereomeric mixture of tetrahydro-11-thiohomofolic acid[22] showed excellent antifolate activity[23] when tested for the inhibition of growth of *S. faecium* (I_{50} = 1.2 × 10^{-9} g/mL) and moderate activity against *L. casei* (I_{50} = 8 × 10^{-7} g/mL). Under similar conditions[2] tetrahydrohomofolate gave an I_{50} value of 0.7 × 10^{-9} g/mL for the growth of *S. faecium*. The growth inhibitory potency for *S. faecium* (1.2 ng/mL) indicates that *dl*,L-tetrahydro-11-thiohomofolate to be a potent antifolate of the series related to tetrahydrohomofolate. Tetrahydro-11-thiohomofolic acid was not an inhibitor of *L. casei* thymidylate synthetase.[21] From these studies, it is apparent that it is possible to design and develop synthetic substrates of DHFR which are analogues of folic acid altered in the C^9–N^{10} bridge region and that the tetrahydro derivatives of such compounds could interfere at some level of folate metabolism. Since the catalytic reduction product is a mixture of the two diastereomers, it is unclear whether both of these isomers or only one of these is exhibiting the antifolate activity against these organisms. Investigations are currently under progress toward the isolation and purification of the enzymatic reduction product of 7,8-dihydro-11-thiohomofolic acid and evaluation of the antifolate activity of this isomer. An appreciable quantity of this material is being accumulated for in vivo antitumor evaluation against MIX-resistant leukemic mice.

Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer, and NMR spectra were run in $CDCl_3$ or CF_3COOH on a 90-MHz Perkin-Elmer R-32 spectrometer with Me_4Si as internal lock signal. Field strength 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, the center of which is given. UV spectra were determined on a Beckman Model 25 spectrophotometer.

Ion-exchange 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 either by Galbraith Laboratories, Inc., Knoxville, Tenn., or Integral Microanalytical Lab, Raleigh, N.C. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values. Yields represent the amount of pure compound isolated.

1-Phthalimido-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone (3). (A) Preparation of 10. Method a. 1-Hydroxy-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone (**11**) was prepared by the Michael addition of hydroxymethyl vinyl ketone (**15**) and *p*-(carbomethoxy)thiophenol according to the following procedure. An aqueous solution of hydroxymethyl vinyl ketone was prepared from 2,4-butynediol according to the procedure of Hennion and Kupiecki using HgOAc as a catalyst.[13] A solution of 1.68 g (1 mmol) of *p*-(carbomethoxy)thiophenol was prepared in 80 mL of CH_3OH under nitrogen, and an excess of hydroxymethyl vinyl ketone was added to it under stirring, followed by 1 mL of triethylamine. After 1 h, methanol was evaporated off under reduced pressure, and the white solid thus obtained was recrystallized from benzene: mp 91–92 °C; yield 2.2 g (86.6%). Anal. ($C_{12}H_{14}O_4S$) C, H, S. In a similar manner, reaction of *p*-(carbomethoxy)-thiophenol with acetoxy methyl vinyl ketone yielded **12** in ~90% yield, mp 98–99 °C. Anal. ($C_{14}H_{16}O_5S$) C, H, S.

A solution of 254 mg (1 mmol) of **11** in 10 mL of chloroform was refluxed with a mixture of 0.2 mL of thionyl chloride in 10 mL of pyridine for 4 h. After this period, the reaction mixture was allowed to stand overnight at room temperature. Evaporation of this mixture under reduced pressure gave a residue which was crystallized from acetone–hexane: mp 94–95 °C; NMR of the product ($CDCl_3$) 7.90, 7.26 (d, 4, aromatic), 4.1 (s, 2, chloromethyl), 3.96 (s, 3, carbomethoxy), 3.25 and 2.95 (a, t, 2, 2, ethylene) ppm; yield 200 mg. Anal. ($C_{12}H_{13}ClO_3S$) C, H, Cl, S.

Method b. (1) Preparation of 3-[*p*-(Carbomethoxy)-thiophenoxy]propionic Acid (20). A mixture of equimolar amounts of 3-bromopropionic acid and *p*-(carbomethoxy)thiophenol was placed in a round-bottom flask. The flask was heated slowly with the aid of an oil bath. When the mixture melted, giving a homogeneous solution, 2 equiv of pyridine was added and the reaction was allowed to proceed under an atmosphere of nitrogen at 80 °C for 1 h. After this period, the product was dissolved in ethyl acetate and extracted repeatedly with aqueous bicarbonate. Acidification of the bicarbonate layer gave colorless crystals: mp 137–138 °C; yield 76%.

