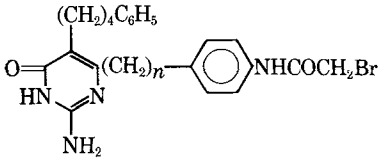
Irreversible Enzyme Inhibitors LXX

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase IV. Substituted 1-Phenyl-1,2-dihydro-*s*-triazines

By B. R. BAKER and BENG-THONG HO

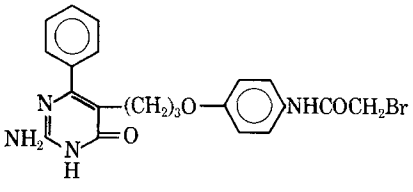
4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazines substituted on the phenyl ring by *p*-chloroacetyl (IV*a*), *m*-chloroacetyl (IV*c*), and 4-chloro-3-butanone-1-yl (IV*b*) groups were synthesized from the appropriate aromatic amine hydrochlorides, cyanoguanidine, and acetone. All three compounds were good reversible inhibitors of dihydrofolic reductase. However, none of the three compounds inactivated dihydrofolic reductase when incubated with the enzyme; this lack of active-site-directed irreversible inhibition by compounds of type IV has been attributed to the complexing of the phenyl group to the hydrophobic bonding region on dihydrofolic reductase—a region not apt to have attackable nucleophilic groups present.

SEVERAL active-site-directed irreversible inhibitors (1–3) of dihydrofolic reductase were successfully designed once the strong hydrophobic bonding area on the enzyme was discovered (4) and its effect on the mode of pyrimidine binding was explored (1, 5–10). When a hydrocarbon-type 5-side-chain was allowed to complex to the hydrophobic bonding region on the enzyme, the



I, $n = 2$

II, $n = 4$



III

6-side-chain of I and II could project to a hydrophilic area on the enzyme and form a covalent bond within the enzyme–inhibitor complex (11); similarly, the 6-phenyl group of III could complex to the hydrophobic region of dihydrofolic reductase with the result that the 5-side-chain projected into the hydrophilic area and formed a covalent bond with the enzyme (12).

Received August 5, 1966, from the Department of Chemistry, University of California, Santa Barbara, 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo 14214.

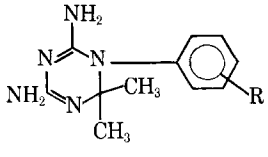
Accepted for publication September 27, 1966.

This investigation was supported by grants CA-05867, CA-06624, and CA-08695 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

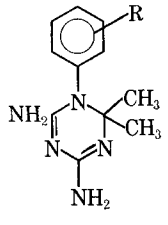
Previous paper: Baker, B. R., and Santi, D. V., *J. Pharm. Sci.*, unpublished data.

DISCUSSION

Prior to the synthesis and enzymic evaluation of I–III, over 30 candidate irreversible inhibitors for dihydrofolic reductase failed to inactivate the enzyme; most of these failures can now be rationalized on the basis that these heterocycles with a single large side chain bearing a covalent-forming group had their side chain complexed with the hydrophobic region of the enzyme where there was not apt to be a nucleophilic group on the enzyme that could be covalently linked with the inhibitor. One such unsuccessful class was derived from the 1-phenyl-1, 2-dihydro-*s*-triazine (IV) where the covalent forming group, R, was placed on the phenyl ring; these candidates failed since the dihydro-*s*-triazines presumably assumed a different conformation, IV*B*, rather than IV when complexed to the enzyme, due to the strong hydrophobic bonding of the phenyl group being determinate (10).



IV



IV*B*

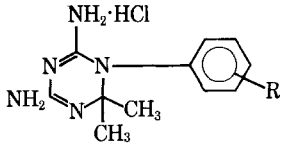
a, R = *p*-$COCH_2Cl$

b, R = *p*-$(CH_2)_2COCH_2Cl$

c, R = *m*-$COCH_2Cl$

The enzymic evaluations of IV*a–c* are summarized in Table I; a successful active-site-directed irreversible inhibitor [II (11)] is included for comparison. Note that IV*a* and IV*c* are still excellent reversible inhibitors of dihydrofolic reductase even though they are $^1/_{12}$ and $^1/_{17}$ as effective as the unsubstituted 1-phenyl-*s*-triazine (V). This decrease in binding by IV*a* and IV*c* can now be rationalized from a study on the mode of phenyl binding (6) subsequent to the evaluation of IV*a* and *c*. The phenyl group is most probably complexed to a hydrophobic region on the enzyme; it was previously

