Department of Medicinal Chemistry, School of Pharmacy,
State University of New York at Buffalo

Analogs of Tetrahydrofolic Acid . XXI. Synthesis of 6-Bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine and its Evaluation as a Dihydrofolic Reductase Inhibitor (1,2)

B. R. Baker and Johannes H. Jordaan

The title compound (IV) has been synthesized from *p*-chlorophenylacetonitrile by Claisen condensation with ethyl ethoxyacetate, conversion to an enol ether, then reaction with guanidine to give the key intermediate 5-(*p*-chlorophenyl)-2,4-diamino-6-ethoxymethylpyrimidine (V). Cleavage of the ethyl ether of V with hydrogen bromide in acetic acid gave IV. The bromomethylpyrimidine (IV) was a good reversible inhibitor of dihydrofolic reductase from pigeon liver, but IV was not an irreversible inhibitor of the enzyme.

As a result of a recent study on the mode of binding of some 2,4-diaminopyrimidines to dihydrofolic reductase, it was proposed that an imidazole group in the active site of the enzyme would be a likely proton donor for reduction of the 5,6 and 7,8 double bonds of folic acid by hydride transfer from TPNH (3). If this imidazole group were juxtapositioned face-to-face (I) with the pyrazine moiety of folic acid, it could transfer a proton to either N-5 or N-8 of folic acid without shifting position in the active site (3). To test the possible proximity of an imidazole moiety to the N-8 position of folic acid when the latter is complexed with the active site of (dihydro)folic reductase, a potential active-site-directed irreversible inhibitor (4) was designed that could link covalently with this imidazole if it were located within close proximity to N-8; such a possible compound is 6-bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (IV). The synthesis and enzymic evaluation of IV is the subject of this paper.

The key intermediate 6-ethoxymethylpyrimidine (V) was synthesized by suitable modification of the procedures of Russell and Hitchings (5). Claisen condensation of *p*-chlorophenylacetonitrile (II) with ethyl ethoxyacetate to III with ethanolic sodium ethoxide proceeded in 61% yield, providing the isolation procedure (5a) was modified by acidification as the first step; water extraction of the soluble sodium salt of III as recommended (5a) led to a decrease in yield to 26% by hydrolytic cleavage of the enolate (III) back to II. Reaction of the crystalline enol (III) with ethyl orthoformate (5b) gave the enol ether (VI) as an oil with proper spectral properties; VI was condensed with guanidine hydrochloride and sodium methoxide in ethanol to give the pure, crystalline diaminopyrimidine (V) in 54% overall yield from III.

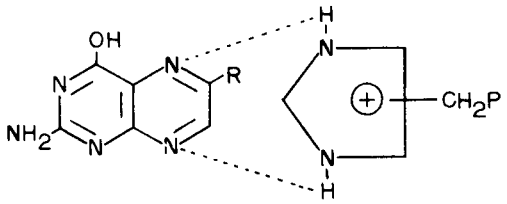
When the 6-ethoxymethylpyrimidine was reacted with boiling, 48% hydrobromic acid, the product was not the desired IV; ultraviolet spectra of the reaction product showed that cleavage of the 4-amino to 4-hydroxy (VII) had occurred, such acid lability of 2,4-diamino-5-arylpyrimidines having been previously observed (6). However, it was reasoned that cleavage of the ethoxy linkage to the bromomethyl should proceed just as rapidly with hydrogen bromide in acetic acid, but that solvolysis of the 4-amino group of the protonated diaminopyrimidine should be much slower with acetic acid than water. When the ethyl ether (V) was refluxed for eight hours in glacial acetic acid containing 10% hydrogen bromide, the cooled reaction mixture deposited 78% of analytically pure crystals of the desired 6-bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine hydrobromide (IV).

The ultraviolet absorption spectra of IV and V, and other 2,4-diamino-5-(*p*-chlorophenyl)-6-R-pyrimidines (5a, 5c, 6b) further illustrated the effect of side-chain substituents on the ultraviolet maxima of pyrimidines (7). These spectra usually consist of two bands, the one at shorter wavelength being attributed by Mason (7) to $\pi \longrightarrow \pi$ transitions of the system and the band at longer wavelength being ascribed to $n \longrightarrow \pi$ excitations of the pyrimidine ring. The position of the $n \longrightarrow \pi$ band is influenced mainly by electronic effects, the theory being that an electron donating substituent at the 6-position of the pyrimidine ring causes a shift to shorter wavelength and an electron withdrawing 6-substituent causes a shift to longer wavelength in the $n \longrightarrow \pi$ band (7). Accordingly the peak at 294 mμ in the spectrum of 2,4-diamino-5-phenylpyrimidine (A) in ethanol is shifted to 288 mμ in the spectrum of 2,4-diamino-5-phenyl-6-ethylpyrimidine (B) (Table I).