Alternately, to a mixture of 1.68 g (1 mmol) of *p*-(carbomethoxy)thiophenol and 1.06 g (1 mmol) of sodium carbonate in 40 mL of acetone, 1.08 g (1.5 mmol) of β-propiolactone was added and stirred overnight. The reaction mixture was evaporated to dryness, and 200 mL of water was added and filtered. Acidification of the clear filtrate with concentrated HCl gave a white precipitate, which was identical with the product obtained by the procedure described above, yield 83%. Anal. ($C_{11}H_{12}O_4S$) C, H, S.

On refluxing a methanolic solution of *p*-(carbomethoxy)-thiophenol with ethyl β-bromopropionate in the presence of an equivalent amount of cesium bicarbonate for 1 h and subsequent workup gave the ethyl ester **23** in quantitative yield, mp 58–60 °C. Anal. ($C_{13}H_{16}O_4S$) C, H, S.

When an etherial solution of **20** was treated with diazomethane, the methyl ester **22** was obtained, mp 70 °C. Anal. ($C_{12}H_{14}O_4S$) C, H, O.

(2) Conversion of 20 to 10. The acid chloride **23b** was prepared by refluxing 2.4 g (10 mmol) of **20** with 40 mL of thionyl chloride under anhydrous conditions for 4 h. Excess thionyl chloride was removed under vacuum and the residue was thoroughly dried under vacuum. This solid was then treated with an ethereal solution of (20 mmol) diazomethane, first at 0 °C for 30 min and then for an additional 30 min at room temperature. Following this treatment, gaseous HCl was bubbled through this solution for 15 min when the solution became saturated with HCl. After keeping the contents for 1 h at room temperature, the solvent was removed under vacuum. The clean white precipitate thus obtained was identical with the product obtained by treating **11** with thionyl chloride, yield 2.5 g (88%).

(3) Conversion of 1-Chloro-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone to 3. Treatment of chloromethyl ketone (**10**) with hydroxylamine hydrochloride in the presence of sodium methoxide[15] gave the corresponding oxime **33**. This oxime was reacted with potassium phthalimide according to a previous procedure described[15] by us yielding **4** in 80% yield, mp 145 °C. This compound was an isomer of **26**. Anal. ($C_{20}H_{18}N_2O_5S$) C, H, N, S.

Treatment of this oxime with a TFA/HCl mixture gave **3**, which was isomeric with **25** as described previously in one of our earlier papers concerning a phthalimide-induced rearrangement.[15] We have also described the preparation and characterization of both compounds **25** and **26** in that paper.

Hydrazinolysis of 4: Preparation of 1-Amino-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone Oxime (5). A slight modification from the original procedure, involving the use of a 25% excess of hydrazine, was employed for this reaction. A methanol solution of 3.98 g of **4** (10 mmol) was deaerated by bubbling through nitrogen for 15 min, a solution of 12.5 mmol of 95% hydrazine hydrate in 20 mL of methanol was added to it, and the mixture was refluxed for 4 h. After stirring the reaction mixture overnight in nitrogen, it was refluxed for an additional hour, cooled, added to 10 mL of 1 N HCl, evaporated under vacuum, and worked up as previously described.[7,11] A similar procedure was adopted for the hydrazinolysis of **26** or **27** to **28** and **29**. The yield of the amine **5** from **4** was 85% while those of **28** and **29** were only 60–65%. TLC examination of the reaction mixture prior to workup revealed that only one isomer of the syn and anti mixture of **28** was cleaved, and cleavage of **29** was exceedingly slow. Prolonged treatment with hydrazine did not improve the yield of either **28** or **29**.

Preparation of 1-[*N*-(2-Amino-4-hydroxy-5-nitropyrimidin-6-yl)amino]-4-[*p*-(carbomethoxy)thiophenoxy]-3-butanone (32). The preparation of oxime **30** and ketal **31** has been described previously.[15] Treatment of either **30** or **31** with a mixture of TFA and 1 N HCl gave the same ketone **32**. In a typical experiment, 844 mg (2 mmol) of **30** was dissolved in 20 mL of TFA at 55–60 °C, and 20 mL of 1 N HCl was added portionwise to this mixture under stirring during a period of 15 min. The mixture was evaporated to remove most of TFA at this temperature, and the addition of 250 mL of ice-cold water precipitated **32** as a yellow solid. This material was filtered, washed, and dried: yield 750 mg (92%); mp 165–167 °C; UV λ_{max} (0.1 N NaOH) 346, 275 nm. Anal. ($C_{16}H_{17}N_5O_6S$) C, H, N, S.

