The recrystallized product gave a negative Bratton–Marshall test for arylamine; the crude product contained 3% Bratton–Marshall positive material calculated as starting material (XXIVb).

N-[1-(2-Amino-4-mercapto-6-methyl-5-pyrimidiyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid (II).—To a hot solution of 98.6 mg. (0.5 mmole) of XVIII in 30 ml. of absolute ethanol was added 133 mg. (0.5 mmole) of *p*-aminobenzoyl-L-glutamic acid. After being refluxed with stirring for 1 hr., the solution was filtered from a trace of insoluble material, then spin-evaporated *in vacuo* leaving 231 mg. (100%) of crude XXIVa; $\lambda_{max}^{pH\ 1}$ 222, 282, 317 mμ, $\lambda_{max}^{pH\ 8.4}$ 307 mμ; $\lambda_{max}^{pH\ 13}$ 312, shoulder at 270 mμ.

A solution of 230 mg. (0.5 mmole) of the crude XXIVa in 20 ml. of reagent methanol and 1.7 ml. of *N* methanolic sodium methoxide was allowed to stand at room temperature protected from moisture for 1 hr. After the addition of 0.7 g. (18.7 mmoles) of sodium borohydride, the mixture was refluxed with stirring for 90 min., then spin-evaporated *in vacuo*. The residue was dissolved in 25 ml. of water and the solution adjusted to pH 3 with 6 *N* hydrochloric acid. The precipitate was collected by centrifugation, then washed successively with two 5-ml. portions of water and two 5-ml. portions of hot water; yield, 146 mg. (69%), which contained 4% of Bratton–Marshall positive material calculated as the intermediate thiopyran (XXIVa). For purification, 142 mg. was dissolved in 20 ml. of 1% aqueous sodium bicarbonate; the filtered solution was acidified to pH 3 and the product collected and washed as before; yield, 119 mg. (55%) of amorphous solid that contained 2.6% Bratton–Marshall positive material and melted at 180–189° dec.; $\lambda_{max}^{pH\ 1}$ 337 mμ (ε 16,200); $\lambda_{max}^{pH\ 8.4}$ 310 (20,200), inflection centering at 350 mμ (ε 7200); $\lambda_{max}^{pH\ 13}$ 312 mμ (ε 24,000).

Anal. Calcd. for $C_{20}H_{25}N_5O_5S$: C, 53.7; H, 5.63; N, 15.6; S, 7.16. Found: C, 53.5; H, 5.87; N, 15.6; S, 7.48.

The pH for reprecipitation of XXVa is critical; precipitation at pH 4.5 caused loss of nearly half the product. In contrast to I, XXVa (II) is somewhat soluble in alcohol and acetone; therefore washing with these solvents was avoided.

Acknowledgment.—We wish to thank Starks Associates, Inc. and the Cancer Chemotherapy National Service Center, National Cancer Institute for large-scale preparation of certain intermediates, mediated by Contract No. SA-43-ph-4346.

Analogs of Tetrahydrofolic Acid. IX.[1,2] Synthesis of N-[1-(2-Amino-4-hydroxy-6-phenyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid, a "Nonclassical" Inhibitor of Some Folic Cofactor Area Enzymes

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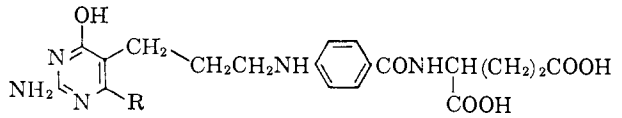
The title compound II has been synthesized in six steps from ethyl benzoylacetate. Due to the restricted rotation of the phenyl ring in 2-amino-5-(3,3-diethoxypropyl)-6-phenyl-4-pyrimidinol (VI), the synthesis of VI from ethyl α-(3,3-diethoxypropyl)benzoylacetate (VII) and guanidine presented difficulty; the rate of reaction of formation of the pyrimidine (VI) was sufficiently slow that alcoholysis of the keto ester VII was the predominant reaction. By use of dimethyl sulfoxide as a solvent, the yield in the condensation reaction was increased from 5 to 52%. II was an excellent inhibitor of folic reductase and was bound 100 times more tightly to the enzyme than the substrate folic acid. In addition, II was a good inhibitor of 5,10-methylenetetrahydrofolate dehydrogenase.

The important B-family vitamin, folic acid, is intracellularly reduced to its cofactor form, tetrahydrofolic acid, by the enzyme, folic reductase. The resultant tetrahydrofolic acid then participates as a cofactor in a series of enzymatic reactions for acceptance and transfer of "one-carbon" fragments involving at least 14 known



I, R = CH_3; II, R = C_6H_5

enzymes.[3-5] In enzymic transfer reactions between substrate and cofactor (or cosubstrate), the atoms to which the transfer group is attached are unlikely to be binding points of the molecule to the enzyme.[6] Therefore, it was postulated that the N-5 of the tetrahydrofolic acid molecule, involved in some of the transfer reactions in the folic cofactor area, should be replaced by a methylene in order to obtain an inhibitor.[7] The first such compounds synthesized were 5,6,7,8-tetrahydroquinazoline analogs of tetrahydrofolic acid[7-9] which showed good inhibition of folic reductase.[5,9] A simpler molecule was needed which could lend itself to the type of molecular manipulation needed for design of "nonclassical antimetabolites" and irreversible inhibitors of the "*exo*-alkylating" type.[6,10] Such a proposed[5] compound was the pyrimidyl analog I of tetrahydrofolic acid; I was synthesized and found to bind to folic reductase about as well as the substrate folic acid.[11]

(1) This work was generously supported by the U. S. Public Health Service by Grant No. CA-06624 and Training Grant No. 2G-555.

(2) For the previous paper of this series see B. R. Baker, C. E. Morreal, and B. Ho, *J. Med. Chem.*, **6**, 658 (1963).