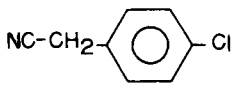
The 6-ethoxymethyl substituent of V being less electron donating than the 6-ethyl of C, causes a shift of the $n \longrightarrow \pi$ band of C at 286 mμ to 295 mμ. The bromide group of IV has still less electron-donating ability than the ethoxymethyl group of V and shifts the 286 mμ peak of C to an even longer wavelength (307 mμ at *p*H 13).

Protonation of the pyrimidine, as in IV, gives a peak at 290 mμ in ethanol and at *p*H 1, but is still at longer wavelength than the spectra of the 6-ethyl (272 mμ) and the 6-ethoxymethyl (273 mμ) in acid solution. This difference can again be attributed to the bromomethyl group being less electron donating than the latter two substituents.

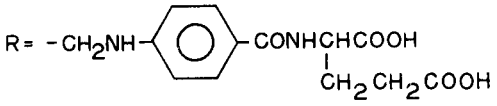
When a compound is to be assayed as a potential active-site-directed irreversible inhibitor (4), it is



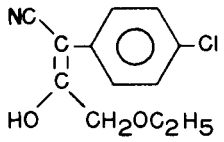
I



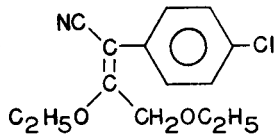
II



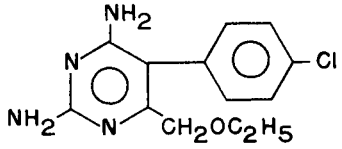
P=Protein



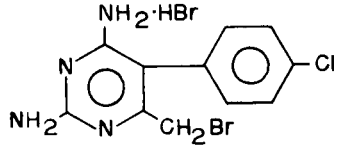
III



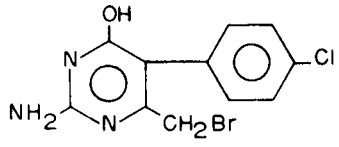
VI



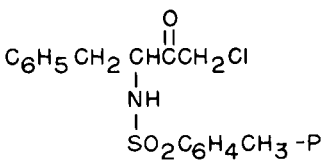
V



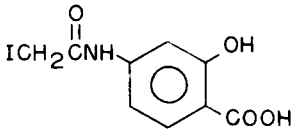
IV



VII



VIII, TPCK



IX

wise to establish three other parameters of the compound before the assay is attempted, namely:

(a) the reversible affinity of the compound for the enzyme by a measurement such as the enzyme-inhibitor dissociation complex (K_i) or the concentration of inhibitor necessary to give 50% inhibition (I_{50}) of the enzyme as a reversible inhibitor (8).

(b) the relative reactivity of the alkylating group - in this case the bromomethyl - compared to known active-site-directed irreversible inhibitors (4) such as 4-iodoacetamidosalicyclic acid (IX) for lactic and glutamic dehydrogenases (8) or L-1-chloro-4-phenyl-3-(*p*-tolylsulfonamido)-2-butanone (TPCK, VIII) (9) for chymotrypsin; in the latter case reaction with one of the two histidines in the enzymic active site has been established (9).

(c) that the alkylating group can survive the conditions of the irreversible assay without appreciable reaction with solvent or buffer.

The determination of these three parameters before the irreversible assays is described in the Experimental section.