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Reversible[a] μM Concn. for 50% Inhibition	Reversible[a] Estimated $K_i \times 10^6$ M[c]	Irreversible[b] μM Concn.	Irreversible[b] % EI[d]	Irreversible[b] Time, min.	Irreversible[b] Inactivation, %
IV*a*	*p*-$COCH_2Cl$	1.2	0.2	1	83	60	0
IV*b*	*p*-$(CH_2)_2COCH_2Cl$	0.025	0.004	0.020	83	60	0
IV*c*	*m*-$COCH_2Cl$	1.7	0.3	1	77	60	0
V	H	0.11[e]	0.02				
VI	*p*-C_4H_9-*n*	0.064[f]	0.01				
VII[g]	*m*-CH_2OH	0.43	0.07				
II[h]		240	40	40	50	18	50
				10	20	25	43

[a] The dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol, as previously described (13). [b] Dihydrofolic reductase was incubated in the absence of TPNH with the inhibitor at 37° in 0.05 M Tris buffer (pH 7.4) containing *no* mercaptoethanol as previously described (14); in each case an enzyme control was run that showed 0–4% thermal inactivation. Similar results with IV*a–c* were obtained in the presence of 12 μM TPNH. [c] Estimated from $K_i = I \times K_m/S$, where I = inhibitor concentration giving 50% inhibition (4, 15); this equation is valid since $S = 6\ K_m > 4\ K_m$. [d] Calculated from $[EI] = [E_t]/[1 + \frac{K_i}{I}]$, where $[E_t]$ = the concentration of total active enzyme, $[EI]$ = the fraction of E_t reversibly complexed by I (1, 16), and I = the inhibitor concentration. [e] Data from *Reference 17*. [f] Data from *Reference 6*. [g] Hydrobromide salt. [h] Data from *Reference 11*.

noted that polar groups on the *m*- or *p*-position of the phenyl (IV) such as *m*-hydroxymethyl (VII, Table I), cyano, carboxylate, carbethoxy, and aminomethyl cause a decrease in binding, presumably by repulsion of these polar groups on the inhibitor from the hydrophobic region of the enzyme (6). Since the carbonyl group of IV*a* and *c* is highly polar, it too could be repulsed; the relative polarity of the C=O group can be calculated to have a Hansch π-constant of −1.1 from the constants for $CH_3C{=}O$ ($\pi = -0.55$) and CH_3 ($\pi = +0.56$).[1] The 4-chloro-3-butanone-1-yl side chain of IV*b* was not repulsed and actually gave additional binding; note that IV*b* is about twice as good a reversible inhibitor than is the *p*-(*n*-butyl) phenyl-*s*-triazine (VI).

When IV*a–c* were incubated at 37° with dihydrofolic reductase at concentrations sufficient to reversibly complex 77–83% of the total enzyme, no inactivation occurred (Table I). In contrast, the irreversible inhibitor (II) gave 50% inactivation in about 18 min. when 50% of the enzyme was reversibly complexed (11). Furthermore, at a concentration of 10 μM, where II reversibly complexes 20% of the total enzyme, 43% inactivation in 25 min. was seen; the 10 μM concentration of II is only 10 times the incubation concentrations of IV*a* and *c*.