(3) T. H. Jukes and H. P. Broquist, "Metabolic Inhibitors," R. M. Hochster and J. H. Quastel Ed., Academic Press, Inc., New York, N. Y., 1963, pp. 481–534.

(4) F. M. Huennekens, M. J. Osborn, and H. R. Whitely, *Science*, **128**, 120 (1958).

(5) B. R. Baker, paper V of this series, Preprints of the Scientific Session of the American Pharmaceutical Association Meeting, Las Vegas, Nevada, 1962.

(6) B. R. Baker, *Cancer Chemotherapy Rept.*, **4**, 1 (1959).

(7) R. Kuehler, L. Goodman, J. DeGraw, and B. R. Baker, *J. Am. Chem. Soc.*, **80**, 5779 (1958); paper I of this series.

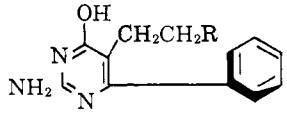
(8) J. I. DeGraw, L. Goodman, and B. R. Baker, *J. Org. Chem.*, **26**, 1156 (1961); paper III of this series.

(9) J. DeGraw, L. Goodman, B. Weinstein, and B. R. Baker, *ibid.*, **27**, 576 (1962); paper IV of this series.

(10) (a) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 454 (1962); (b) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962); (c) B. R. Baker and R. P. Patel, *Biochem. Biophys. Res. Commun.*, **9**, 199 (1962); B. R. Baker, *Biochem. Pharmacol.*, **12**, 293 (1963).

(11) (a) B. R. Baker, and C. E. Morreal, *J. Pharm. Sci.*, **51**, 596 (1962); paper VI of this series; (b) B. R. Baker and C. E. Morreal, *Ibid.*, **52**, 804 (1963); paper VII of this series.

To design an irreversible inhibitor of the *exo*-alkylating type,[6,10] it is necessary to find which areas of the inhibitor are bound to the enzyme, or conversely, which areas of the inhibitor are not in contact with the enzyme surface. The area of the inhibitor not in contact with the enzyme can be found by modification of the inhibitor with large or bulky groups in one or more positions; if these oversize inhibitors (so-called nonclassical antimetabolites) still inhibit the enzyme, then the area of the inhibitor where the oversized group is attached cannot be in contact with the enzyme surface.[12] It is at the area of no-contact between enzyme and inhibitor where covalent bond-forming groups might be placed to obtain irreversible inhibitors.[6,10] The first molecule chosen for this study had a larger substituent at the 6-position of the pyrimidine ring, namely II. The phenyl ring of II constitutes more than just increased planar bulk of the phenyl compared to methyl since the 5-side chain forces the benzene ring out of coplanarity with the pyrimidine ring (III) due to restricted rotation, thus increasing the total volume of the inhibitor in the neighborhood of the 6-position.

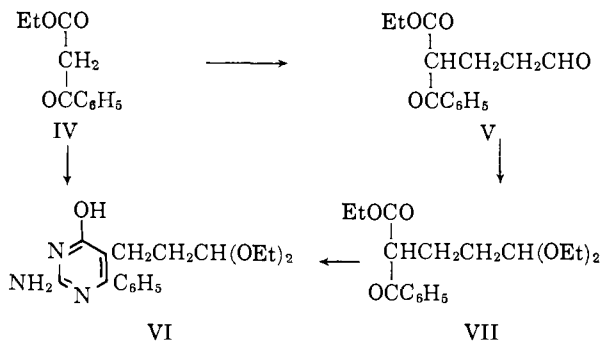


III

The synthesis of II and its inhibition of some of the enzymes in the folic cofactor area is the subject of this paper.

The sequence used for II followed a route similar to that for I with use of ethyl benzoylacetate in place of ethyl acetoacetate.[11] The difficulties and differences in reactions caused by the restricted rotation of the phenyl ring (III) compared to the reactions for synthesis of I are of both theoretical and practical interest. Michael condensation of ethyl benzoylacetate (IV) with acrolein gave the adduct V; considerable loss occurred on high-vacuum distillation, but the pure adduct was obtained in 26% yield. Conversion of V with ethanol to the acetal VII was carried out by ammonium chloride catalysis; again distillation losses were high, but the pure acetal was obtainable in 23% yield. Condensation of guanidine carbonate with VII in boiling ethanol gave a disappointing 5.3% yield of the desired pyrimidine VI; the yield was the same whether the reaction was run for 6 hr. or 24 hr., indicating that the starting material VII was suffering some type of cleavage. The accumulation of low yields, particularly the conversion of VII → VI, pointed out that this series starting with ethyl benzoylacetate involved unknown problems not encountered in the series starting with ethyl acetoacetate[11]; therefore detailed studies of the three reactions were made.

Reaction of acrolein with ethyl acetoacetate under the best conditions has been shown to give a mixture of 50% product, 25% starting material, and presumably 25% of disubstituted acetoacetic ester[11]; the former two were readily separated by fractional distillation. However, the much higher boiling point of V made separation of such a mixture by fractional distillation uneconomical due to decomposition. The decision was made to avoid distillation. Since the acrolein Michael addition compounds such as V are unstable, the crude mixture was treated with ethanol and ammonium chloride to give a mixture of ethyl benzoylacetate (IV), the acetal VII of the monoacrolein adduct, the acetal of the



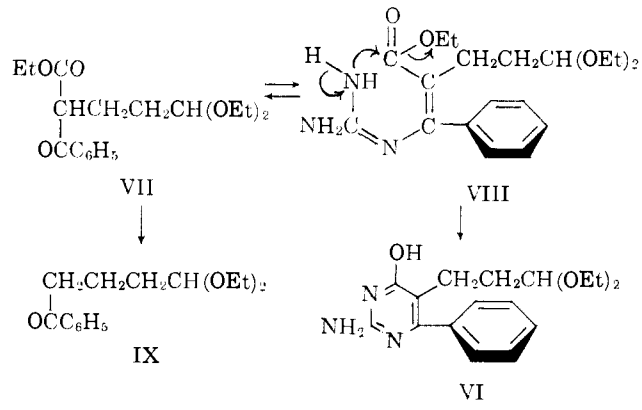
bisacrolein adduct, and possibly unknown impurities. Unchanged ethyl benzoylacetate was removed by washing a benzene solution with cold 3% sodium hydroxide solution. The amount of the desired VII could then be determined by quantitative ultraviolet analysis.