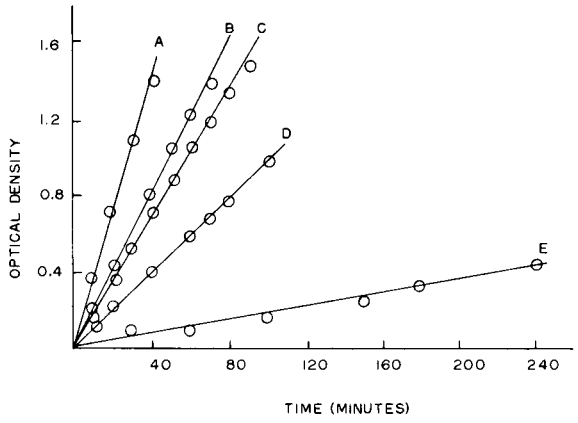


FIGURE 1

Relative rate of reaction of alkylating agents with 66 mM 4-(*p*-nitrobenzyl)pyridine at *p*H 4.2 under the conditions specified in the Experimental section. Curve A: 0.40 mM 6-bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (IV); curve B: 0.40 mM 4-(iodoacetamido)salicylic acid (IX); curve C: 0.20 mM 6-bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (IV); curve D: 0.20 mM 4-(iodoacetamido)salicylic acid (IX); curve E: 0.80 mM L-1-chloro-4-phenyl-3-(*p*-tolylsulfonamido)-2-butanone (TPCK, VIII).

TABLE I

Absorption Spectra of 2,4-Diamino-5-(*p*-R_2-phenyl)-6-R_1-pyrimidines

Compound No.	R_1	R_2	$\lambda_{max}(m\mu)$ ($\epsilon \times 10^{-3}$) Ethanol	*p*H 1	*p*H 13
A (a)	-H	-H	256 (10.5) 294 (8.5)		256 (10.5) 294 (8.5)
B (b)	$-C_2H_5$	-H	250 (8.0) 288 (9.5)		
C (c)	$-C_2H_5$	-Cl		272 (7.75)	286 (9.24)
V	$-CH_2OC_2H_5$	-Cl	255 (7.0) 296 (8.5)	273 (6.25)	246 (7.1) 295 (8.0)
IV	$-CH_2Br$	-Cl	291 (6.0)	290 (6.2)	307 (6.8)

(a) From reference 5a. (b) From reference 5c. (c) From reference 6b.

EXPERIMENTAL

A. Synthetic methods.

Melting points were determined in capillary tubes on a Mel-temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer Model 137B spectrophotometer. Ultraviolet and visible spectra were determined with a Perkin-Elmer Model 202 spectrophotometer.

α-(Ethoxyacetyl)-*p*-chlorophenylacetonitrile (III).

A mixture of 7.6 g. (50 mmoles) of *p*-chlorophenylacetonitrile (II), 2.7 g. (50 mmoles) of sodium methoxide and 6.61 g. (50 mmoles) of ethyl ethoxyacetate in 25 ml. of absolute ethanol was refluxed for 6 hours. The mixture was cooled, acidified with 5 ml. of glacial acetic acid and diluted with 150 ml. of ice water. The mixture was extracted with three 25 ml. portions of dichloromethane. The combined extracts were washed with water, dried over magnesium sulfate, then the solvent was removed *in vacuo*. The oily residue crystallized on cooling and was recrystallized from dichloromethane-petroleum ether (60-110°); yield 7.2 g. (61%); m.p. 91-92°; λ max 3.15 (enol OH), 4.48 (C≡N), 6.09 (enol C=O), 6.25, 6.68 (phenyl), 9.22 (ether C-O-C), 11.96 (*p*-C_6H_4-), 12.97 μ (C-Cl); λ max (H_2O) 258 (ε, 7,500), 304 mμ (ε, 15,900); λ max (*p*H 1) 270 mμ (ε, 6,300); λ max (*p*H 13) 258 (ε, 9,000), 304 mμ (ε, 16,700); λ max (EtOH), 275 mμ (ε, 15,900).

Anal. Calcd. for $C_{12}H_{12}ClNO_2$: C, 60.6; H, 5.09; N, 5.89. Found: C, 60.4; H, 5.31; N, 5.88.

2-(*p*-Chlorophenyl)-3,4-diethoxycrotononitrile (VI).

A solution of 1.63 g. (6.8 mmoles) of III in 5 ml. of ethyl orthoformate was heated at 140° in a flask fitted with a downward condenser for 2 hours, after which time no more low boiling products distilled. The solution was then spin-evaporated *in vacuo*. The remaining oil could not be crystallized, but had λ max (film), 3.30, 3.41 (CH), 4.48 (C≡N), 6.21 (C=C), very broad and strong C-O-C absorption in the 8 to 10 μ region, 12.95 (C-Cl), 13.18 μ (phenyl); λ max (EtOH) 279 mμ.

