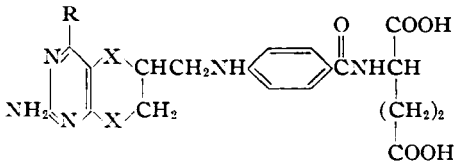
Analogs of Tetrahydrofolic Acid VII

Synthesis of N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid, An Inhibitor of Folic Reductase

By B. R. BAKER and C. E. MORREAL

The title compound (XI) has been synthesized in a six-step sequence using ethyl acetoacetate, acrolein, guanidine, and *p*-aminobenzoyl-L-glutamic acid. This compound inhibits folic reductase and binds to the enzyme better than the substrate, folic acid. It also inhibits dihydrofolic reductase.

FIFTEEN ENZYMES UTILIZING folic acid, tetrahydrofolic acid (I), or derivatives of tetrahydrofolic acid are known (2–4). A number of these enzymes are inhibited (5–8) by aminopterin (4-amino-4-deoxyfolic acid), but nearly as many are not (7, 9–11). In contrast, 5,6,7,8-tetrahydroaminopterin (II) can inhibit some of the enzymes not inhibited by aminopterin (11, 12). 5,8-Dideaza-5,6,7,8-tetrahydroaminopterin (IV) has been recently synthesized (13) and found to have inhibitory properties similar to tetrahydroaminopterin (13, 14). In addition, 5,8-dideaza-5,6,7,8-tetrahydrofolic acid (III) (15) has been found to bind to folic reductase eight times stronger than the substrate, folic acid (16).



I. R = OH, X = —NH—
II. R = NH_2, X = —NH—
III. R = OH, X = —CH_2—
IV. R = NH_2, X = —CH_2—

The folic cofactor area should be a prime target for utilization of recent developments in nonclassical antimetabolite theory (17–19), since larger differential effects on inhibition of these enzymes might be obtained by the bulk principle of specificity (17), the exo-alkylating irreversible inhibition phenomenon (18), and the bridge principle of specificity (19). In order to use these three corollaries of nonclassical antimetabolite theory, it would be advisable to have an inhibitor that can be made by a relatively short sequence and the sequence should be one that lends itself to the placing of substituents in a variety of positions. Synthesis of compound XI, which satisfies both the inhibitor and synthetic requirements, is the subject of this paper.

Although the synthesis of ethyl 2-acetylglutaraldehydate (V) by Michael addition of ethyl acetoacetate to acrolein has been described by Moe and Warner (20), but no yield recorded, their procedure gave 5–20% yields in this laboratory. After considerable experimentation, consistent yields were obtained when it was finally realized that the reaction of ethyl acetoacetate with acrolein was so rapid that a 2:1 adduct between acrolein and the keto ester was being obtained before an individual drop could be adequately mixed. By addition of an ethanolic solution of acrolein to the vortex of a stirred ethanol solution of a 30% excess of ethyl acetoacetate containing a trace of sodium ethoxide, consistent yields of about 47% of V were obtained. The aldehyde function of V was then selectively converted to a diethyl acetal (VI)[1] in 56% of yield by reaction of V with boiling ethanol containing ammonium chloride, under which conditions the ketone function was not converted to an enol ether.

Reaction of VI with guanidine hydrochloride and sodium methoxide in boiling absolute ethanol gave the crystalline pyrimidine VII in 76% yield; guanidine carbonate was equally effective. In order to complete the synthesis of XI *via* the anil X, it was considered necessary to block the 2-amino group of VII, before the aldehyde function was unblocked in order to avoid polymerization of the amino aldehyde (13, 15, 21). Acetylation of VII with hot acetic anhydride (13, 15, 21) gave small and variable yields of the crystalline acetamido acetal, VIII; the oily by-products appeared to contain *O*-acetate bands in the infrared, suggesting that some acetolysis of the acetal function was taking place in the mildly acidic medium. As a result of this apparent side reaction, acetylation was run with acetic anhydride in pyridine at 85° when consistent yields of crystalline VIII in the order of 57% were obtained.