The analytical sample of the acetal VII showed λ_{max} 245 mμ (ε 14,400) in absolute ethanol; when converted to the enol with 0.1 *N* ethanolic sodium ethoxide, VII had λ_{max} 290 mμ (ε 11,900). Fractions boiling somewhat lower than VII showed lower molecular extinctions; these slightly lower boiling fractions are most likely contaminated with ω-diethoxyvalerophenone (IX), the product formed by pyrolytic loss of the carbethoxy group; this pyrolytic loss of the carbethoxy group of a β-keto ester is normally accompanied by considerable tar formation.[13] Crude VII, obtained in 79% yield from ethyl benzoylacetate (IV), showed λ_{max} 292 mμ (ε 8630) in 0.1 *N* ethanolic sodium ethoxide, thus showing a purity of 73%, or a maximum actual yield of 58% compared to 6% over-all when both V and VII are distilled.

Chronologically, two explanations were considered for the low 5% yield in the conversion of pure VII to the pyrimidine VI. For the sake of continuity, the second and correct explanation with the resultant increase in yield of VI will be considered first. The yield of VI remained at 5% whether the guanidine condensation in ethanol was run for 6 hr. or 24 hr., indicating that by 6 hr. all the keto ester VII not forming VI had been converted to some other product; in contrast, the corresponding acetoacetate gave a 76% yield of pyrimidine. Investigation of the oil left in the filtrate from VI by infrared and ultraviolet spectra showed that this material no longer contained a β-keto ester, but did contain carbonyl and phenyl absorption. It appeared that this material was either ω-diethoxyvalerophenone (IX) or a mixture of IX and ethyl 5,5-diethoxyvalerate formed by alcoholysis of the keto ester VII. Since the phenyl keto ester VII is more enolic than the corresponding methyl keto ester, alcoholysis of VII might be expected to be more rapid than in the corresponding acetoacetate series. In addition, condensation of VII

(12) For the approach and solution of this type of problem on lactic dehydrogenase and glutamic dehydrogenase see B. R. Baker, W. W. Lee, W. L. Skinner, A. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960); B. R. Baker, W. W. Lee, E. Tong, L. Ross, and A. Martinez, *J. Theort. Biol.*, **3**, 446 (1962).

(13) B. R. Baker, M. V. Querry, S. R. Safir, and S. Bernstein, *J. Org. Chem.*, **12**, 138 (1947).



with guanidine to give VI presumably proceeds through VIII; in order for VIII to ring close further, the limitation of possible conformations in the transition state due to restricted rotation will slow the conversion of VIII to VI.

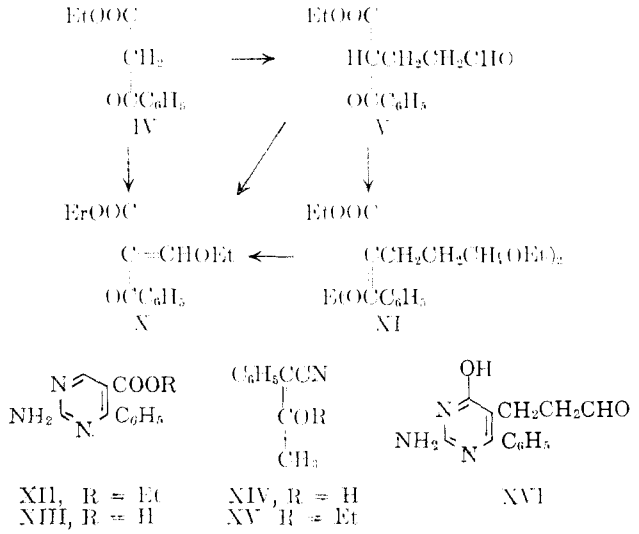
If the foregoing explanation is correct, then a solvent which could not cleave the β-keto ester VII to IX would be necessary; this poses the problem of the incompatability of guanidine carbonate with nonpolar solvents. Three solvents were selected for study. *t*-Butyl alcohol, which does not cause alcoholysis due to steric hindrance, was effective, although reaction was slow. The maximum yield of VI from pure VII was reached after 60 hr. of boiling; the yield of 38% was a considerable improvement over the 5% obtained in ethanol. Surprisingly, with N,N-dimethylformamide as solvent at 80° for 16 hr., no pyrimidine VI could be isolated. In contrast, another dipolar-aprotic solvent, dimethyl sulfoxide at 80°, was effective; the maximum yield of 45% of VI was reached after 48 hr. Since 1 mole of alcohol and 2 moles of water are formed for each mole of pyrimidine VI formed, it is possible for even these low concentrations of alcohol and water might cause some cleavage of VII to IX. When the calculated amount of hexamethyldisilazane, a scavenger for alcohol, or 2,2-dimethoxypropane, a scavenger for water, were included in the reaction mixture, the yield was not further increased; the use of 1:3 benzene–dimethyl sulfoxide as solvent, and periodical distillation and replacement of the benzene to remove alcohol and water, raised the yield to 52%. This latter reaction was slowed by the decrease in solubility of the guanidine carbonate in the mixed solvent medium; the method was considered too cumbersome for the 7% increase in yield obtained.