The predominance of enol bands in III indicated that the compound existed almost exclusively in the enol form. The appearance of additional C-O-C bands in the infrared (8.0 to 10.0 μ) and disappearance of an enol OH band in the 3.2 μ region showed that VI was the required enol ether. The crude VI was used in the following reaction:

5-(*p*-Chlorophenyl)-2,4-diamino-6-ethoxymethylpyrimidine (V).

To a solution of crude VI from 1.63 g. of III (6.8 mmoles) in 10 ml. of absolute ethanol was added 0.72 g. (7.5 mmoles) of guanidine hydrochloride and 0.41 g. (7.5 mmoles) of sodium methoxide. The mixture was refluxed with magnetic stirring for 2 hours, then cooled and poured into 50 ml. of water. The product was collected on a filter and washed with water. Recrystallization from ethanol gave 1.03 g. (54% based on III) of white crystals, m.p. 237-239°; λ max 2.88, 3.00, 3.12 (NH); 6.10, 6.15, 6.31, 6.42 (NH, pyrimidine, phenyl), 9.10, 9.18 (C-O-C), 12.05 μ (*p*-C_6H_4).

Anal. Calcd. for $C_{13}H_{15}ClN_4O$: C, 56.0; H, 5.43; N, 20.1. Found: C, 55.8; H, 5.61; N, 19.9.

6-Bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine Hydrobromide (IV).

A solution of 2.00 g. (7.2 mmoles) of V in 40 ml. of glacial acetic acid containing 10% hydrogen bromide was refluxed for 8 hours. After standing for about 15 hours at room temperature, the mixture was filtered and the crystalline product was washed with 10 ml. of glacial acetic acid, then with acetone; yield, 2.2 g. (78%) of nearly white crystals, m.p. greater than 300°; λ max 3.05; 3.20 (NH, broad); 6.10, 6.15, 6.22, 6.63 (NH, C=N, C=C); no strong C-O-C band in 9.1-9.2 region, 12.08 μ (*p*-C_6H_4).

Anal. Calcd. for $C_{11}H_{11}Br_2ClN_4$: C, 33.5; H, 2.81; N, 14.2; total halogen (as Br) 56.1. Found: C, 33.5; H, 2.62; N, 14.2; Br, 57.3.

B. Kinetic Methods.

Reversible Inhibition of Dihydrofolic Reductase.

The enzyme preparation was a 45-90% ammonium sulfate fraction isolated from pigeon liver acetone powder and assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described (10). Compound IV showed 50% inhibition (I_{50}) at a concentration of 0.26 μM; in comparison, 5-(*p*-chlorophenyl)-2,4-diamino-6-methylpyrimidine had an I_{50} of 0.85 μM. The K_i values for these compounds can be estimated (11) from the I_{50} to be in the range of 10^{-8} molar. Incubations for irreversible inhibitors were performed at 5×10^{-7} M which would convert greater than 50% of the enzyme into an enzyme-inhibitor reversible complex since the enzyme is 50% complexed when the concentration of the inhibitor is equal to K_i (8c).

Chemical Reactivity of the Alkylating Group.

In the past, the relative reactivity with thiosulfate has been used (8b); this method suffers from the disadvantage that relatively large quantities of an inhibitor is required (250 μmoles). 4-(*p*-Nitrobenzyl)-pyridine has recently been used as a sensitive colorimetric assay for determination of nitrogen mustards in animal tissues and blood (12 a-c) and the assay has been modified (12 d) for determination of the relative reactivity of various nitrogen mustards and aziridines. The latter method has now been further modified to be more compatible with the solubility and greater reactivity of compounds such as IV and IX and requires only 10-80 μmoles of sample. The method involved treatment of a known concentration of alkylating agent in aqueous 2-methoxyethanol containing phthalate buffer (*p*H 4.2) at 37° with a large excess of 4-(*p*-nitrobenzyl)pyridine so that *pseudo*-first order kinetics would be followed. The resultant quaternary salt of the 4-(*p*-nitrobenzyl)-pyridine was converted into its quinoid-like free base with potassium hydroxide, then the liberated dye was quantitated by its visible absorption between 550 and 650 mμ (12 a). The following reagents were used: (a) a 5% solution of 4-(*p*-nitrobenzyl)pyridine in 2-methoxyethanol. (b) 0.05 M potassium phthalate buffer (*p*H 4.2) in water. (c) 0.2 N potassium hydroxide in 90% aqueous 2-methoxyethanol. (d) the test compound (10-80 μmoles) in 25 ml. of 2-methoxyethanol; the higher concentrations are used for less active alkylating agents.