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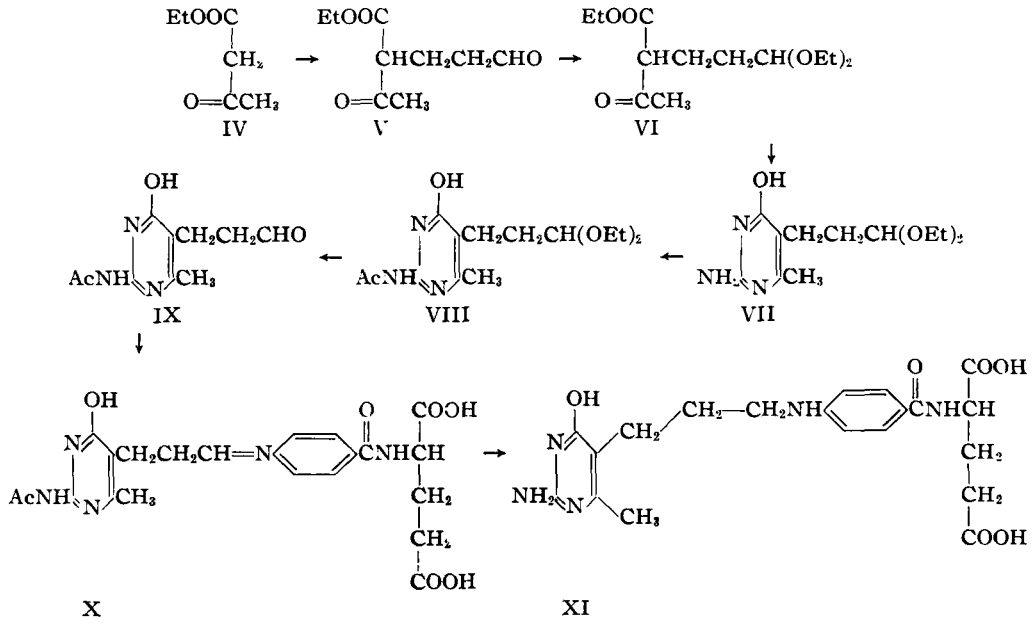
[1] Attempts to prepare VI by condensation of ethyl acetoacetate with β-chloropropionaldehyde diethyl acetal in absolute ethanolic sodium ethoxide with or without the presence of sodium iodide were unsuccessful. The reaction was slow, the products boiled over a wide range and examination of various fractions by analysis and infrared spectra did not give data compatible with structure VI.



Hydrolysis of the acetal function of VIII with 90% formic acid (13, 15, 21) gave variable yields of the crystalline aldehyde IX; unfortunately, the best yields were obtained on quantities of less than 1 Gm. of VIII. However, short boiling in water was sufficient to hydrolyze the acetal group of VIII to IX which was readily purified in consistent yields of 45–50%.

When a solution of the aldehyde IX and *p*-aminobenzoyl-L-glutamic acid in ethanol was refluxed for 1 hour, an amorphous precipitate separated from solution that resisted purification and gave poor analytical values for the supposed anil, X. In addition, the maximum amount of *p*-aminobenzoyl-L-glutamic acid, as determined quantitatively by the Bratton-Marshall reaction (22), that could be obtained on mild acid hydrolysis was 37–44 mole%; the analytical figures and the Bratton-Marshall values indicated that some de-N-acetylation of IX or X or both had occurred in the absolute alcohol, followed by the formation of higher condensation products. Although the anil was highly impure, this material did serve for working out suitable conditions for reduction and de-N-acetylation of X to the desired folic acid analog, XI.

The reduction of crude X with sodium borohydride was followed by a combination of change in ultraviolet spectrum at pH 1 and the amount of Bratton-Marshall dye obtained after short acid hydrolysis. The reduced product XI, had a peak at 222 mμ[2] that was absent in the anil, X, but present in *p*-aminobenzoyl-L-glutamic acid at 224 mμ with lower extinction coefficient; thus the reaction could be followed by the increase in extinction. The reduction of X in aqueous sodium bicarbonate with a large excess of sodium borohydride was complete in 1 hour at room temperature; the reaction was followed by periodic removal of aliquots, short hydrolysis in 1.5 *N* acid to remove the N-acetyl group, then measurement of the extinction coefficient at 222 mμ in 0.1 *N* acid. In this way the first sample of pure XI was obtained in about 15% yield.

