Reaction took place with strong liberation of gas and was completed after 3 min (disappearance of IR band at 2250 cm^{-1}). The precipitate from the cold reaction mixture was collected by filtration and washed with toluene. The dry solid was washed with water and dried over P_2O_5 in vacuo to give compound 8: yield 1.9 g (70%); mp 182–185 °C dec; $[\alpha]^{25}_D$ −1.9° (c 1, DMF); TLC R_f (B) 0.53, R_f (E) 0.62. Anal. ($C_{25}H_{31}N_3O_6$) C, H, N.

Boc-Phe-gPhe-mGly-Leu-Met-NH_2 (9). To a solution of 8 (1.7 g, 3.6 mmol) in DMF (15 mL) were added HOBt (1.06 g, 7.8 mmol), NMM (0.4 mL, 3.6 mmol), and the amino component **5b** (1.1 g, 3.6 mmol). A solution of DCC (0.82 g, 3.9 mmol) in DMF (5 mL) was cooled and added to the stirred reaction mixture kept in ice bath. The coupling procedure and the workup followed general procedure B. The crude product was loaded on a silica gel column (5 × 25 cm, 70–230 mesh) and eluted by a gradient of MeOH in $CHCl_3$ (5 to 10%). The product obtained from chromatography was recrystallized from MeOH–ether. The precipitate was collected by filtration, and the white solid **9** was dried over P_2O_5 in vacuo: yield 0.95 g (35%); $[\alpha]^{25}_D$ −13.4° (c 1.0, DMF); mp 226–228 °C; TLC R_f (A) 0.32; R_f (E) 0.64, R_f (H) 0.67; HPLC (H_2O–MeOH, 25:75 and 30:70) $k' = 3.87$, $k' = 8.75$, respectively. Anal. ($C_{36}H_{52}N_6O_7S$) C, H, N. Amino acid analysis calcd for Phe/Leu/Met/NH_3, 1:1:1:3; found, 0.93:1:1:2.80.

HCl·H-Phe-gPhe-mGly-Leu-Met-NH_2 (9a). Compound **9** (0.45 g, 0.62 mmol) was deprotected according to general procedure A. The crude product was recrystallized from MeOH–ether to give **9a** as a solid: yield 0.31 g (77%); $[\alpha]^{25}_D$ −3.0° (c 0.3, AcOH); mp 223–225 °C dec; TLC R_f (B) 0.59; R_f (D) 0.64; R_f (E) 0.52. Anal. ($C_{31}H_{45}N_6O_5SCl$) C, H, N.

pGlu-Phe-gPhe-mGly-Leu-Met-NH_2 (III). The coupling and workup followed general procedure A. To a solution of pGlu-OH (0.166 g, 1.29 mmol) in DMF (2.5 mL) was added NMM (0.144 mL, 1.29 mmol). After cooling of the reaction mixture, IBCF (0.15 mL, 1.2 mmol) was added. A solution of the amino component **9a** (0.26 g, 0.4 mmol) in HMPA (3 mL) was treated with NMM (0.045 mL, 0.4 mmol), cooled, and added to the reaction mixture. The analogue III was obtained from the reaction mixture as a white powder: yield 0.26 g (90%); $[\alpha]^{25}_D$ −24° (c 0.1, dimethyl sulfoxide); mp 230–231 °C (full characterization of analogue III is summarized in Table I).

In Vitro Bioassay. Guinea Pig Ileum Assay. The activity of analogues I–III was assayed on an isolated guinea pig ileum as described by Rossel et al.[34] with the following modifications. The ileum was suspended in a 10-mL organ bath containing Tyrode solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 11 mM D-glucose), thermostated at 32 °C, and bubbled with 95% O_2 and 5% CO_2. The segment of the ileum was left to equilibrate for 30 min, before introduction of the tested compounds, and washed every 10 min with Tyrode solution. Isotonic contractions of the longitudinal muscles were measured with a smooth-muscle transducer purchased from Harvard Apparatus and recorded on a chart mover. We prepared fresh stock solutions of the peptides (10^{-3} M) by dissolving the peptides in Me_2SO. Further dilutions (up to 10^{-9} M) were performed with Me_2SO. The final concentration of Me_2SO in the organ bath did not exceed 0.1% (v/v).

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Registry No. 1, 32159-21-0; **1a**, 82379-40-6; **1b**, 82379-41-7; **1c**, 66488-77-5; **2**, 66488-70-8; **2a**, 40759-90-8; **3**, 82379-43-9; **3a**, 3709-27-1; 4 (isomer 1), 83815-83-2; 4 (isomer 2), 83803-08-1; **4a**, 82379-45-1; **5a**, 58172-54-6; **5b**, 2131-00-2; **6**, 13122-89-9; **7**, 40099-25-0; **7a**, 82379-46-2; **7b**, 82379-47-3; **8**, 83803-09-2; **9**, 82379-49-5; **9a**, 83860-21-3; II, 79775-20-5; III, 82379-39-3; Boc-Phe-OH, 13734-34-4; H-Phe-OMe·HCl, 7524-50-7; pGlu-OH, 98-79-3; ethyl chloroformate, 541-41-3; 2-benzylmalonic acid, 616-75-1; malonic acid, 141-82-2; acetone, 67-64-1.