CHEMISTRY

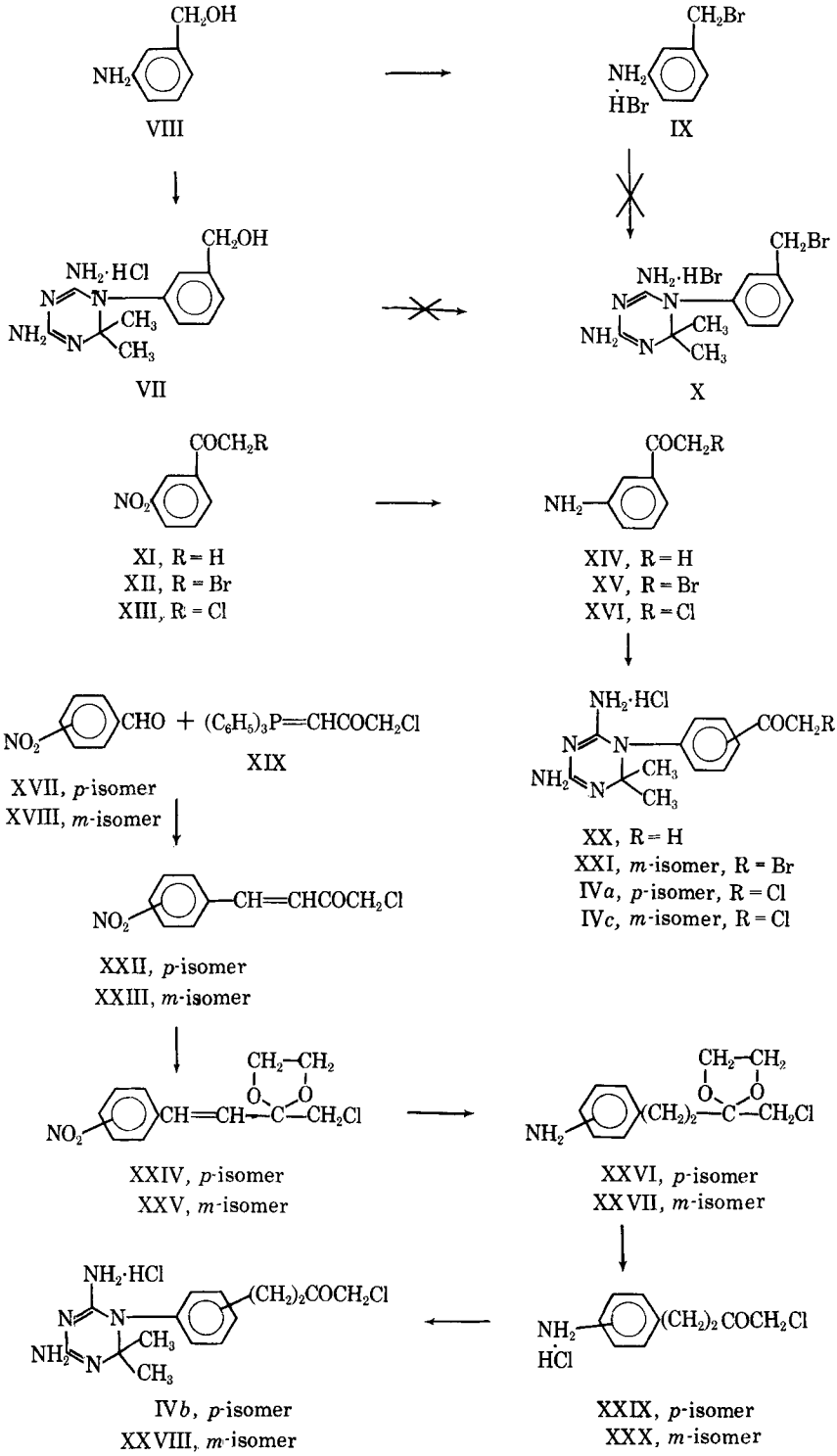
Methods

The first candidate active-site-directed irreversible inhibitor for dihydrofolic reductase chosen in this series was the *m*-bromomethylphenyl-*s*-triazine (X). *m*-Aminobenzyl alcohol (VIII) (19) was smoothly converted to the corresponding benzyl bromide, isolated as its hydrobromide salt (IX) in 76% yield, with hot 10% anhydrous hydrogen bromide in acetic acid. When attempts were made to convert IX to a dihydro-*s*-triazine (X) by the three component method of Modest using cyanoguanidine and acetone (20), a gummy, intractable (polymeric?) mass rapidly formed; apparently these conditions were not compatible with the high chemical reactivity of the benzylic bromide. The benzyl alcohol (VIII) was readily convertible to the *m*-hydroxymethylphenyl-*s*-triazine (VII) by the three component method (20) in 78% yield; unfortunately, treatment of VII with 10% anhydrous hydrogen bromide in acetic acid to give X—the type of conditions used for the conversion of VIII to IX—cleaved the triazine ring as shown by changes in the ultraviolet spectrum. Treatment of VII with anhydrous hydrogen bromide in acetic acid or 48% aqueous hydrobromic acid, both at 25°, gave oily mixtures.