Since the formation of the anil X in absolute ethanol led to side-reactions involving alcoholysis of the N-acetyl group, the anil was prepared *in situ* by reaction of IX with *p*-aminobenzoyl-L-glutamic acid in dimethylformamide; without isolation, the anil was reduced with sodium borohydride after the addition of methanol. In this way, XI was prepared in 44% yield (based on IX) containing about 5% of Bratton-Marshall positive material. Final purification afforded 21% of XI (based on IX) of pure product giving a negative Bratton-Marshall test.

The folic acid analog, XI, inhibited folic reductase (16) and had a $K_i = 2 \times 10^{-6}$, which is one-fifth the K_m value of folic acid. This compound also inhibited the dihydrofolic reductase from amethopterin-resistant Ehrlich ascites; an 0.2 m*M* concentration of XI gave a 66% inhibition of the enzymatic reaction run in the presence of 0.02 m*M* dihydrofolic acid (23). Since this compound is constructed from ethyl acetoacetate, acrolein, guanidine, *p*-aminobenzoic acid, and L-glutamic acid, it is obvious that a variety of substituted derivatives of XI can be made by modifying the five components or by transformations of VII; in this way, compounds could be obtained that—by use of non-classical antimetabolite theory (17–19)—might selectively inhibit some of the 15 enzymes in the folic acid cofactor area. In addition, considerable information could be obtained about the relative binding and conformational requirements of these substrates when complexed to their respective enzymes (14). Such a program is continuing in these laboratories.

EXPERIMENTAL[3]

Ethyl 2-Acetylglutaraldehydate (V).—To a vigor-

[2] It could be anticipated that the ultraviolet spectrum of XI and III (15) would be similar, since their chromophores are identical.

[3] Melting points were determined on a Kofler Heizbank and are corrected. Ultraviolet spectra were obtained on a Cary 11 recording spectrophotometer and infrared spectra on a Perkin-Elmer 137B recording spectrophotometer.

ously stirred solution of 130 Gm. (1 mole) of ethyl acetoacetate and 0.1 Gm. of sodium methoxide in 350 ml. of absolute ethanol was added dropwise to the vortex, a solution of 46.4 Gm. (0.83 mole) of acrolein in 100 ml. of absolute ethanol; the addition time was 70 minutes, the temperature was maintained at 10–13° by adequate cooling, and the solutions were protected from moisture. After standing for an additional 30 minutes without cooling, the solution was neutralized with acetic acid, then spin-evaporated to a syrup *in vacuo*. A solution of the syrup in 300 ml. of chloroform was washed with water, dried with magnesium sulfate and evaporated *in vacuo*. Distillation of the residue gave 66.2 Gm. (47%) of a colorless oil, b.p. 89–101° (0.5 mm.), that was suitable for the next step. Redistillation of a similar preparation gave pure material, b.p. 99–101° (0.5 mm.); $\lambda_{max.}^{film}$ 3.68 (aldehyde CH); 5.79 (aldehyde and ester C=O); 5.88 (ketone C=O); 6.12 (weak enolic C=C); 8.09, 8.20 μ (ester C—O—C).

Ethyl 2-Acetylglutaraldehydate Diethyl Acetal (VI).—To a solution of 66.2 Gm. (0.36 mole) of V in 450 ml. of absolute ethanol was added 2 Gm. of ammonium chloride. After being refluxed for 2 hours, the mixture was spin-evaporated to a syrup *in vacuo*. The residue was partitioned between 300 ml. of methylene chloride and 50 ml. of water. Washed again with 50 ml. of water and dried with magnesium sulfate, the organic solution was evaporated *in vacuo* and the residue distilled. After a forerun of 7.7 Gm. of unchanged V, the product distilled as a colorless oil, b.p. 107–124° (0.3 mm.) that was suitable for the next step; yield, 46.3 Gm. (50%). Redistillation gave the pure product, b.p. 110–112° (0.2 mm.); $\lambda_{max.}^{film}$ 5.77 (ester C=O); 5.84 (ketone C=O); 6.12 (weak enolic C=C); 8.09 (ester C—O—C); 9.45 μ (ether C—O—C). When the reaction was run for 4.5 hours, the yield was increased to 56%.

Anal.—Calcd. for $C_{13}H_{24}O_5$: C, 60.1; H, 9.64. Found: C, 60.0; H, 9.70.