Folate Analogues. 20. Synthesis and Antifolate Activity of 1′,2′,3′,4′,5′,6′-Hexahydrohomofolic Acid[1]

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The synthesis of 1′,2′,3′,4′,5′,6′-hexahydrohomofolic acid (**3**), a close analogue of homofolic acid (**2**), has been carried out by replacement of the benzene ring of **2** with a cyclohexane ring. The synthetic methods employed here were based on the Boon–Leigh strategy to obtain products with unambiguous structures. Based on a number of chemical and spectral observations, a tentative cis stereochemistry was assigned to the 1,4-substituents of the cyclohexane ring of both the homopteroate analogue **13** and the target compound **3**. We investigated hexahydrohomopteroic acid (**13**), hexahydrohomofolic acid (**3**), and their 7,8-dihydro and *d,l*-5,6,7,8-tetrahydro derivatives for antifolate activities employing several biological test systems. The dihydro and tetrahydro derivatives of both **13** and **3** were active against *Streptococcus faecium*, whereas they were inactive against *Lactobacillus casei*. These compounds were neither substrates nor inhibitors of *L. casei* dihydrofolate reductase or thymidylate synthase.

Thymidylate synthase (EC 2.1.1.45) catalyzes the terminal step in the de novo synthesis of thymidylic acid, which is required exclusively for DNA synthesis. Because of the unique feature of this enzyme, it continues to be a prime target for cancer chemotherapy. Although potent inhibitors of thymidylate synthase (TS) of the substrate class, such as 5-FUdR, are well known[2] and are being used in the clinical treatment of various human cancers, such inhibitors belonging to the coenzyme class [analogues of the vitamin folic acid (**1**, Chart I)] are relatively rare. The tetrahydro derivative of homofolic acid (**2**) synthesized by DeGraw[3,4] was the first known example of a thymidylate synthase inhibitor of the coenzyme class. Goodman and

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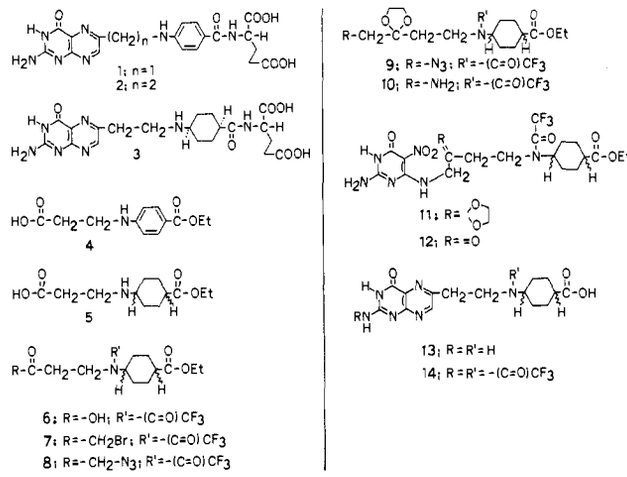
0022-2623/83/1826-0135$01.50/0

Chart I



co-workers converted homofolate to dihydrohomofolate by dithionite reduction and then further reduced it enzymatically using mouse leukemia dihydrofolate reductase (EC 1.5.1.3). The enzymatically formed tetrahydrohomofolate was shown to be a potent inhibitor of *E. coli* thymidylate synthase.[4] The most potent coenzyme class inhibitor of TS reported thus far is 5,8-dideaza-N^{10}-propargylfolate, recently described by Jones and associates.[5] This antitumor quinazoline inhibitor exhibited excellent activity against L1210 leukemia in mice, inhibiting TS from these cells competitively, with respect to the substrate methylenetetrahydrofolate, with an inhibition constant of ~1 nM.

Soon after the initial report of Goodman et al.[4] regarding the inhibitory potency of tetrahydrohomofolate toward *E. coli* TS, a large number of homofolate analogues were synthesized and evaluated with enzymes from different sources.[6-9] A few analogues of homofolate that were altered in the C^9–N^{10} bridge region, such as 11-thiohomofolate[10] and 11-oxahomofolate,[11] were also synthesized in our laboratories. We hoped that these compounds might function as substrates for dihydrofolate reductase and that the resulting tetrahydro derivatives would be capable of interfering with folate metabolism. Although some success in this regard was achieved with 11-oxahomofolate,[11] neither the parent compounds nor their reduced derivatives inhibited *L. casei* TS to any appreciable extent. As an extension of our continuing efforts in developing synthetic substrates of DHFR that are potential inhibitors of TS after enzymatic reduction, we were interested in examining the effects of increasing the basicity of the 11-amino group of homofolate with regard to its cofactor or inhibitory activity toward TS. Therefore, replacement of the benzene ring of homofolate with a cyclohexane ring was considered, and this paper describes the chemical synthesis and biological evaluation of such an analogue (**3**).

At the outset it was realized that even if one starts with either of the pure *cis*- or *trans*-4-aminocyclohexanecarboxylate derivative **5**, epimerization at the 1- and 4-positions cannot be precluded during the remaining synthetic steps, which required treatment of the various intermediates with strong acid and strong base at some stage. Since the major objective of this work was to examine the effect of enhanced basicity of the 11-amino group of a homofolate analogue on its interaction with TS, without strictly defining the stereochemical disposition of the 1,4-substituents on the cyclohexane ring, it was decided to proceed with the synthesis first and then to undertake separation and structural characterization of the stereoisomers, if mixtures were indeed formed during the synthesis, subject to encouraging preliminary biological results.