Preparation of 1-[*N*-(2-Amino-4-hydroxy-5-nitropyrimidin-6-yl)amino]-4-[*p*-(carbomethoxy)thiophenoxy]-2-butanone (35). In our previous paper concerning the rearrangement[15] we have described the preparation of both **33** and **34**. Deprotection of **34** to **35** was carried out by procedures identical with those described for the preparation of **32**. The cream-colored solid thus obtained showed a single λ_{max} in 0.1 N NaOH at 312 nm: yield ~94%; mp 245 °C. Anal. ($C_{16}H_{17}N_5O_6S$) C, H, N, O.

Conversion of 35 to 11-Thiohomopteroic Acid (38). (a) Dithionite Reduction of 35. This reduction was carried out according to procedures described by us previously.[7,8,11] Briefly, 407 mg (1 mmol) of **35** was dissolved in minimum amount of DMF at 50–55 °C, and 4.07 g of sodium dithionite was added to it under stirring. This was followed by portionwise addition of distilled water during a period of 5–10 min at this temperature until all the dithionite has gone into solution. Addition of crushed ice and water (~300 g) to this solution gave a precipitate, which was washed and dried to obtain a cream-colored solid which showed λ_{max} at 282 nm with a shoulder around 320 nm in a 2:1 ratio.

In a similar manner, **32** was reduced with dithionite, but addition of crushed ice to the mixture as described previously failed to yield a precipitate but gave a turbid solution. This solution was filtered, and the filtrate, on standing for several days in the refrigerator, gave yellow crystals, mp 148 °C. The NMR spectrum of this material in TFA exhibited all the characteristic signals expected of the reduction product. The compound showed a single λ_{max} in 0.1 N NaOH at 277 nm with no shoulder.

(b) Conversion of 35 to 38. The dithionite reduction product of **35** was suspended in 100 mL of a deaerated solution containing equal volumes of pyridine and methanol whose pH was adjusted

to 5.0 with the aid of concentrated HCl. This material was cyclized by refluxing for 1 h under an atmosphere of N_2. After cooling, the solvents were removed by evaporation under vacuum. The orange residue thus obtained was triturated with 50 g of ice and filtered. The residue was washed with distilled water and dried to give a product, whose UV spectrum in 0.1 N NaOH indicated that it is a mixture of the 7,8-dihydro derivative and an oxidized pteridine (presence of λ_{max} at 260, 275, 320, and 365 nm).

In order to complete the oxidation, the product was dissolved in DMF and heated to 100–120 °C for 2 h as described previously.[8] Examination of the UV spectrum of the solution at this stage indicated complete oxidation. Removal of solvent under vacuum gave the methyl ester **37**, which was hydrolyzed with the use of 0.33 N NaOH in acetonitrile for 4–5 h. After evaporation of acetonitrile, the pH of the clear solution was adjusted to pH 7.2 and the mixture was subjected to ion-exchange chromatography over DEAE-cellulose in the chloride form. A linear NaCl gradient eluted the product, which was accompanied by several degradation products. The major product was the desired pteroic acid analogue **38**, as evidenced by its UV and NMR spectral characteristics. All the column effluents corresponding to this material were pooled and evaporated to a small volume. Acidification of this concentrate with glacial HOAc precipitated a cream-colored solid, which was collected by filtration and washed with water to obtain the analytical sample: mp >300 °C; yield ~30% based on **35**. The compound showed λ_{max} at 368 and 256 in 0.1 N NaOH with a shoulder at 276 nm. The UV absorption spectrum of **38** in 0.1 N HCl showed λ_{max} at 298 and 250 nm and at pH 7.0 350 and 247 nm. It is noteworthy that the spectral characteristics of 11-thiohomopteroic acid are drastically different from those of 10-thiopteroic acid in 0.1 N HCl. This compound showed relevant NMR signals in TFA at 8.78 (s, l, C_7), 8.07, 7.4 (2 d, 2, 2, aromatic) and 3.55 (t, 4, ethylene bridge) ppm. Anal. ($C_{15}H_{13}N_5O_3S$) C, H, N, S.