β-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-propionaldehyde Diethyl Acetal (VII).—To a mixture of 1.52 Gm. (0.016 mole) of guanidine hydrochloride and 0.86 Gm. (0.016 mole) of sodium methoxide in 40 ml. of absolute ethanol was added 4.16 Gm. (0.016 mole) of VI. After being refluxed for 19 hours, the solvent was spin-evaporated *in vacuo*. The residue was partitioned between 50 ml. of chloroform and 30 ml. of water. The separated chloroform layer was dried with magnesium sulfate, then evaporated to dryness *in vacuo*. Recrystallization of the residue from ethyl acetate gave 3.1 Gm. (76%) of product, m.p. 176–177°, that was suitable for the next step. Further recrystallization from ethyl acetate afforded white crystals, m.p. 179–180°; $\lambda_{max.}^{KBr}$ 2.93, 3.14 (OH, NH); 6.10, 6.29 (NH, pyrimidine ring); 9.45 μ (ether C—O—C).

Anal.—Calcd for $C_{12}H_{21}N_3O_3$: C, 56.6; H, 8.31; N, 16.5. Found: C, 56.5; H, 8.10; N, 16.7.

β-(2-Acetamido-4-hydroxy-6-methyl-5-pyrimidyl)propionaldehyde Diethyl Acetal (VIII).—A solution of 15 Gm. of VII in 60 ml. of reagent pyridine and 40 ml. of acetic anhydride was heated in a bath at 80–90° for 1 hour protected from moisture. The solution was spin-evaporated to a syrup *in vacuo;* the evaporation was then repeated after the addition of 60 ml. of toluene. Recrystallization of the residue from ethyl acetate gave 10 Gm. (57%) of product, m.p. 148–150°. Further recrystallization from ethyl acetate afforded white crystals, m.p. 150°; $\lambda_{max.}^{KBr}$ 3.15 (NH); 6.05 (amide I); 6.28, 6.38 (NH and pyrimidine ring); 9.44, 9.66 μ (ether C—O—C).

Anal.—Calcd. for $C_{14}H_{23}N_3O_4$: C, 56.6; H, 7.81; N, 14.1. Found: C, 56.8; H, 7.94; N, 14.3.

β-(2-Acetamido-4-hydroxy-6-methyl-5-pyrimidyl)propionaldehyde (IX).—A solution of 1.00 Gm. (4.5 mmoles) of VIII in 50 ml. of water was refluxed for 1 hour, then spin-evaporated to dryness *in vacuo*. Two recrystallizations from ethyl acetate gave 0.34 Gm. (45%) of pure IX, m.p. 158–160°; $\lambda_{max.}^{pH\ 1}$ 228 (ϵ 8880), 270 mμ (ϵ 8270); $\lambda_{max.}^{pH\ 8.4}$ 270 mμ (ϵ 5350); $\lambda_{max.}^{pH\ 13}$ 278 mμ (ϵ 10,500); $\lambda_{max.}^{KBr}$ 3.16 (OH, NH); 3.65 (aldehyde CH); 5.87 (aldehyde C=O); 6.16 (amide I and pyrimidine ring); 6.74 (NH and pyrimidine ring); no ether C—O—C near 9.5 μ.

Anal.—Calcd. for $C_{10}H_{13}N_3O_3$: C, 53.8; H, 5.87; N, 18.8. Found C, 53.6; H, 5.95; N, 18.6.

N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-p-aminobenzoyl-L-glutamic Acid (XI).—A solution of 100 mg. (0.5 mmoles) of IX and 120 mg. (0.5 mmoles) of *p*-aminobenzoyl-L-glutamic acid in 2 ml. of dimethylformamide was allowed to stand for 10 minutes, then diluted with 15 ml. of reagent methanol. The solution was then treated with 0.20 Gm. of sodium borohydride portionwise with stirring over a period of 30 minutes, then stirred overnight at room temperature. After the addition of 5 ml. of 0.1 *N* sodium hydroxide, the solution was spin-evaporated *in vacuo* to 5 ml., then acidified to pH 5 with 3 *N* hydrochloric acid. After being chilled for several hours, the mixture was centrifuged and the white solid washed successively with water (2 × 5 ml.), absolute alcohol (2 × 5 ml.) and dry ether (5 ml.); yield, 84 mg. (44%) that contained 5% of Bratton-Marshall positive material calculated as the anil (X). Recrystallization from dimethylformamide by addition of water gave 40 mg. (21%), m.p. >250°, of pure material, as determined by its ultraviolet spectra and negative Bratton-Marshall test. A similar preparation was analyzed and had $\lambda_{max.}^{pH\ 1}$ 222 (ϵ 27,800), 270 (ϵ 19,200), and 303 mμ (ϵ 11,400); $\lambda_{max.}^{pH\ 8.4}$ 295 mμ (ϵ 15,100); $\lambda_{max.}^{pH\ 13}$ 284 mμ (ϵ 20,500).