When crude, undistilled keto ester VII was condensed with guanidine carbonate in dimethyl sulfoxide, VI was obtained in 20% yield; the yield was 27% based on the estimated maximum purity of VII of 73% (by ultraviolet analysis). In a preparative-sized run the over-all yield of the pyrimidine VI for the three steps from ethyl benzoylacetate was 11.8%, a practical increase above the initial over-all yield of 0.3% when the intermediates were isolated and a standard guanidine condensation was used in the terminal step.

Some exploratory work was done on the use of *t*-butyl alcohol as a general solvent for synthesizing pyrimidines from guanidine carbonate and β-keto esters. Ethyl benzoylacetate, ethyl α-allylacetoacetate, ethyl α-aceto-γ-(1,3-dioxolan-2-yl)butyrate,[2] and ethyl acetoacetate gave 62, 55, 59, and 79% yields, respectively, of the corresponding 2-amino-4-pyrimidinols; these yields were about the same as could be obtained in absolute ethanol as a solvent. Apparently, these four keto esters do not undergo alcoholysis and no advantage is gained. However, as a general method, *t*-butyl alcohol or dimethyl sulfoxide can be preferable to ethanol since the chance for cleavage of the β-keto ester in sluggish reactions is largely avoided.

The second possible explanation for the low yield of the pyrimidine VI from the keto ester VII was based on some observations of Russell and Whittaker[14] on α-acetobenzylcyanides of type XIV. They observed that this type of compound failed to condense with guanidine, but condensed smoothly if first converted to an enol ether such as XV; they attributed the inability of the type XIV compound to condense with guanidine to the highly enolic character of XIV. Such an explanation might also have accounted for the low yield and low reaction rate in the conversion of VII to VI. When the crude keto aldehyde V reacted with ethyl orthoformate[13] *without acid catalysis*, the resultant crude enol ether XI gave a 12% yield of VI when condensed with guanidine; this was about three times the yield obtained from VII.

In order to try to force the conversion of V to XI with ethyl orthoformate, catalysis by *p*-toluenesulfonic acid was investigated. Distillation of the product



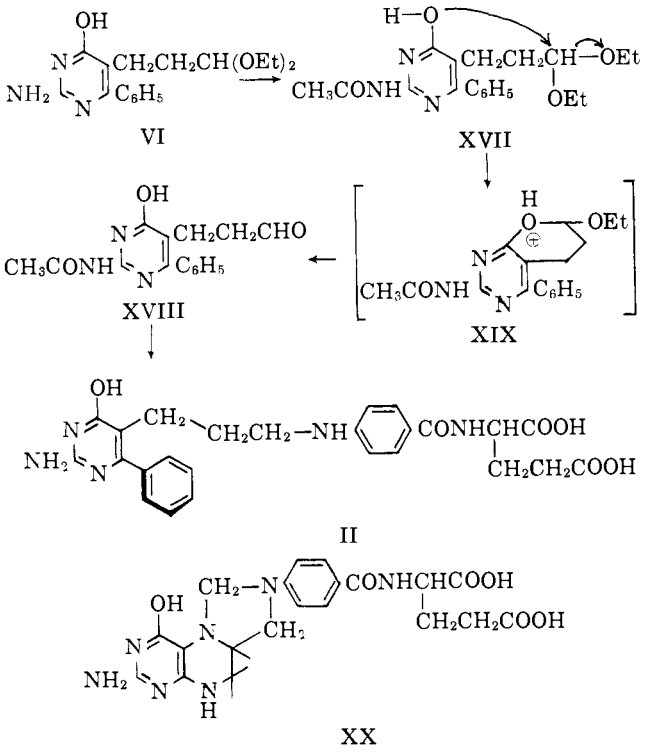
afforded an oil which ultimately was shown to be primarily ethyl α-ethoxymethylenebenzoylacetate (X). Since unchanged ethyl benzoylacetate had been removed earlier by base extraction, X must surprisingly arise by an acid-catalyzed reverse Michael condensation of V back to IV. A trial with the keto acetal VII showed this was also converted to X under acidic conditions.

Condensation of X with guanidine afforded 2-amino-5-carbethoxy-6-phenylpyrimidine (XII), analogous to the reaction of guanidine with ethyl α-ethoxymethyleneacetoacetate.[15] Initially the formation of XII was confusing since it was isomeric to 2-amino-4-hydroxy-6-phenyl-5-pyrimidinepropionaldehyde (XVI), and an apparent reverse Michael condensation of V to IV was unexpected and inconspicuous. That this product was not XVI was shown by its incompatible ultraviolet spectrum and its lack of aldehyde properties. Pure

(14) P. B. Russell and N. Whittaker, *J. Am. Chem. Soc.*, **74**, 1310 (1952).
(15) C. C. Price, N. J. Leonard, and R. H. Reitsema, *ibid.*, **68**, 767 (1946).

ethoxymethylene derivative X was then made from ethyl benzoylacetate (IV), ethyl orthoformate, and acetic anhydride. Condensation of X with guanidine afforded the carbethoxypyrimidine XII, identical with the sample prepared earlier; XII was further characterized by saponification to XIII. Thus, partial conversion of V or VII to the enol ether XI with ethyl orthoformate *without* acid catalysis did lead to an increase in yield of the pyrimidine, VI; however, this approach was discontinued when the condensation of the keto acetal VII with guanidine in dimethyl sulfoxide was found to be satisfactory.