Attention was therefore directed to the synthesis of the *m*-bromoacetylphenyl-*s*-triazine (XXI). Reduction of the nitro group of *m*-nitro-α-bromoacetophenone (XII) with copper powder in sulfuric acid also caused reduction of the halogen and led to *m*-aminoacetophenone (XIV) rather than the desired *m*-amino-α-bromoacetophenone (XV); these conditions had been used for the conversion of the corresponding α-chloro ketone (XIII) to *m*-amino-α-chloroacetophenone (21). The desired *m*-amino-α-bromoacetophenone (XV) was prepared by bromination of *m*-aminoacetophenone (XIV) in glacial acetic acid containing an excess of ethanesulfonic acid which protonated the amine and, in turn, stabilized the ring against halogenation; the product (XV) was isolated as the hydrobromide salt but

[1] The Hansch π-constant (18) is a measure of hydrophobic character on a log scale, where H is assigned $\pi = 0$.

direct bromination of XIV in acetic acid containing hydrogen bromide failed because of the insolubility of the hydrobromide salt of XIV. Attempts to convert XV to the dihydro-*s*-triazine, XXI, by the three component method (20) rapidly formed a brownish gummy (polymeric?) mass.



Scheme I

Since the attempts to prepare the dihydro-*s*-triazines (X and XXI) containing an active bromine appeared to fail because of the excessive chemical reactivity of this halogen, attention was directed toward the less reactive chloroacetyl-*s*-triazines (IV*a* and *c*). Commercially available *p*-amino-α-chloroacetophenone readily formed the requisite *p*-chloroacetylphenyldihydro-*s*-triazine (IV*a*) by the three component method (20) in 88% yield. Similarly, *m*-amino-α-chloroacetophenone (21) formed the *m*-chloroacetylphenyldihydro-*s*-triazine (IV*c*) in 42% yield. Although the *m*-acetylphenyldihydro-*s*-triazine (XX) was readily prepared from *m*-aminoacetophenone, bromination experiments to convert XX to XXI were discontinued when IV*a* and *c* were successfully synthesized.

4 - (*p* - Aminophenyl) - 1 - chloro - 2 - butanone hydrochloride (XXIX) was prepared in crystalline form by hydrolysis of the previously prepared dioxolane (XXVI) (22). Condensation of XIX with cyanoguanidine and acetone by the three component method (20) afforded the chlorobutanonyl-*s*-triazine (IV*b*) in 67% yield. The corresponding *m*-aminophenyl dioxolane (XXVII) was prepared *via* XXIII and XXV. The hydrochloride salt of XXX formed after acid hydrolysis of XXVII could not be purified; furthermore, the *s*-triazine (XXVIII) obtained from impure XXX by the three component method (20) also could not be purified. (Scheme I.)

Synthesis

Melting points were taken on a Fisher-Johns apparatus and those below 230° were corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 recording spectrophotometer.

m-Aminobenzyl Bromide Hydrobromide (IX).—A solution of 0.550 Gm. (4.47 mmoles) of VIII (19) in 6 ml. of 10% anhydrous hydrogen bromide in glacial acetic acid was refluxed for 8 hr. The solution was cooled to about 20°; the product was collected on a filter and washed with a small amount of glacial acetic acid, then ether; yield, 0.91 Gm. (76%) of an off-white solid, m.p. 244° dec., with browning at 230°; $\nu_{max.}$ 2950, 2650 (NH, NH^+); 1600, 1575, 1550, 1525 (NH, NH^+, C=C); 885, 783 $cm.^{-1}$ (*m*-C_6H_4).

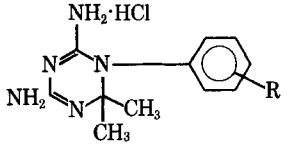
Anal.—Calcd. for $C_7H_8BrN \cdot HBr$: C, 31.5; H, 3.40; N, 5.25. Found: C, 31.6; H, 3.50; N, 5.40.

α-Chloro-m-nitroacetophenone (XIII).—To a solution of 1.65 Gm. (10 mmoles) of XI in 15 ml. of chloroform was added 2 ml. of sulfuryl chloride. After 24 hr. protected from moisture, the solution was spin-evaporated *in vacuo;* the crystalline residue was triturated with ether; yield, 1.34 Gm. (67%), m.p. 97–99°. Recrystallization from petroleum ether (b.p. 60–110°) with only slight loss gave light yellow crystals, m.p. 101–103°.