The preparation of *N*-(*p*-carbethoxyphenyl)-β-alanine (**4**) has been described previously by DeGraw.[3] Catalytic hydrogenation of **4** with PtO_2 in acetic acid at 45 psig resulted in the formation of the fully reduced, noncrystalline product, which was homogeneous by TLC criteria. The splitting patterns of the carbethoxy functionality in the NMR spectrum of **5** also indicated (clean quartet and triplet) that the catalytic reduction product **5** is not a mixture of the cis and trans isomers. However, more positive evidence regarding the stereochemistry of this product, such as evaluating the splitting pattern of the 1 and 4 hydrogens under the 1,4-substituents, could not be carried out because these signals were buried under the cyclohexyl protons. Since **5** was obtained by catalytic hydrogenation, by analogy it was presumed that this compound has the cis configuration and was subsequently converted to the *N*-trifluoroacetyl derivative **6** by standard procedures.[12] The conversion of **6** to the bromomethyl ketone **7** was carried out by modification of the Arndt–Eistert synthesis.[13,14] Reaction of **7** with NaN_3 in aqueous MeOH gave the azido ketone **8**, which was protected at the ketone functionality as the ethylene ketal **9**, with *p*-toluenesulfonic acid as a catalyst. Hydrogenation of **9** with 5% Pd/C gave the desired masked α-amino ketone **10**, which was reacted with 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine in absolute MeOH. The pyrimidine intermediate **11** thus obtained was deprotected with an aqueous mixture of TFA/HCl to the ketone **12**.

Conversion of the 6-substituted pyrimidine intermediate **12** to the pteroic acid intermediate **13** was carried out by procedures developed in our laboratory. Briefly, the 5-nitro group of **12** was reduced with sodium dithionite in aqueous DMF,[15] and the dried reduction product was subjected to the simultaneous cyclization oxidation procedure with pyridine and pyridine hydrochloride in ethanol.[16] The progress of the reaction was monitored by the characteristic shift of the UV absorption maximum of the 6-substituted 7,8-dihydropteridine derivative from 325 to 365 nm in 0.1 N NaOH, which represents the long-wavelength UV maximum of a 6-alkyl-substituted, fully oxidized pteridine. The NMR spectrum of the ethyl homopteroate analogue thus obtained exhibited resonances of the 1,4-protons of the cyclohexane ring in an axial equatorial relationship.[17] These protons were clearly resolved

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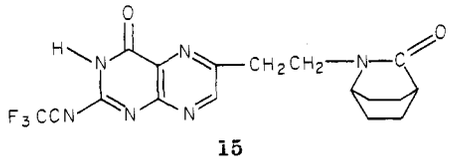
in this compound, with the equatorial proton resonating at 2.9 ($\omega_{1/2}$ = 6 Hz) and the axial at 3.5 ($\omega_{1/2}$ = 15 Hz) ppm. Individual assignment of these protons, however, could not be made because on hydrolysis to **13** these resonances shifted considerably and were buried under the methylene envelope of the cyclohexane ring or the resonances of the bridge methylene groups. Hydrolysis of this derivative to **13** was carried out with NaOH in aqueous acetonitrile.[18]

When attempts were made to purify the crude hydrolysate by ion-exchange chromatography over DEAE–Cl resin, compound **13** was not adsorbed on the column. This mobility of **13** on the column could be attributed to the lack of a net negative charge on the molecule at pH 7.5. Purification was, nevertheless, achieved simply by passing the hydrolysate through the column. Minor impurities and highly colored materials that were present in the hydrolysate were removed by this procedure, and the light yellow effluents from the column, containing **13**, were pooled, evaporated to a small volume, and acidified. The yellow precipitate of **13** in TFA exhibited resonances due to the C^7 proton of the pteridine ring as a sharp singlet at 8.9, the bridge ethylene protons as two broad multiplets at 3.9 and 3.5, and the cyclohexyl protons as a complex set of signals appearing between 2.5 and 1.5 ppm; these spectral data were in complete agreement with the required structure.

To complete the synthesis of **3**, the only remaining step was the glutamate conjugation of **13**. This necessitated the protection of the highly basic 11-amino group. It has been known from our previous work[11,15] that the trifluoroacetyl group is an excellent choice for protecting reactive functionalities of pteroate analogues during the glutamate coupling procedures. These protective groups on the pteroate framework also render them more soluble in solvents commonly used for such coupling, either by the solid phase or solution chemistry methods. When **13** was stirred with excess trifluoroacetic anhydride and worked up as usual, a creamy white product was obtained that was presumed by analogy to have the required structure **14**. This product (**15**) also exhibited NMR resonances in TFA that were compatible with structure **14**. However, when **15** was used for the next step, it failed to undergo the glutamate coupling reaction. On further examination, it was discovered that **15** was insoluble in 0.1 N NaOH, indicating the absence of the carboxy group. Therefore, it was concluded that upon treatment with trifluoroacetic anhydride, a peptide bond was formed between the 11-amino group and the carboxy group of compound **13**, resulting in the formation of **15**.