Acetylation of the 2-aminopyrimidine VI with acetic anhydride in pyridine at 85–90° gave the crystalline 2-acetamidopyrimidine (XVII) in 60% yield. When boiled in water for 1 hr., the acetal group of XVII was hydrozlyed to give an 87% yield of pure pyrimidylpropionaldehyde (XVIII); this ease of hydrolysis of the



acetal group was also noted with the corresponding 2-acetamido-6-methyl pyrimidine.[11] Since the 2-aminopyrimidine acetal VI and the corresponding 6-methyl derivative are stable to boiling water for this length of time, the increased rate of hydrolysis of the acetal group of the 2-acetamidopyrimidine XVII must be accounted for. Such rate accelerations are characteristic of anchimeric assistance by a neighboring group. The explanation that the 4-hydroxyl of the 2-acetamidopyrimidine (XVII) is more enolic than that of the 2-aminopyrimidine VI is not lacking support; if the hydroxyl group of XVII is more enolic, then anchimeric assistance by the hydroxyl group can increase the rate of hydrolysis *via* a cyclic intermediate such as XIX, which in turn would rapidly hydrolyze further. Such cyclic intermediates have actually been isolated when a 4-mercapto group was present on the pyrimidine.[2] The change in the nature of the hydroxyl group in molecules of type XVII compared to type VI has been noted previously in that the 4-hydroxyl group of a 2-acetamido-4-pyrimidinol is readily replaced by chlorine under conditions where a 2-amino-4-pyrimidinol does not even react.[2]

Condensation of the pyrimidylpropionaldehyde XVIII with *p*-aminobenzoyl-L-glutamic acid in dimethylformamide, reduction of the resultant anil with sodium borohydride in methanol, and basic hydrolysis of the N-2 acetyl group afforded the phenylated folic acid analog II in 36% yield, as previously described for the synthesis of I,[11] but with some modification of the purification method.

This phenyl analog II was investigated as an enzyme inhibitor of the tetrahydrofolate type. At pH 6.1, compound II inhibited folic reductase from rat liver[16,17] much more effectively than the corresponding methyl analog I; II had an inhibitor–enzyme dissociation constant (K_i) of 9×10^{-8}, whereas I had a K_i of 2×10^{-5} and the substrate–folic acid complex had a dissociation constant (K_m) of 1×10^{-5}. Thus compound II is the most potent inhibitor of folic reductase known that does not have 2,4-diamino substituents of the aminopterin type.

At pH 7.4, II also was a good inhibitor of the dihydrofolic reductase from pigeon liver; in the presence of 6 μM dihydrofolate, II showed 50% inhibition at 20 μM concentration. The 5,10-methylenetetrahydrofolate dehydrogenase from pigeon liver was also inhibited; in the presence of 31 μM 5,10-methylenetetrahydrofolate (XX), II showed 50% inhibition at 100 μM, but I required 700 μM.[17,18]

The fact that II is a good inhibitor of folic (dihydrofolic) reductase and a fair inhibitor of 5,10-methylenetetrahydrofolate (XX) dehydrogenase shows that when the respective substrate–enzyme complexes are formed, the N-5-group of XX and folate must be part of the substrate surface *not in contact* with the enzyme surface. Thus compound II could be further substituted on the 6-phenyl with covalent bond-forming groups that might give good irreversible inhibitors[6,10] of these two enzymes.

The added bonus of II forming an enzyme-inhibitor complex with folic reductase that has 0.005 times the dissociation of the enzyme I-complex merits comment. Since part of the enzyme binding of folate, I, and of II is contributed by the 2-amino-4-hydroxypyrimidine moiety, the predominant tautomeric form of this moiety involved in binding to the enzyme must be favorably influenced by the 6-phenyl group. Further studies in this Laboratory are underway which might possibly shed light on this interesting and potentially useful phenomenon.

Experimental[19]

Ethyl α-Benzoylglutaraldehydate (V).—Condensation of 20 g. (0.10 mole) of ethyl benzoylacetate with 4.75 g. (0.085 mole) of acrolein in absolute alcohol, as described for the preparation of ethyl α-acetylglutaraldehydate,[11] gave an oil, which was distilled

(16) We wish to thank Dr. W. C. Werkheiser, Roswell Park Memorial Institute, for the assays on folic reductase from rat liver.

(17) Details will be published elsewhere.

(18) The technical assistance of Maureen Vince and Dorothy Ackerman for the assays on dihydrofolic reductase and 5,10-methylenetetrahydrofolate dehydrogenase is gratefully acknowledged.

(19) Melting points were taken in capillary tubes in a Mel-temp block. Melting points below 230° are corrected; those above are not. Boiling points are uncorrected. Infrared spectra and ultraviolet spectra were determined on Perkin-Elmer spectrophotometers, Models 137B and 202, respectively.

to give a fraction boiling at 125–150° (0.4 mm.). Redistillation and collection of the fraction boiling at 142–144° (0.3 mm.) afforded 5.66 g. (26%) of colorless oil; $\lambda_{max}^{6\%}$ 3.53, 3.68 (aldehyde CH), 5.81 (aldehyde and ester C=O), 5.97 (ketone C=O), 8.10 (ester C–O–C), 14.5 μ (benzoyl CH).

Anal. Calcd. for $C_{14}H_{16}O_4$: C, 67.7; H, 6.52. Found: C, 67.8; H, 6.65.

Considerable decomposition occurred in both distillations; as a result, on a larger scale, the per cent yield was lower.

Ethyl α-Benzoylglutaraldehydate Diethyl Acetal (VII).—A mixture of 25 g. (0.10 mole) of V, 150 ml. of absolute ethanol, and 0.54 g. of ammonium chloride was refluxed with magnetic stirring for 3 hr.; then the solvent was removed by spin-evaporation *in vacuo*. The residual oil was dissolved in 100 ml. of dichloromethane, washed with two 25-ml. portions of water, then dried over magnesium sulfate, and the solvent was removed *in vacuo*. Distillation through a short Vigreux column gave a middle fraction, b.p. 124–132° (0.2 mm.). Redistillation afforded 7.3 g. (23%) of colorless oil, b.p. 122–126° (0.15 mm.); λ_{max}^{EtOH} 245 mμ (ε 14,400); $\lambda_{max}^{0.1\ N\ NaOEt}$ 290 mμ (ε 11,900); $\lambda_{max}^{6\%}$ 5.84 (ester C=O), 5.96 (ketone C=O), 8.20 (ester C–O–C), 9.50 (ether C–O–C), 14.5 (benzoyl CH), and no aldehyde CH at 3.5–3.7 μ; $n^{29}D$ 1.5093.