Anal.—Calcd. for $C_{20}H_{23}N_5O_6$: C, 55.8; H, 5.85; N, 16.2. Found: C, 56.2; H, 5.66; N, 16.5.

The Bratton-Marshall test was performed in the following way. A 1-ml. aliquot in 5% aqueous sodium bicarbonate, estimated to contain 0.2 to 0.5 mg. of XI, was diluted with 1 ml. of 3 *N* hydrochloric acid, then heated on a steam-bath for 15 minutes to hydrolyze any anil (X) to *p*-aminobenzoyl-L-glutamic acid. This solution was then cooled, diazotized and coupled with N-(1-naphthyl)-ethylenediamine as described by Bratton and Marshall (22). The amount of dye was determined by its extinction at 560 mμ using *p*-aminobenzoyl-L-glutamic acid as a standard.

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Nonaqueous Titrimetric Analysis of Aminophylline

By THOMAS MEDWICK and FREDERICK SCHIESSWOHL

A nonaqueous potentiometric titration is described by which ethylenediamine and theophylline, the components of aminophylline, are titrated differentially as a mixture of bases. Acetic acid is used as the solvent for the ethylenediamine neutralization after which acetic anhydride is added to the system to allow titration of the theophylline, a very weak base. This method was applied to the analysis of aminophylline powder, tablets, ampuls, and suppositories. When compared with the U.S.P. XVI analyses, the nonaqueous approach is simpler since no elaborate sample treatment is needed. The precision of the one titration, nonaqueous procedure is about the same as the U.S.P. XVI analyses which require two titrations to obtain the same data. An alternative approach is suggested for cases, for example, some tablets, where the nonaqueous method is not successful.

VARIOUS APPROACHES to the analysis of aminophylline, a mixture of theophylline and ethylenediamine, have been reported. In the majority, these procedures measure the theophylline which is present and (by appropriate calculation) express the result as aminophylline. Connors (1) discussed the argentometric, ultraviolet spectrophotometric, and other methods which have been used. In the U.S.P. XVI (2–5) the theophylline content of aminophylline in its various forms is determined by an argentometric titration procedure involving several preliminary steps. In aminophylline powder and ampuls only (2, 3) in addition to the theophylline analysis, an ethylenediamine assay is specified.

The xanthines, theophylline, theobromine, and caffeine possess analytically useful acid-base properties. All three xanthines have been found to be very weakly basic, pKb's (aqueous) > 13 (6), and, in addition, theophylline and theobromine are weakly acidic, pKa (aqueous) = 8.6, pKa (aqueous) = 10, respectively. Acetic anhydride has been used as a solvent component in the titration of these compounds as bases. The titrant used exclusively was acetous perchloric acid. Theophylline has been titrated in a 4:1 nitromethane-acetic anhydride solvent (7) and theobromine, dihydroxypropyltheophylline, and caffeine were titrated in mixed solvents containing acetic anhydride and nitromethane, benzene, toluene, or dioxane (8, 9). Similarly, Anastasi, Gallo, and Novacic (10) reported the satisfactory titration of caffeine in an acetic anhydride–acetic acid solvent. Recent work by Ellert, Jasinski, and Pawelczak (11) showed that, although theophylline did not titrate as a base in propionic acid, a propionic acid–propionic anhydride solvent permitted satisfactory titration as a base. Employing the acidic behavior of theophylline, McEniry (12) was able to determine the theophylline content of aminophylline by titrating a sample in dimethylformamide with sodium methoxide titrant.

Although nonaqueous titrimetric methods have been applied to theophylline determination, no work appears to have been done in regard to measuring both the theophylline and ethylenediamine by this means. Since ethylenediamine

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