15

The formation of **15** required the orientation of the 1',4'-substituents in **13** to be cis, and since no other base-soluble trifluoroacetyl derivative was isolated from the trifluoroacetylation reaction, it was concluded that compound **13** is the pure cis derivative. Recalling the axial–equatorial relationship exhibited by the 1,4-hydrogens of the N^{11}-trifluoroacetylethyl ester of **14**, one must assume that base hydrolysis of the ethyl ester did not epimerize the 1' hydrogen. Although these observations permitted us to make the stereochemical assignments of the 1,4-substituents on the cyclohexane ring of **13** to be cis, the major problem of glutamate coupling of **13** remained to be studied.

At this stage it occurred to us that in order to form the internal peptide bond between the 11-amino group and the carboxy group of **13**, activation of the carboxy group to the trifluoroacetyl mixed anhydride[19] should precede the trifluoroacetylation of the 11-amino group. Indeed, it has been shown previously from our laboratory that the trifluoroacetyl mixed anhydride of pteridine-6-carboxylic acid reacts with various amines to form peptide bonds.[19] Therefore, we felt that if this trifluoroacetylation reaction is carried out in excess trifluoroacetic acid, *N*-trifluoroacetylation will predominate over mixed anhydride formation, and the internal peptide bond formation can be prevented or suppressed.

In actuality, selection of such conditions permitted the preparation of the trifluoroacetyl derivative **14** in excellent yield. However, there is a possibility that under these conditions the cis configuration of **13** might have been altered to a trans configuration by epimerization at the 1' position by TFA. To rule out this possibility, the trifluoroacetyl groups on **14** were hydrolyzed with 0.1 N NaOH to a homopteroic acid, which was found to be indistinguishable from **13** by UV or NMR spectroscopy. Coupling of the L-glutamate moiety to **14** was carried out in DMF by the isobutyl chloroformate method, and the resulting diethyl ester was hydrolyzed to the title compound **3** in ~40% yield. Final purification of **3** was achieved by ion-exchange chromatography over DEAE-cellulose. The purity of **3** was judged by its sharp symmetrical elution from a DEAE column when monitored at 245 nm. The compound appeared to be stereochemically pure as judged by the elution pattern as well as the sharp signal of the C^7 pteridine proton and the undistorted triplet due to the C^2 glutamate proton in the 220-MHz NMR spectrum of **3**. If target compound **3** was a mixture of the cis and trans isomers, some broadening of these signals would have been expected. It has been previously established from chemical and spectral observations that **13** had the cis configuration, and due to the absence of evidence of epimerization during the glutamate coupling of **13** and its subsequent hydrolysis, we must conclude that the target compound **3** also has the 1,4-substituents on the cyclohexane ring oriented in a cis configuration. Although the evidence presented regarding the stereochemistry is convincing, these stereochemical assignments should be considered tentative.

Biological Evaluation and Discussion

The 7,8-dihydro derivatives of **13** and **3** were prepared in aqueous solution by dithionite reduction of their potassium salts as described previously for the preparation of dihydro-11-oxahomofolate.[11] These derivaties were examined for their ability to function as synthetic substrates of dihydrofolate reductase with the *L. casei* enzyme. Even at 10 times the dihydrofolate reductase concentrations over the controls, both the homopteroate and the homofolate analogues failed to show any significant substrate activity. Controls with dihydrofolate and dihydrohomofolate showed activity. The substrate concentration used in all cases was 1×10^{-4} M. In addition, we examined the parent compound **3** for substrate activity toward DHFR, employing a system that was previously shown to

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Table I. Bacterial Growth Inhibition of 3, 13, and Their Reduced Derivatives[a]

compd	concn, ng/mL, for 50% inhibn: *S. faecium* ATCC 8043	concn, ng/mL, for 50% inhibn: *S. faecium* MTX resistant
1',2',3',4',5',6'-hexahydrohomopteroic acid (13)	>2000	>2000
7,8-dihydro-1',2',3',4',5',6'-hexahydrohomopteroic acid	130	>2000
d,l-5,6,7,8-tetrahydro-1',2',3',4',5',6'-hexahydrohomopteroic acid	260	>2000
1',2',3',4',5',6'-hexahydrohomofolic acid (3)	2000	>2000
7,8-dihydro-1',2',3',4',5',6'-hexahydrohomofolic acid	30	>2000
d,l-L-5,6,7,8-tetrahydro-1',2',3',4',5',6'-hexahydrohomofolic acid	38	>2000

[a] None of the above compounds supported growth in the absence of folate at 200 ng/mL.

reduce folate to tetrahydrofolate.[20] However, in this system also, **3** was found to be inactive.

Next, the catalytic reduction products of **13** and **3** were investigated for their ability to function either as inhibitors or pseudocofactors of *L. casei* thymidylate synthase. These *d,l*-tetrahydro derivatives at a concentration of 3×10^{-4} M did not show any detectable pseudocofactor activity even at enzyme levels 10 times those normally used. Neither of these tetrahydro derivatives inhibited the standard thymidylate synthase assay when added to the assay medium at 1×10^{-4} M.