Into a series of ten test tubes were pipetted 2.00 ml. of test compound solution (d), 1.00 ml. of buffer, and 1.00 ml. of solution (a). A similar set of control tubes were run where solution (d) was replaced with 2.00 ml. of 2-methoxyethanol. The assay and control tubes were placed in a 37° water bath and a pair of tubes (with and without alkylating agent) were removed at the proper time intervals and cooled briefly in an ice bath, one pair being removed at near zero time. To the cooled tube was then added 0.40 ml. of potassium hydroxide solution and the spectrum at 550-650 mμ was recorded. The optical density of the peak was corrected by the absorption in the control tube with no alkylating agent (usually near zero) and the corrected optical density was recorded against time as shown in Figure 1.

TPCK (VIII) was given an arbitrary rate constant of 1, since it is commercially available (Mann Research Laboratories) and is known to react with a single histidine at the active site of chymotrysin with a reaction half-life of 50 minutes at 37° (9). From Figure 1, it can be seen that the bromomethylpyrimidine (IV) has a relative rate of 40 and 4-iodoacetamidosalicylic acid (IX) (8) a relative rate of 21, both being considerably more reactive than TPCK (VIII). Note in Figure 1 that doubling the concentration of IV or IX from 0.20 mM to 0.40 mM gives a doubling of the rate and note that TPCK (VIII) was run at 0.80 mM; the relative rates of IV:IX:VIII of 40:21:1 are corrected for concentration.

If a tube was heated in a boiling water bath for 20 minutes, the reaction was totally complete. The optical density reading after addition of KOH could then be used to determine the concentration of an alkylating agent in solution. By this procedure it could be shown that less than 10% of IV had reacted with buffer or solvent during the 1 hour incubation procedure described below.

Incubation Procedure to Detect Chemical Inactivation of Dihydrofolic Reductase.

A 45-90% ammonium sulfate precipitate from extraction of pigeon liver acetone powder was redissolved in 0.05 M Tris buffer (*p*H 7.4) as previously described (10). This solution precipitated some denatured protein other than dihydrofolic reductase when incubated for 1 hour at 37°; this turbidity subsequently interfered with the determination of the remaining dihydrofolic reductase. Therefore the previously described solution (10) was incubated for 1 hour at 37°, refrigerated overnight at 3-5°, then clarified by filtration through a small Celite pad; no further precipitation occurred if this solution was incubated for one more hour. This solution gave no extraneous reaction with dihydrofolate or TPNH.

In two test tubes were placed 0.100 ml. of 0.372 mM TPNH in 0.05 M Tris buffer (*p*H 7.4), 0.675 ml. of Tris buffer, enzyme solution, and sufficient water to give a total of 1.50 ml. of solution. The two test tubes were placed in a 37° bath. When the two tubes had reached 37°, to one tube was added 0.015 ml. of a 51.5 μM solution of IV in 50% aqueous dimethylformamide; to the second tube was added 0.015 ml. of 50% aqueous dimethylformamide to serve as a control. After 3 minutes, 0.7 ml. was removed from each tube and placed in an ice-bath to quench any reaction between enzyme and inhibitor; these two tubes were labeled C-1 and I-1 for control and inhibitor solutions, respectively. After 60 minutes at 37°, the remainder in the two tubes were also placed in an ice-bath and labeled C-2 and I-2. The enzyme content of each of the four solutions were measured in duplicate as soon as possible as follows:

In a cuvette were placed 200λ of enzyme incubation solution (same

as C-1), 0.100 ml. of 0.372 mM TPNH and 2.7 ml. of 0.05 mM Tris buffer (*p*H 7.4 containing 10 mM mercaptoethanol and 1 mM Versene). When the optical density baseline had become constant, 50 λ of 0.372 mM dihydrofolate in 0.05 M Tris buffer (*p*H 7.4 containing 10 mM mercaptoethanol and 1 mM Versene) was added. The initial change in optical density units per minute was recorded (10).