Alternate Synthesis of 11-Thiohomopteroic Acid (38). A mixture of 2.4 g (1.01 mmol) of 2,5,6-triamino-4-pyrimidinol and 2.72 g (2 mmol) of sodium acetate in 500 mL of DMF was deaerated by bubbling through N_2 for 15 min. A solution of 2.54 g (10 mmol) of the hydroxy ketone **11** in 100 mL of DMF was also deaerated by this procedure. The two solutions were mixed, and stirring was continued under an atmosphere of N_2 for 4 h. After this period, nitrogen was cut off and the stirring was carried out under aerobic conditions for 18 h. Then the mixture was refluxed for 2 h and DMF was removed under vacuum. The dark-brown residue thus obtained was triturated with water and filtered. The precipitate thus obtained after washing several times with distilled water was hydrolyzed as described previously for **37**. The yield of the crude pteroic acid fraction was 400 mg. The mixture was then chromatographed on DEAE-cellulose. All fractions containing **38** were pooled, concentrated to a small volume, and acidified, and the precipitate thus obtained was rechromatographed twice to obtain pure **38**, yield 60 mg. The UV and NMR spectra of this compound was identical with those of **38** prepared by the unambiguous procedure in all respects. The UV and NMR spectra of this compound was identical with those of **38** prepared by the unambiguous procedure in all respects. In addition, alkaline permanganate oxidation of this material, according to a literature procedure previously employed[19] for the oxidation of 6-alkylpteridines, gave pteridine-6-carboxylic acid, which was identical in all respects with an authentic sample prepared by the oxidation of folic acid.

Preparation of 11-Thiohomofolic Acid (2) from 11-Thiohomopteroic Acid (38). Method A. Solid-Phase Coupling Procedure. A solution of 171.5 mg (0.5 mmol) of **38** was made in 25 mL of dry Me_2SO by heating to 100 °C. This solution was cooled to room temperature and an equal volume of THF was added. The mixture was chilled in an ice bath and treated with 0.07 mL (0.625 mmol) of freshly distilled *N*-methylmorpholine. After keeping 15 min at this temperature, 0.065 mL (0.5 mmol) of freshly distilled isobutyl chloroformate was added. After 15 min at this temperature, the mixed anhydride was allowed to couple with an excess of resin-bound L-glutamate for 18 h as described previously from this laboratory.[7,8,11]

Cleavage of the product from the resin by earlier procedures using 2 N NaOH and dioxane and subsequent chromatography gave only 17 mg of the desired product. Most of the pteridine fractions were decomposition products. Modifications of this cleavage procedure, substituting acetone for dioxane, did not improve the yield of the product. Subsequent experimentations revealed that neither the pteroate nor the homofolate analogue is stable toward 2 N NaOH under the cleavage conditions, and therefore this method of coupling was abandoned.

Method B. The mixed anhydride **39** from 0.5 mmol of **38** was made in Me_2SO at room temperature without the use of THF. To this, a solution of 1 mmol of diethyl L-glutamate hydrochloride dissolved in 10 mL of Me_2SO and 0.113 mL (1 mmol) of *N*-methylmorpholine was added and stirred for 18 h. After this period, the reaction mixture was made 0.33 N with respect to NaOH by the addition of 1 N NaOH and hydrolyzed for 4 h. The pH of the hydrolysate was adjusted to 7.2 and the mixture chromatographed on a DEAE-cellulose column. Three products were eluted from the column. The least polar fraction was the monoethyl ester of **2** (NMR) and the most polar fraction was the desired product. The middle fraction consisted of unreacted starting material **38**. The monoethyl ester could be rehydrolyzed to **2** in a solution of 0.33 N NaOH in acetonitrile for 4 h. The total yield of **2** obtained by this procedure was ~40%. However, the recovered starting material could be recycled to get additional amounts of **2**.

The final product was isolated as a light yellow powder by the following procedure. The combined column effluents were concentrated to a small volume and acidified with glacial acetic acid, whereupon **2** precipitated from the solution. It was filtered and washed with distilled water, containing a trace of acetic acid, several times and dried under vacuum overnight at room temperature over P_2O_5. Compound **2** showed λ_{max} in 0.1 N NaOH at 366 (ϵ 7888) and 256 (ϵ 29 389) with a shoulder at 275 (ϵ 19 288) nm. In 0.1 N HCl it showed λ_{max} at 290 (ϵ 14 911) and 255 (ϵ 15 151) nm and it showed relevant NMR signals at 8.80 (s, 1, C_7), 7.8, 7.45 (d, J = 9, 2, 2, aromatic), 3.55 (br, 4, ethylene), 2.85 and 2.5 (c, 4, glutamic acid) ppm, in complete agreement with the required structure. Anal. ($C_{20}H_{20}N_6O_6S \cdot 5H_2O$) C, H, N, O, S.