Although results obtained in vitro with *L. casei* DHFR and *L. casei* TS were disappointing, the antifolate activities of both **13** and **3** and their respective dihydro and tetrahydro derivatives were evaluated in two folate-requiring microorganisms. These organisms were the MTX-sensitive and MTX-resistant strains of *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecium* (ATCC 8043). The results with *S. faecium* are summarized in Table I. None of the compounds showed inhibition of either strain of *L. casei* up to a concentration of 2 μg/mL. This inactivity against *L. casei* can be explained in terms of the lack of inhibition of *L. casei* DHFR and *L. casei* TS by these derivatives. On the other hand, the dihydro and tetrahydro derivatives of both **13** and **3** showed significant inhibition of the growth of *S. faecium*. Although there exists a possibility that these derivatives may function as inhibitors of DHFR and TS derived from *S. faecium*, and thereby offer an explanation of their activities against the growth of *S. faecium*, such conclusions should await further in vitro studies. Homofolate derivatives are known to exhibit species specificity. 1-L-Tetrahydrohomofolate has been shown to be an effective inhibitor of *Escherichia coli* TS,[4] whereas it did not show inhibition of the *L. casei* and mammalian enzymes.[7,8] We have previously shown that 1-L-tetrahydro-11-oxahomofolate prepared by the enzymatic reduction of its dihydro derivative with *L. casei* DHFR inhibited the growth of *L. casei*, whereas this tetrahydro derivative prepared by the enzymatic reduction with human KB cell DHFR did not inhibit the KB cell growth.[21] Since various previously synthesized homofolate analogues[7,8,11,12] exhibited species specificity with regard to their antifolate activities,[7,8,21] it appears that evaluation of the biological activities of **13**, **3**, and their reduced derivatives utilizing mammalian enzymes and mammalian cell lines in culture is of interest. Such studies are in progress.

Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer. 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 strengths of the various proton resonances are expressed in δ units (parts per million) and coupling constants in hertz. Peak multiplicity is depicted as follows: s, singlet; d, doublet; t, triplet, q, quartet; br, broadened singlet or unresolved multiplet, and c, complex signal, 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 2.5 × 20 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 Galbraith Laboratories, Ind., Knoxville, TN. 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.

Preparation of *N*-(4-Carbethoxycyclohexyl)-β-alanine (5). The starting material, *N*-(4-carbethoxyphenyl)-β-alanine (4), was prepared according to the procedure of DeGraw.[3] We carried out the hydrogenation of 4 to 5 by dissolving 10 g of **4** in 75 mL of HOAc and hydrogenating at 45 psig for 18 h using 500 mg of PtO_2. The product was freed from the catalyst by filtration, and upon evaporation and evacuation, the glassy residue of **5** was obtained in quantitative yield: NMR ($CDCl_3$) δ 4.15 (q, 4 H, ethoxy), 3.2 and 2.65 (2 t, 4 H, ethylene), 2.4–1.45 (c, 10 H, cyclohexyl), 2.05 (s, 6 H, 2 CH_3COOH), 1.28 (t, 3 H, ethoxy). Anal. ($C_{12}H_{21}NO_4 \cdot 2CH_3COOH$) C, H, O.

Conversion of 5 to *N*-(Trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)-β-alanine (6). Ten grams of **5** was added to a chilled solution of 100 mL of trifluoroacetic anhydride in a 250-mL round-bottomed flask placed in an ice bath. This was stirred for 2 h at 0 °C. The bath was removed, and the reaction was allowed to proceed at room temperature (RT) for 18 h and then evaporated. The residue was treated with 500 mL of ice-cold water, and the product was extracted in 300 mL of ethyl acetate. The ethyl acetate layer was washed several times with distilled water, dried with Na_2SO_4, and evaporated. The gummy product was dissolved in 40 mL of benzene and applied on a column made of 200 g of silica gel CC7 in benzene. The column was eluted with methylene chloride, and ten 100-mL fractions were collected. Fractions 2–9 showed a single spot on silica gel TLC plates. These fractions were pooled and evaporated to obtain the colorless glassy product **6** in 90% yield. Anal. ($C_{14}H_{20}F_3NO_5$) C, H, F.

Preparation of 1-Bromo-4-[*N*-(trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)amino]butane-2-one (7). To a solution of 6.78 g (20 mmol) of **6** in 100 mL of benzene was added 1.8 mL (25 mmol) of thionyl chloride. This was refluxed for 1 h under strictly anhydrous conditions. The reaction mixture was evaporated to dryness and evacuated for 2 h. This acid chloride was dissolved in 30 mL of anhydrous ether and was added while stirring to a chilled solution of 65 mmol of diazomethane dissolved in 125 mL of ether. This mixture was stirred at 25 °C for 1 h, followed by bubbling HBr through the solution until saturated. The reaction mixture was evaporated to dryness at 30 °C by rotary evaporation. The brownish viscous product thus obtained was dissolved in 200 mL of ethyl acetate, washed several times with distilled water, dried over Na_2SO_4, and evaporated. Upon examination by TLC, this crude product showed two major compounds (silica gel GF 250, chloroform); the less polar minor component was identified to be the methyl ester of **6** by comparison with an authentic sample, which was freed from the desired bromo ketone **7** by column chromatography over silica

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gel CC7. The pure noncrystalline **7** was obtained in 80% yield by this procedure: NMR ($CDCl_3$) δ 4.25 (q, 2 H, ethoxy), 3.9 (s, 2 H, bromomethyl), 3.58 and 3.0 (2 t, 4 H, methylenes), 2.6–1.5 (c, 10 H, cyclohexyl), 1.25 (t, ethoxy). The ethoxy quartet and triplet were slightly broadened in this spectrum, indicating the presence of an isomeric compound, although the bromomethyl signal was sharp. Anal. ($C_{15}H_{21}BrF_3NO_4$) C, H, Br.