Anal. Calcd. for $C_{18}H_{26}O_5$: C, 67.1; H, 8.19. Found: C, 67.2; H, 8.18.

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionaldehyde Diethyl Acetal (VI). (A). In Ethanol.—To a solution of 169 mg. (3.6 mmoles) of sodium methoxide in 50 ml. of absolute ethanol was added 296 mg. (3.1 mmoles) of guanidine hydrochloride and 1.00 g. (3.1 mmoles) of VII. The mixture was refluxed with magnetic stirring for 24 hr., then spin-evaporated to residue *in vacuo*. The residue was partitioned between 50 ml. of chloroform and 30 ml. of water. The separated chloroform layer was washed with 30 ml. of water, dried with magnesium sulfate, and was spin-evaporated to residue *in vacuo*. The gummy residue was dissolved in a minimum of boiling ethanol and then refrigerated. The white powder was collected and recrystallized twice from ethanol; yield, 52 mg. (5.3%), m.p. 226–228°; $\lambda_{max}^{pH\ 1}$ 230 (ε 14,100), 275 mμ (ε 9900); $\lambda_{max}^{pH\ 7}$ 229 (ε 15,400) 292 mμ (ε 7000); $\lambda_{max}^{pH\ 13}$ 225 (ε 14,800), 287 mμ (ε 6600); λ_{max}^{KBr} 2.95 (NH), 6.12, 6.40 (pyrimidine, NH), 9.50 (ether C–O–C), 14.3 μ (phenyl CH).

Anal. Calcd. for $C_{17}H_{23}N_3O_3$: C, 64.2; H, 7.25; N, 13.2. Found: C, 64.3; H, 7.26; N, 13.4.

The same yield was obtained when an equivalent amount of guanidine carbonate (1.6 mmoles) was used in place of guanidine hydrochloride and sodium methoxide.

(B). In *t*-Butyl Alcohol.—A mixture of 0.280 g. (1.5 mmoles) of guanidine carbonate, 1.00 g. (3.1 mmoles) of VII, and 10 ml. of *t*-butyl alcohol was refluxed with magnetic stirring for 60 hr., then cooled, and filtered. The product was washed successively with water, acetone, and hexane; yield, 0.368 g. (38%), m.p. 232–240° dec. A weak carbonate bond was present at 11.5 μ; otherwise the spectrum was identical with that obtained in method A.

(C). In Dimethyl Sulfoxide.—A mixture of 0.280 g. (1.5 mmoles) of guanidine carbonate, 1.00 g. (3.1 mmoles) of VII, and 3.0 ml. of dimethyl sulfoxide was magnetically stirred in a bath at 80° for 48 hr. The cooled reaction mixture was poured into a vigorously stirred mixture of 25 ml. of water and 25 ml. of benzene. The product was collected on a filter, then washed successively with water, acetone, and hexane; yield, 0.441 g. (45%), m.p. 236–238° dec., which was identical with the product from preparation B. Recrystallization from ethanol did not remove the weak carbonate band at 11.5 μ, but reprecipitation from a basic solution did.

If the reaction solution was poured directly into water, it was more difficult to free the product of oily impurities.

(D). Preparative Method.—To the vortex of a vigorously stirred solution of 100 g. (0.52 moles) of ethyl benzoylacetate and 0.1 g. of sodium methoxide in 300 ml. of absolute ethanol was added dropwise with ice-cooling over a period of 1 hr., a solution of 24.1 g. (0.43 moles) of acrolein in 50 ml. of absolute ethanol. The mixture was stirred for an additional hr. without cooling, then neutralized with 0.12 ml. of glacial acetic acid. The solvent was removed by spin-evaporation *in vacuo*. The residual crude V was dissolved in 300 ml. of absolute ethanol, 1 g. of ammonium chloride was added, and the mixture was refluxed with stirring for 3 hr. The solvent was spin-evaporated *in vacuo;* the residual oil was dissolved in a mixture of 200 ml. of benzene and 25 ml. of toluene, and the solution decanted from the ammonium chloride. The organic solution was washed five times with 100-ml. portions of ice-cold 3% sodium hydroxide solution then with three 100-ml. portions of water. The organic solution, dried with magnesium sulfate, was spin-evaporated *in vacuo* leaving crude VII, 104 g.

The 104 g. of crude VII was dissolved in 300 ml. of dimethyl sulfoxide (practical grade), 38.3 g. (0.213 moles) of guanidine carbonate was added, and the mixture was stirred in a bath at 80° for 48 hr. Most of the solvent was removed by distillation *in vacuo* (220 ml. recovered); the resulting still hot sirup was poured into a vigorously stirred mixture of 300 ml. of benzene and 300 ml. of water. After being stirred in an ice bath for 30 min., the mixture was filtered, and the product was washed successively with water, acetone, and hexane. The crude product was heated to boiling with about 250 ml. of ethanol; the insoluble product (19.5 g.) was collected by filtration, and the filtrate on being cooled deposited an additional 3.2 g. Recrystallization of the combined fractions from boiling ethanol gave 19.3 g. (11.8% based on IV) of product, m.p. 230–240° dec., which was suitable for further transformations.

Ethyl α-(Ethoxymethylene)benzoylacetate (X).—A mixture of 5.7 g. (30 mmoles) of ethyl benzoylacetate (IV), 4.4 g. (30 mmoles) of ethyl orthoformate, and 6.1 g. (60 mmoles) of acetic anhydride was refluxed for 90 min. Volatile materials were removed by spin-evaporation *in vacuo*. Distillation of the residue gave, after a low boiling forerun, 2.85 g. (38%) of product as a colorless oil, b.p. 128–130° (0.55 mm.); $\lambda_{max}^{6\%}$ 5.80 (ester C=O), 6.00 (ketone C=O), 7.83, 8.33 (ester C–O–C), 9.10 (ether C–O–C), 14.5 μ (phenyl).