Sufficient enzyme solution is used in the incubation so that the C-1 solution when assayed as just described will give 0.005-0.010 optical density units change per minute. In a separate experiment it was shown that 100 λ and 50 λ of the C-1 solution would give one-half and one-quarter the optical density change per minute, thus showing that the assay as performed was measuring the amount of enzyme remaining in solutions C-2, I-1, and I-2. Thus, if I-2 had one-half of the optical density change per minute as I-1, then 50% of the enzyme would have been destroyed.

With the 6-bromomethylpyrimidine, IV, no appreciable difference in enzyme content was noted with the four solutions, thus showing that IV was not an irreversible inhibitor of dihydrofolic reductase under the conditions specified.

DISCUSSION

Although 6-bromomethyl-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (IV) was (a) a good reversible inhibitor of dihydrofolic reductase, (b) was stable to the irreversible assay conditions and (c) had an alkylating group even more reactive than that of the chloromethyl ketone (TPCK, VIII) which is known to react with a histidine at the active site of chymotrypsin (9), IV did not inactivate dihydrofolic reductase from pigeon liver. It is therefore reasonably certain that a histidine residue in this dihydrofolic reductase is not sufficiently near the bromomethyl group of IV to form a covalent linkage within the enzyme-inhibitor complex. Furthermore it is clear that there is no other enzymic nucleophilic group (4) with sufficient nucleophilicity and proper spatial relation to IV in the complex to react with the bromomethyl methyl group of IV.

There is still a good probability that the enzyme has a proton donor at the active site in order to aid the TPNH reduction of the 5,6 and 7,8 double bonds of folic acid. If the picture depicted in structure I has a fairly rigidly held imidazole ring, then it may be possible that a two atom bridge from the 6-position of the pyrimidine is necessary for bridging to the histidine, that is, the N-8 of folic acid bridges to the proton of the imidazole, not the imidazole *per se*. Furthermore, it is possible to depict other positions for this proton donor where it is near N-5 or N-8, but not near both. Further search is continuing for active-site-directed irreversible inhibitors of dihydrofolic reductase which may covalently link to the proton donor presumably present in the active site of this enzyme.

REFERENCES

(1) This work was supported in part by Grant Nos. CA-05867 and CA-06624 from the National Cancer Institute, U. S. Public Health Service. J. H. Jordaan also wishes to thank the Atomic Energy Board, Republic of South Africa, for a fellowship. We wish to thank Gail Westley and Shirley Herrmann for technical assistance with the kinetic measurements.

(2) For the previous paper of this series see reference 13.

(3) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, 53, 1457, (1964); paper XV of this series.

(4) B. R. Baker, *ibid.*, 53, 347 (1964).

(5a) P. B. Russell and G. H. Hitchings, *J. Am. Chem. Soc.*, 73, 3763 (1951); (b) P. B. Russell and N. Whittaker, *ibid.*, 74, 1310 (1952); (c) P. B. Russell, *J. Chem. Soc.*, 2951 (1954).

(6a) G. H. Hitchings, P. B. Russell, and N. Whittaker, *ibid.*, 1019 (1956); (b) R. B. Trattner, G. B. Elion, G. H. Hitchings, and D. M. Sharefkin, *J. Org. Chem.*, 29, 2674 (1964).

(7) S. F. Mason, *J. Chem. Soc.*, 1247 (1959).

(8a) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez and E. Tong, *J. Med. Pharm. Chem.*, 2, 633 (1960); (b) B. R. Baker, W. W. Lee, E. Tong, and L. O. Ross, *J. Am. Chem. Soc.*, 83, 3713 (1961); (c) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, 3, 459 (1962).

(9) G. Schoellmann and E. Shaw, *Biochemistry*, 2, 252 (1963).

(10) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, 1, 79 (1964); paper XII of this series.

(11a) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, 52, 927, (1962); (b) B. R. Baker and R. P. Patel, *ibid.*, 53, 714 (1964).

(12a) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, 27, 1435 (1955); (b) O. Klatt, A. C. Griffin and J. S. Stahlin, Jr., *Proc. Soc. Exptl. Biol. Med.*, **104**, 629 (1960); (c) O. M. Friedman and E. Boger, *Anal. Chem.*, 33, 906 (1961); (d) T. J. Bardos, N. Datta-Gupta, P. Hebborn and D. J. Triggle, *J. Med. Chem.*, in press.

(13) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, 54, in press (1965).

Received December 19, 1964 Buffalo, New York 14214