Conversion of 7 to 1-Azido-4-[*N*-(trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)amino]butane-2-one (8). In a 250-mL Erlenmeyer flask was dissolved 2.91 g (7 mmol) of bromo ketone **7** in 70 mL of methanol and stirred with a solution of 5.19 g (78 mmol) of sodium azide in 16 mL of H_2O at RT for 3.5 h. The reaction mixture was evaporated almost to dryness and diluted to 200 mL with distilled water, and the product was extracted into 150 mL of ethyl acetate. The ethyl acetate layer was washed three times with water, dried over Na_2SO_4, and evaporated to a golden yellow syrup. The infrared spectrum of this crude product, when run neat, showed the characteristic intense absorption of the azide group at 2110 cm^{-1}. Subsequent purification of this material was achieved by column chromatography over a Florisil column, eluting the column with methylene chloride: yield 2.38 g (91%); this product could not be crystallized; NMR ($CDCl_3$) δ 4.15 (q, 2 H, ethoxy), 3.95 (s, 2 H, azidomethyl), 3.6 and 2.7 (2 t, 4 H, methylenes), 2.6–1.5 (c, 10 H, cyclohexyl), 1.3 (t, 3 H, ethoxy). This spectrum was also very similar to that of **7**, indicating broadening of the quartet and triplet due to the ethoxy signal, presumably reflecting the presence of an isomer that could not be resolved by chromatography. Anal. ($C_{15}H_{21}F_3N_4O_4$) C, H, N.

Preparation of 1-Azido-4-[*N*-(trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)amino]-2,2-(ethylenedioxy)butane (9). In a 500-mL round-bottomed flask were added 600 mL of *p*-toluenesulfonic acid, 15 mL of ethyleneglycol, and 5.67 g (15 mmol) of **8**, and the mixture was refluxed with 300 mL of benzene with a Dean–Stark apparatus. During the 6-h reflux period, the benzene in the flask was changed three times by withdrawal of the distillate through the apparatus and addition of fresh dry benzene to the flask. At the end of the 6-h period, most of the benzene was removed from the flask, and the flask and its contents were allowed to cool to ~30 °C and treated with 2 g of $KHCO_3$. The contents of the flask were diluted with 500 mL of 10% $KHCO_3$, and the product was immediately extracted with 200 mL of ethyl acetate. After washing, drying, and evaporating the ethyl acetate layer as usual, we obtained the crude product **9** in 70% yield. This product was freed from minor impurities by column chromatography on Florisil, eluting with CH_2Cl_2: NMR ($CDCl_3$) δ 4.5 (q, 2 H, ethoxy), 4.03 (s, 4 H, ethylenedioxy), 3.21 (s, 2 H, azidomethyl). Anal. ($C_{17}H_{25}F_3N_4O_5$) C, H, F.

Hydrogenation of 9 and Preparation of 1-[(2-Amino-4-hydroxy-5-nitropyrimidin-6-yl)amino]-4-[*N*-(trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)amino]-2,2-(ethylenedioxy)butane (11). In a Parr hydrogenation flask was dissolved 4.22 g (10 mmol) of **9** in 75 mL of MeOH, and 500 mg of 10% palladium on carbon was added. This mixture was hydrogenated at 10 psi for 18 h; during this time, all the azide **9** was converted to the more polar amino compound, as evidence by TLC. The product was removed from the catalyst by filtration, and upon evaporation of the filtrate, a gummy product was obtained. The IR spectrum of this material showed the complete disappearance of the azide band. The amino compound thus obtained was redissolved in 75 mL of MeOH, mixed with a solution of 2.14 g (11.2 mmol) of 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine, and refluxed for 1 h. At this stage, 1.68 mL (15 mmol) of *N*-methylmorpholine was added to the reaction mixture, and the reflux continued for 3 h, followed by evaporation to ~50 mL. Upon cooling, crystals of **11** were formed. These were separated by filtration and washed successively with water, ethanol, and ether: yield 4.12 g (66%); mp 140–145 °C dec. Anal. ($C_{21}H_{29}F_3N_6O_8$) C, H, N.

Preparation of 1-[(2-Amino-4-hydroxy-5-nitropyrimidin-6-yl)amino]-4-[*N*-(trifluoroacetyl)-*N*-(4-carbethoxycyclohexyl)amino]butan-2-one (12). We carried out the deprotection of **11** to **12** by dissolving 2.75 g (5 mmol) of **11** in 75 mL of trifluoroacetic acid at 55–60 °C and gradually adding 75 mL of 0.1 N HCl over a period of 20 min. When the addition was complete, the reaction mixture was evaporated to dryness under vacuum and triturated with 100 g of ice. The yellow solid thus obtained was filtered, washed with water, and dried: yield 2.5 g (94%); mp 186 °C; UV (0.1 N NaOH) λ_{max} 340 nm. Anal. ($C_{19}H_{25}F_3N_6O_7 \cdot 0.5HCl$) C, H. F.