Anal. Calcd. for $C_{14}H_{16}O_4$: C, 67.7; H, 6.45. Found: C, 67.5; H, 6.79.

This procedure is similar to that used for the preparation of ethyl α-(ethoxymethylene)acetoacetate.[20] Replacement of the acetic anhydride with 15 mg. *p*-toluenesulfonic acid, then distillation of the generated ethyl formate and ethyl alcohol over a period of 4 hr. gave a yield of 56% of product, b.p. 122–124° (0.2 mm.), that had an identical infrared spectrum. Reaction of crude V (free of IV) with ethyl orthoformate and *p*-toluenesulfonic acid in the same manner gave a 52% yield of X, b.p. 130–134° (0.4 mm.), which gave an infrared spectrum quite similar to pure X.

Anal. Found: C, 67.8; H, 7.23.

Similar results were obtained with crude VII (free of IV). All four distilled preparations of X gave XII on reaction with guanidine carbonate.

2-Amino-5-carbethoxy-6-phenylpyrimidine (XII). (A).—A mixture of 1.00 g. (4.53 mmoles) of X (prepared from IV), 10 ml. of absolute ethanol, and 0.545 g. (3.03 mmoles) of guanidine carbonate was refluxed with magnetic stirring for 18 hr., then the solvent was removed by spin-evaporation *in vacuo*. The residue was partitioned between 20 ml. of dichloromethane and 10 ml. of water. After drying with magnesium sulfate, the organic layer was evaporated *in vacuo* leaving 0.58 g. (59%) of product, m.p. 154°. Recrystallization from ethyl acetate gave white crystals, m.p. 156°, which had an infrared spectrum identical with the analytical sample in preparation B.

(B).—Similarly, reaction of 21.1 g. of X, prepared from V, gave 4.2 g. (28%) of recrystallized product, m.p. 155°; λ_{max}^{KBr} 2.95, 3.13 (NH), 5.97 (ester C=O), 6.13, 6.41 (NH, aromatic double bonds), 7.83 (ester C–O–C), 13.2 μ (phenyl); $\lambda_{max}^{0.1\ N\ HCl}$ 248 (15,500), 305 mμ (ε 5600); λ_{max}^{EtOH} 265 (23,100) 300 mμ (ε 4900); $\lambda_{max}^{0.1\ N\ KOH}$ 264 (18,200), 308 mμ (ε 2900) (the three ultraviolet curves were run in ethanol).

Anal. Calcd. for $C_{13}H_{13}N_3O_2$: C, 64.2; H, 5.41; N, 17.3. Found: C, 64.0; H, 5.22; N, 17.2.

2-Amino-6-phenylpyrimidine-5-carboxylic Acid (XIII).—A solution of 500 mg. (2.06 mmoles) of XII in 80 ml. of water and 25 ml. of 10% ethanolic potassium hydroxide was heated on a steam bath for 15 min., then acidified to pH 2 with hydrochloric acid. On being cooled, the solution deposited silky, white needles which were collected on a filter, then washed successively with water and acetone; yield, 332 mg. (75%), m.p. 271–274° dec.; λ_{max}^{KBr} 3.03 (NH), 4.10, 5.35 (NH^+), 5.95 ($C{=}NH^+$), 6.15, 6.35 (NH, double bonds), 7.85 μ (COO^-).

Anal. Calcd. for $C_{11}H_9N_3O_2$: C, 61.2; H, 4.18; O, 15.1. Found: C, 60.9; H, 4.50; O, 14.7.

The infrared spectrum indicated a dipolar ion structure. The found nitrogen analysis was about 2% low, indicating difficult combustion.

(20) L. Claisen, *Ann.*, **297**, 1 (1897).

2-Acetamido-4-hydroxy-6-phenyl-5-pyrimidylpropionaldehyde Diethyl Acetal (XVII).—A solution of 5.0 g. (15.8 mmoles) of VI, 50 ml. of pyridine, and 15 ml. of acetic anhydride was heated in a bath at 85–90° for 1 hr. Solvent was removed by spin-evaporation *in vacuo*. The residue was dissolved in 50 ml. of warm toluene and again spin-evaporated *in vacuo*. The toluene treatment was repeated twice more to remove the last of the pyridine. Recrystallization from benzene gave 3.39 g. (60%) of product, m.p. 133–140°, that was suitable for further transformations. Two further recrystallizations from di-*n*-butyl ether gave white crystals (77% recovery), m.p. 142–144°; λ_{max}^{KBr} 3.12 (NH), 6.05, 6.15, 6.43 (C=O, aromatic double bonds, NH), 9.45 (ether C–O–C); 12.9 μ (phenyl).

Anal. Calcd. for $C_{19}H_{25}N_3O_4$: C, 63.6; H, 6.99; N, 11.7. Found: C, 63.7; H, 6.99; N, 11.6.

2-Acetamido-4-hydroxy-6-phenyl-5-pyrimidylpropionaldehyde (XVIII).—A stirred mixture of 1.00 g. (2.78) mmoles) of XVII and 50 ml. of water was refluxed for 1 hr. after solution took place, solution requiring 15 min. Spin-evaporation to dryness *in vacuo* left 0.747 g. (91%) of product, m.p. 148–152°. Recrystallization from benzene gave white crystals, m.p. 149–152°; yield, 0.689 g. (87%); λ_{max}^{KBr} 3.15 (NH), 5.89 (aldehyde C=O), 6.20 (broad) 6.47, 6.73 (amide C=O, aromatic double bonds, NH), 14.3 (phenyl), no acetal at 9.45 μ.