This procedure is considerably simpler than the direct treatment of molten XI with chlorine gas (23). [Lit. (23) m.p. 103°.]

m - Amino - α - bromoacetophenone (XV) Hydrobromide.—A mixture of 540 mg. (7 mmoles) of XIV, 5 ml. of glacial acetic acid, and 0.65 (8 mmoles) of ethanesulfonic acid was warmed to complete solution, then cooled to room temperature. After the addition of 0.21 ml. of bromine, the solution was magnetically stirred for 12 hr. with ultraviolet light irradiation. Some of the hydrobromide salt separated during this time. The mixture was treated with 3 ml. of 30% anhydrous hydrogen bromide in acetic acid, then diluted with about 5 vol.

TABLE II.—PHYSICAL PROPERTIES OF



Compd.[a]	R	Yield, %	M.p., °C.	Anal. Calcd.	Anal. Found
IV*a*	*p*-$COCH_2Cl$	88	197–199[b]	C, 44.8[c] H, 5.50 N, 20.1	C, 44.7 H, 5.47 N, 19.9
					C, 44.8 H, 5.51 N, 20.3
IV*b*	*p*-$(CH_2)_2COCH_2Cl$	81	204–206[b]	C, 50.3 H, 5.91 N, 19.5	C, 49.9 H, 5.89 N, 19.2
IV*c*	*m*-$COCH_2Cl$	43[d]	174–176	C, 47.3 H, 5.19 N, 21.2	C, 47.0 H, 5.21 N, 21.0
VII	*m*-CH_2OH	78	191–192[e,f]	C, 43.9[f] H, 5.53 N, 21.3	C, 43.7 H, 5.51 N, 21.4
XX	*m*-$COCH_3$	41	195–197[f,g]	C, 46.0 H, 5.34 N, 20.6	C, 46.4 H, 5.10 N, 20.4

[a] All compounds had ultraviolet and infrared spectra in agreement with their assigned structures. [b] Recrystallized from methanol–ether. [c] Monohydrate. [d] Yield after crystallization of the amorphous product from methanol–ether. [e] Recrystallized from absolute ethanol–ether. [f] Hydrobromide salt; 4 mmoles of aromatic amine was reacted with cyanoguanidine in acetone containing 4 mmoles 48% aqueous hydrobromic acid. [g] Recrystallized from glacial acetic acid–ether.

of ether. The product was collected and washed with ether; yield, 608 mg. (52%); the compound gradually decomposes above 190° without definite melting. Recrystallization of 100 mg. from absolute ethanol–ether gave 50 mg. of beige crystals with unchanged melting behavior; $\lambda_{max.}$ 2950–2850, 2600 (NH, NH^+); 1700 (C=O); 1625, 1600, 1550, 1500 (NH^+, C=C); 798 $cm.^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_8H_8BrNO \cdot HBr$: C, 32.6; H, 3.08; N, 4.75. Found: C, 32.3; H, 2.96; N, 4.61.

Chloromethyl m-Nitrostyryl Ketone (XXIII).—A mixture of 0.705 Gm. (2 mmoles) of XIX (24) and 0.378 Gm. (2.5 mmoles) of *m*-nitrobenzaldehyde in 18 ml. of benzene was refluxed 18 hr., then spin evaporated *in vacuo*. The residue was triturated with petroleum ether, then recrystallized from absolute alcohol to give 0.333 Gm. (74%) of light yellow crystals, m.p. 120–121°; $\nu_{max.}$ 1700 (C=O); 1625, 1575 (C=C); 1525, 1340 $cm.^{-1}$ (NO_2).

Anal.—Calcd. for $C_{10}H_8ClNO_3$: C, 53.2; H, 3.57; N, 6.21. Found: C, 53.3; H, 3.70; N, 6.00.

2 - Chloromethyl - 2 - (m - nitrostyryl) - 1,3-dioxolane (XXV).—A mixture of 4.52 Gm. (20 mmoles) of XXIII, 35 ml. of benzene, 10 ml. of ethylene glycol, and 40 mg. of *p*-toluenesulfonic acid was refluxed under a Dean-Stark trap for 18 hr. The solution was washed with water, then spin evaporated *in vacuo*. Trituration of the residue with ether gave 5.2 Gm. (97%) of a pale yellow solid, m.p. 87–89°. Recrystallization from absolute ethanol gave 5.07 Gm. (94%) of pale yellow crystals, m.p. 90–91°; $\nu_{max.}$ 1530, 1350 (NO_2); 1040 (C—O—C); no C=O near 1700 $cm.^{-1}$.