Conversion of 12 to 1',2',3',4',5',6'-Hexahydrohomopteroic Acid (13). A. Dithionite Reduction of 12. A solution of 1.75 g (3.3 mol) of **12** in 35 mL of DMF in an Erlenmeyer flask was placed in a water bath maintained at 55 °C. Fifteen grams of sodium dithionite was added carefully to the solution and vigorously stirred. Over a period of 20 min, 35 mL of distilled water was added to the stirring suspension. When the addition of water was almost complete, a homogeneous solution was obtained, and the yellow color of the original solution almost disappeared. Addition of 200 g of crushed ice to this mixture induced the precipitation of the reduction product, which was filtered and dried: yield 1.1 g (70%); UV (0.1 N NaOH) λ_{max} 325 and 280 nm; mp 158 °C.

B. Cyclization.[15,16] This dithionite reduction product was suspended in 100 mL of a 1:1 mixture of pyridine and methanol containing 0.5 mL of concentrated HCl and refluxed under nitrogen for 1 h. The nitrogen and heat were cut off, and the reaction mixture was allowed to stir for 4 h under aerobic conditions. During this time, the UV λ_{max} at 325 nm (in 0.1 N NaOH) slowly disappeared, and a new λ_{max} at 360 nm appeared. No UV spectral change was observed after 3 h, and the oxidation was judged complete. The solution was evaporated to dryness, and 100 g of crushed ice was added. The bright yellow precipitate of the ethyl pteroate analogue was filtered, washed, and dried: yield 850 mg; UV (0.1 N NaOH) λ_{max} 253 and 360 nm; NMR (TFA) δ 8.9 (s, 1 H, pteridine), 4.32 (q, 2 H, ethoxy), 4.05 (c, 4 H, methylenes), 3.5 and 2.9 (2 s, 2 H, 2', 4' hydrogens of cyclohexane), 2.5–1.5 (c, 8 H, cyclohexane), 1.4 (t, ethoxy).

C. Hydrolysis. We hydrolyzed this cyclization product by stirring 800 mg of the product with 100 mL of 0.1 M NaOH and 25 mL of acetonitrile for 6 h at 25 °C. The acetonitrile was evaporated off at 30 °C with rotary evaporation under vacuum, and the clear aqueous solution of **13** thus obtained was adjusted to pH 7.5 with 1 N NCl, diluted to 500 mL, and applied on a column of DEAE chloride (20 × 5 cm bed). When the application was complete, the column was washed with 200 mL of 0.05 N NaCl in 0.005 M phosphate buffer, pH 7. All the effluents from the column were pooled and evaporated to ~50 mL. Upon acidification with glacial HOAc to pH 4.0, a bright yellow precipitate was formed. After cooling overnight in the refrigerator, the thick precipitate was filtered, washed several times with ice-cold distilled water previously adjusted to pH 4.0 by HOAc, and dried over P_2O_5: yield 550 mg; mp >300 °C; UV (0.1 N NaOH) λ_{max} 365 and 253 nm; NMR (TFA) δ 8.9 (s, 1 H, pteridine), 3.9 (br, 2 H, methylene), 3.7 (c, 3 H, methylene and 1 H of cyclohexane), 2.5–1.5 (c, cyclohexyl protons). Anal. ($C_{15}H_{20}N_6O_3 \cdot H_2O$) C, H, N, O.

Preparation of 1',2',3',4',5',6'-Hexahydrohomofolic Acid (3). A. Trifluoracetylation of 13. In a round-bottomed flask was stirred 125 mg of **13** with 25 mL of trifluoroacetic anhydride for 72 h and then evaporated. On trituration with ice, a cream-colored product was formed, which was separated by filtration and dried. The NMR spectrum of this compound was almost identical with **13**, with the C^7 pteridine proton appearing at δ 8.85. This trifluoroacetyl derivative (**15**) was insoluble in 0.1 N NaOH and did not undergo the glutamate coupling reaction, mp >300 °C. Anal. ($C_{17}H_{17}F_3N_6O_3$) C, H, F.

In the next attempt, 400 mg of **13** was dissolved in 15 mL of trifluoroacetic acid and stirred for 15 min. To this solution was added 5 mL of trifluoroacetic anhydride, and stirring was continued for 6 h. Upon evaporation, a gummy product was obtained, which on trituration with 5 g of ice gave **14** as a white solid. This was filtered and dried: yield 480 mg (93%). This compound was soluble in Na_2CO_3 solution.