N-[1-(2-Amino-4-hydroxy-6-phenyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid (II).—A solution of 500 mg. (1.75 mmoles) of XVIII and 465 mg. (1.75 mmoles) of *p*-aminobenzoyl-L-glutamic acid in 10 ml. of N,N-dimethylformamide was stirred for 20 min., then diluted with 75 ml. of reagent methanol. After the addition of 1.0 g. of sodium borohydride over a period of about 10 min., the reaction mixture was magnetically stirred for 18 hr. To the mixture was added 25 ml. of 0.1 *N* sodium hydroxide, then the solution was spin-evaporated *in vacuo* to about 20 ml. and diluted with 50 ml. of water. The solution was acidified to pH 5 with 3 *N* hydrochloric acid. After being chilled, the mixture was filtered and the product washed with water. The crude material was dissolved in 15 ml. of 3 *N* hydrochloric acid and heated on a steam-bath for 15 min. to hydrolyze most of the contaminating intermediate "anil" that was present. The cooled solution was brought to pH 8 with 10% sodium hydroxide, and the solution was clarified by filtration. The filtrate was acidified to pH 5 with 3 *N* hydrochloric acid; the product was collected by centrifugation and washed successively with four 5-ml. portions each of water, ethanol, and dichloromethane; after being dried overnight *in vacuo*, the product weighed 0.31 g. (36%). The product was further purified by solution in hot N,N-dimethylformamide, addition of water to turbidity, and chilling; the recovery was 68%. For analysis the precipitation from N,N-dimethylformamide was repeated twice more. The compound retains traces of solvent tenaciously; acceptable combustion values were only obtained after drying the sample at 100° under high vacuum. The recovery of material was 60–75% in each reprecipitation. Ultraviolet data showed that the second and third reprecipitations gave material with constants essentially identical with the material obtained in the first reprecipitation from N,N-dimethylformamide. The constants were as follows: $\lambda_{max}^{pH\ 1}$ 226 (ϵ 21,900), 285 mμ (15,300); $\lambda_{max}^{pH\ 7}$ 220 (ϵ 26,900), 298 mμ (19,700); $\lambda_{max}^{pH\ 14}$ 294 mμ (ϵ 20,250); the Bratton-Marshall test showed[11] 2.6% "anil" was present.

Anal. Calcd. for $C_{25}H_{27}N_5O_6$: C, 60.9; H, 5.48; N, 14.2; O, 19.5. Found: C, 61.1; H, 5.46; N, 14.1; O, 19.6.

The Synthesis of Antineoplastic Agents. XXXII. N-Nitrosoureas.[1] I.

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A number of N-nitrosoureas have been synthesized and evaluated for activity against Leukemia L1210. The most active member of the series thus far evaluated, 1,3-bis(2-chloroethyl)-1-nitrosourea, is highly active in a number of other experimental animal tumor systems.

The reported ability of 1-methyl-3-nitro-1-nitrosoguanidine to increase the life span of mice implanted intraperitoneally with Leukemia L1210[2] prompted us to investigate the anticancer activity of the closely related compound, 1-methyl-1-nitrosourea.[3] Although this compound showed only borderline activity against Adenocarcinoma 755 and Sarcoma 180, it proved even more effective, in our hands, against Leukemia L1210 than 1-methyl-3-nitro-1-nitrosoguanidine, increasing the life span by a factor of 2. These significant results caused 1-methyl-1-nitrosourea to be selected, along with other compounds of known activity against L1210 such as amethopterin, for evaluation against L1210 implanted intracerebrally in mice.[5] Of the compounds evaluated in this test system prior to this study, only 1-methyl-1-nitrosourea has shown significant activity.[6] These results stimulated our interest in the preparation of a number of congeners of 1-methyl-1-nitrosourea[7] for screening against experimental animal neoplasms, particularly Leukemia L1210.

Chemistry.—Ureas and N-nitrosoureas of varied structure have been prepared in this continuing study. Table I summarizes the syntheses of those ureas that were used in the preparation of the previously undescribed N-nitrosoureas of Table II; in addition, Table I includes several new ureas, the attempted nitrosations of which have not led thus far to the isolation of pure N-nitroso derivatives. The synthetic procedures used in the preparation of the ureas are adapted from known methods, which are indicated in the footnotes to Table I. Examples of unusual variations of these procedures are described in the Experimental section.

Each of the tabulated nitrosations involved the use of a cold acidic medium and either aqueous sodium nitrite solution of variable concentration or solid sodium

(1) This work was supported by funds from the C. F. Kettering Foundation and the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Contract No. SA-43-ph-1740. Part XXXI: C. Temple, Jr., C. L. Kussner, and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 866 (1962).

(2) Personal communication from Dr. Howard Bond of the Cancer Chemotherapy National Service Center. See also ref. 4.

(3) B. R. Baker and co-workers have investigated a large number of derivatives of 1-methyl-3-nitro-1-nitrosoguanidine itself.[4]

(4) W. A. Skinner, H. F. Gram, M. O. Greene, J. Greenberg, and B. R. Baker, *J. Med. Pharm. Chem.*, **2**, 299 (1960).

(5) A possible explanation of the failure of leukemias to respond to drug therapy is the sequestering of leukemic cells in the brain where they cannot be reached by drugs that fail to cross the so-called "blood-brain barrier." Compounds effective against experimental animal leukemias implanted in the brain might be of value in the treatment of the human disease.

(6) H. E. Skipper, F. M. Schabel, Jr., M. W. Trader, and J. R. Thomson, *Cancer Res.*, **21**, 1154 (1961).

(7) Degradation studies indicate that streptozotocin, a new broad spectrum antibiotic, contains an N-methyl-N-nitrosoamide or N-methyl-N-nitrosourea function.[8]

(8) E. R. Garrett, *J. Am. Pharm. Assoc. Sci. Ed.*, **49**, 767 (1960).