Anal.—Calcd. for $C_{12}H_{12}ClNO_4$: C, 53.4; H, 4.49; N, 5.19. Found: C, 53.7; H, 4.81; N, 5.15.

Catalytic reduction of XXV as previously described for XXIV (22) proceeded smoothly to XXVII which was obtained as an oil that moved as a single spot on thin-layer chromatography (22); its ultraviolet spectrum showed that the double bond had been reduced.

4 - (p - Aminophenyl) - 1 - chloro - 2 - butanone Hydrochloride (XXIX).—A solution of 600 mg. (2.5 mmoles) of XXVI (22) in 6 ml. of ethanol and 6 ml. of 1 *N* aqueous hydrochloric acid was refluxed for 2 hr., then spin evaporated *in vacuo*. The solid residue was triturated with acetone, then collected on a filter and washed with ether; yield, 290 mg. (50%) of a beige-white solid, m.p. 160–162°; $\nu_{max.}$ 3450 (NH), 2850, 1950, 1570 (NH^+); 1730 (C=O); 1615, 1500 (C=C); 840, 810 $cm.^{-1}$ (*p*-C_6H_4). No suitable solvents for recrystallization could be found.

A similar hydrolysis of XXVII gave a gummy product that showed a C=O bond at 1730 $cm.^{-1}$, but could not be crystallized.

1 - (p - Chloroacetylphenyl) - 4,6 - diamino - 1,2-dihydro - 2,2 - dimethyl - s - triazine Hydrochloride (IVa).—A mixture of 848 mg. (5 mmoles) of *p*-amino-α-chloroacetophenone (Eastman), 450 mg. (5.35 mmoles) of cyanoguanidine, 3 ml. of acetone, and 0.42 ml. (5 mmoles) of 1 *N* aqueous hydrochloric acid was refluxed with magnetic stirring. Solution was complete when the b.p. was reached, then within 10 min. the product began to separate. An additional 5 ml. of acetone was added, then the mixture was refluxed for 17 hr. After 1 hr. at 0°, the mixture was filtered and the product was washed with acetone; yield, 1.46 Gm. (88%), m.p. 200–202°. Recrystallization from methanol–ether gave 587 mg. (36%) of white crystals, m.p. 198–199°; $\nu_{max.}$ 3450, 3350, 3200 (NH); 1700 (C=O); 1670, 1640, 1600, 1550, 1540, 1520, 1500 (NH, C=NH^+, C=N, C=C); 830 $cm.^{-1}$ (*p*-C_6H_4); $\lambda_{max.}$ (H_2O): 247 mμ (ε 17,200). See Table II for analytical data and additional compounds prepared by this method (20), except the second addition of acetone was omitted.

REFERENCES

(1) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active Site," John Wiley & Sons, Inc., New York, N. Y., to be published.
(2) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(3) Baker, B. R., *Cancer Chemotherapy Rept.*, **4**, 1(1959).
(4) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(5) Baker, B. R., and Jordaan, J. H., *ibid.*, **54**, 1740(1965).
(6) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(7) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(8) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
(9) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).
(11) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417(1966).
(12) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422(1966).
(13) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(14) Baker, B. R., and Jordaan, J. H., *ibid.*, **2**, 21(1965).
(15) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.*, **53**, 714(1964).
(16) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(17) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **53**, 1137(1964).
(18) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(19) Phillips, A. P., and Maggiolo, A., *J. Org. Chem.*, **15**, 659(1950).
(20) Modest, E. J., *ibid.*, **21**, 1(1956).
(21) Catch, J. R., *et al.*, *J. Chem. Soc.*, **1949**, 552.
(22) Baker, B. R., and Jordaan, J. H., *J. Med. Chem.*, **8**, 35(1965).
(23) Baker, J. W., *J. Chem. Soc.*, **1931**, 2416.
(24) Hudson, R. F., and Chopard, P. A., *J. Org. Chem.*, **28**, 2446(1963).