B. Preparation of 3. The glutamate coupling reaction was carried out as follows. A solution of 446 mg (1 mmol) of **14** in 30 mL of dry DMF was prepared in a 250-mL round-bottomed flask. To this solution was added 0.14 mL (1.25 mmol) of freshly distilled *N*-methylmorpholine and 0.13 mL (1 mmol) of isobutyl chloroformate, and it was then stirred for 35 min. During this interval, 480 mg (2 mmol) of diethyl L-glutamate hydrochloride was dissolved in 30 mL of DMF in another flask, and 0.23 mL (2 mmol) of *N*-methylmorpholine was added. These two solutions

were combined and allowed to stir at 25 °C for 18 h and then evaporated to dryness at 60 °C under vacuum. One-hundred grams of ice was added, and the contents were triturated. After the ice had melted, the gummy product was separated from water by decantation and hydrolyzed with 100 mL of 0.1 N NaOH and 25 mL of acetonitrile at 25 °C for 6 h. Most of the acetonitrile was removed by rotary evaporation under vacuum. The solution was adjusted to pH 7.5 with 1 N HCl, diluted to 450 mL with distilled water, and applied on a DEAE column. The unreacted pteroic acid **13** was eluted during the application. When the application was complete, the column was washed with 200 mL of 0.015 M NaCl in 0.005 M phosphate buffer, pH 7.0. The column effluents were pooled for the recovery of unreacted **13**. After removal of **13**, the gradient was started, and a clean, pure, UV-absorbing material was eluted. Fractions corresonding to this material were pooled, evaporated to 15 mL, chilled, and acidified to obtain the light yellow product **3**, which was separated by filtration, washed with ice-cold water at pH 4.0 (adjusted with HOAc) several times to remove inorganic materials, and dried: yield 180 mg (39%); mp >300 °C; NMR 220 MHz (TFA) δ 8.85 (s, 1 H, C^7 proton), 4.8 (t, 1 H, α proton of glutamate), 3.8 and 3.5 (2 t, bridge ethyl), 3.4 (c, 1 H, 1′-H or 4′-H), 2.8–1.6 (cyclohexane).

A small amount of this material (5 mg) was dissolved in 0.5 mL of 0.005 N NaOH, diluted to 50 mL, adjusted to pH 7.5 with 0.1 N HCl, and chromatographed on a 5 × 25 cm DEAE column with a linear NaCl gradient. Compound **3** eluted from the column at 0.03 M NaCl as a single, symmetrical, UV-absorbing peak, establishing its purity. Anal. ($C_{20}H_{27}N_7O_6 \cdot H_2O$) C, H, N, O.

Biological Evaluation. The methods of preparation of the 7,8-dihydro and *d,l*-tetrahydro analogues of **13** and **3** were identical with those employed previously in our laboratories for the preparation of such derivatives of 11-oxa- and 11-thiohomofolate. Details of these procedures have been published.[10,11] Thymidylate synthase assays were carried out according to the procedure of Wahba and Friedkin.[22] Microbiological and dihydrofolate reductase assays were performed as described.[23,24]

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Registry No. **3**, 83704-88-5; 7,8-dihydro-**3**, 83719-42-0; *dl*-dihydro-**3**, 83705-02-6; **4**, 6959-76-8; **5**, 83704-89-6; **6**, 83704-90-9; **6** acid chloride, 83704-91-0; **7**, 83704-92-1; **8**, 83704-93-2; **9**, 83704-94-3; **10**, 83704-95-4; **11**, 83704-96-5; **12**, 83704-97-6; **12** dithionite reduced, 83704-98-7; **13**, 83705-00-4; 7,8-dihydro-**13**, 83705-01-5; *dl*-dihydro-**13**, 83710-12-7; **13** ethyl pteroate analogue, 83704-99-8; **14**, 83710-11-6; diazomethane, 334-88-3; 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine, 1007-99-4; diethyl L-glutamate hydrochloride, 1118-89-4; thymidylate synthase, 9031-61-2; dihydrofolate reductase, 9002-03-3.

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Conformational Requirements for Histamine H_2-Receptor Inhibitors: A Structure–Activity Study of Phenylene Analogues Related to Cimetidine and Tiotidine†

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Two series of compounds related to cimetidine and tiotidine were synthesized as part of a study to evaluate the importance of conformational parameters in binding at histamine H_2 receptors. The flexible methylthioethyl connecting chain was replaced by a conformationally restricting phenylene unit. These compounds were evaluated for antagonism of the dimaprit-stimulated chronotropic response in the guinea pig atrium and inhibition of histamine stimulated secretion of gastric acid in the dog. In both series, biological activity is markedly dependent on the *m*-phenylene regioisomers. Histamine H_2-receptor activity is retained in both series; however, in the tiotidine series, gastric antisecretory activity is significantly improved. Regardless of the end group, *N*-cyanoguanidine (**1b**), 1,1-diamino-2-nitroethene (**2b**), or 3,4-diamino-1,2,5-thiadiazole 1-oxide (**3b** and **4b**), each 3-(2-guanidino-4-thiazolyl)phenyl analogue was ca. 8 and 90 times more potent intraveneously than tiotidine and cimetidine, respectively. The electronic influences of the phenylene unit on biological activity were also evaluated. It was concluded that the geometric constraints imposed by the *m*-phenylene connecting element were more important than electronic factors in binding events at the histamine H_2 receptor.

The development of the clinically useful histamine H_2-receptor antagonist cimetidine as a gastric antisecretory drug has provided the stimulus in the search for more active agents.[1] Two highly potent histamine H_2-receptor inhibitors, ranitidine[2] [1-(methylamino)-1-[[2-[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]-amino]-2-nitroethane] and tiotidine[3] [*N*-cyano-*N*′-methyl-*N*″-[[2-[[(2-guanidino-4-thiazolyl)methyl]thio]-ethyl]amino]guanidine] evolved from such endeavors.

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