Preparation of 7,8-Dihydro-11-thiohomofolic Acid. In a test tube, 47.2 mg of **2** (0.1 mmol) was suspended in 5 mL of distilled water and stirred. Very small amounts of $KHCO_3$ were added to this mixture portionwise until a clear solution was obtained. A UV spectrum of this solution was recorded, which showed a λ_{max} at 365; then this solution was treated with 50 mg of sodium dithionite. After 5 min, the spectrum was recorded again and no appreciable change had occurred to the 365-nm peak. This treatment and observation of the spectrum was repeated until the λ_{max} at 365 nm completely disappeared. At this point, which required 200 mg of dithionite and a duration of 20 min at room temperature, the solution was cooled in an ice bath and a few drops of glacial HOAc were added until the solution became acidic (pH ~4.5). The precipitated dihydro derivative was filtered, washed three times with water, and dried under vacuum: yield 35 mg; UV λ_{max} (0.1 N NaOH) 327, 285 nm. The ratio of the optical density of the 285-nm peak to the 327-nm peak was 2.71. When the reduction was carried out above room temperature, **2** was converted to the tetrahydro derivative identified by comparison with an authentic sample prepared by catalytic reduction (vide infra) of **2**.

Methods Used for Biological Testing. The preparation of *dl*,L-tetrahydro-11-thiohomofolic acid was carried out as described previously for the preparation of *dl*,L-tetrahydrofolic acid using platinum oxide and hydrogen at atmospheric pressure. The catalytic reduction product showed a single UV absorption maximum at 290 nm when run at pH 7.4. The material was homogeneous by DEAE-cellulose chromatography. The dithionite reduction product obtained by treating **2** with dithionite above 40 °C was identical with this catalytic reduction product.

When 7,8-dihydro-11-thiohomofolate was substituted for the natural substrate in the assay medium previously described for the assay of *L. casei* DHFR, it was reduced by NADPH to the tetrahydro derivative. The velocity of this reduction was approximately 25% of 7,8-dihydrofolic acid. Dihydrofolate reductase,[20] thymidylate synthetase,[21] and microbiological assays were carried out as described.[23]

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(24) A molecular ion having an m/e value of 404 had been inadvertently reported for this compound in the previous paper.[15]

2-Acetylpyridine Thiosemicarbazones. 1. A New Class of Potential Antimalarial Agents[1]

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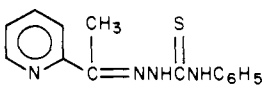
Based on the antimalarial properties observed for 2-acetylpyridine 4-phenyl-3-thiosemicarbazone (1), an extensive series of related thiosemicarbazones was prepared and tested against *Plasmodium berghei* in mice. Screening results indicated that the presence of the 2-pyridylethylidene group was critical and that certain phenyl, benzyl, phenethyl, or cycloalkyl groups at N^4 of the thiosemicarbazone moiety also contribute to antimalarial activity.

Thiosemicarbazones, a class of compounds possessing a wide spectrum of medicinal properties, have been studied for activity against tuberculosis,[2] leprosy,[3] bacterial[4] and viral[5] infections, psoriasis,[6] rheumatism,[7] trypanosomiasis,[8] and coccidiosis.[9] In the past few years, thiosemicarbazones derived from 2-formylpyridine and related aldehydes have been of great interest because of their reported antineoplastic action.[10]

Among the thousands of compounds submitted for antimalarial screening by numerous contributors to the Division of Experimental Therapeutics have been several hundred thiosemicarbazides and thiosemicarbazones. Virtually all were devoid of activity, including the well-known tuberculostat, *p*-acetamidobenzaldehyde 3-thiosemicarbazone (Thiacetazone, Tibione). One thiosemicarbazone, however, namely, 2-acetylpyridine 4-phenyl-3-thiosemicarbazone (1),[11] attracted our attention because



1

it showed activity in our primary screen. It was decided to exploit this interesting lead by ascertaining the molecular features essential for activity and utilizing them to develop a new class of antimalarial agents.

The influence on biological action was observed when the structure of 1 was modified as follows: (1) the thiocarbonyl group was replaced by a carbonyl group; (2) the pyridine moiety was replaced by another heterocyclic, aromatic, or cycloaliphatic ring system; (3) the point of attachment of the ethylidene group to the pyridine ring was changed to the 3 and 4 positions; (4) the methyl of the ethylidene group was replaced by other alkyls or hydrogen; (5) the phenyl ring at the terminal (N^4) position of the thiosemicarbazone was replaced by various substituted phenyls, other cyclic structures, and various so-called antimalarial aliphatic side chains.

This paper is one of the first to report on thiosemicarbazones possessing antimalarial activity.[12] In it, we limit our discussion to those compounds which are monosubstituted at N^4 of the thiosemicarbazone moiety.

Additional reports are in preparation which are devoted to related 2-acetylpyridine thiosemicarbazones that are disubstituted at N^4 and also to the antibacterial properties of this general class of compounds.