REFERENCES

(1) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(2) 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.
(3) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(4) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(5) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(6) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
(7) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.*, **55**, 302(1966).
(8) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 308(1966).
(9) Baker, B. R., and Jordaan, J. H., *ibid.*, **55**, 1417(1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.*, **55**, 1422(1966).
(11) Baker, B. R., *J. Med. Chem.*, **5**, 654(1962).
(12) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).
(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 Jordaan, J. H., *J. Med. Chem.*, **8**, 35(1965).
(18) Baker, B. R., Patel, R. P., and Almaula, P. I., *J. Pharm. Sci.*, **52**, 1051(1963).

(19) Schoellmann, G., and Shaw, E., *Biochemistry*, **2**, 252(1963).
(20) Schoellmann, G., and Shaw, E., *Biochem. Biophys. Res. Commun.*, **7**, 36(1962).
(21) Baker, B. R., Schwan, T. J., and Ho, B.-T., *J. Pharm. Sci.*, **56**, 38(1967).
(22) Schaeffer, H. J., and Odin, E., to be published.
(23) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **54**, 1740(1965).
(24) Baker, B. R., and Morreal, C. E., *ibid.*, **52**, 840(1963).
(25) Baker, B. R., and Shapiro, H. S., *J. Med. Chem.*, **6**, 664(1963).
(26) Baker, B. R., Santi, D. V., Almaula, P. I., and Werkheiser, W. C., *ibid.*, **7**, 24(1964).
(27) Baker, B. R., Santi, D. V., and Shapiro, H. S., *J. Pharm. Sci.*, **53**, 1317(1964).
(28) Baker, B. R., and Shapiro, H. S., *J. Med. Chem.*, **8**, 283(1965).
(29) Baker, B. R., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1252(1965).
(30) *Ibid.*, unpublished data.
(31) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, December(1966).
(32) Fox, H. H., *J. Org. Chem.*, **13**, 438(1948).
(33) Borovicka, M., Sedivy, Z., Jilek, J., and Protiva, M., *Coll. Czech. Chem. Commun.*, **20**, 437(1955).
(34) Baker, B. R., and Ho, B.-T., *J. Pharm. Sci.*, **56**, 28(1967).

# Irreversible Enzyme Inhibitors LXXII

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase VI. Derivatives of Hydrophobically Bonded *p*-Alkyl and *p*-Aralkyl Benzoic Acids

By B. R. BAKER, THOMAS J. SCHWAN, and BENG-THONG HO

Since *p*-(bromobutyl)benzoic acid (IV), *N*-(*m*-aminobenzyl)-*p*-aminobenzoic acid (V), and *p*-benzylbenzoic acid (VI) showed good hydrophobic bonding to dihydrofolic reductase (6), four candidate active-site-directed irreversible inhibitors related to structures IV–VI were synthesized that contained a terminal alkylating function. Two of the candidates, *p*-(4-bromoacetamidobutyl)benzoic acid (IX) and *N*-(*m*-bromoacetamidobenzyl)-*p*-aminobenzoic acid (X), lost their ability to form a reversible complex with the enzyme. In contrast, α-(*p*-chloroacetylanilino)-*p*-toluic acid (XI) and α-[*p*-(4-chloro-1-buten-3-one-1-yl)anilino]-*p*-toluic acid (XII) formed reasonably good reversible complexes with dihydrofolic reductase and could inactivate the enzyme; chloroacetone under comparable conditions showed no inactivation, thus affording strong evidence that XI and XII inactivated the enzyme *via* a neighboring-group reaction within a reversible complex with the enzyme.

THE DISCOVERY of hydrophobic bonding to dihydrofolic reductase (1) with its conformational implications on the mode of binding of pyrimidines (2–8) soon led to the proper design of active-site-directed irreversible inhibitors of this enzyme (9–11). These successful candidates, such as I (9) and IIa (10), were designed on the principle that the 5-phenylbutyl group of I could complex to the hydrophobic region on dihydro-

folic reductase which then projected the 6-side-
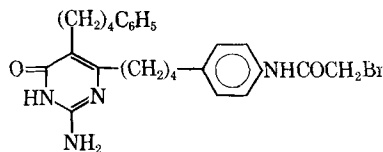
I
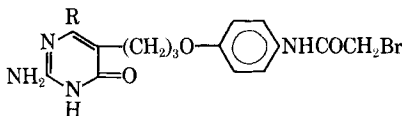
II

a, R = C₆H₅
b, R = CH₃

chain into a hydrophilic area on the enzyme, then formed a covalent bond (9); similarly, the 6-phenyl group of II*a* could complex with the hydrophobic region, thus projecting the 5-side-chain bearing an alkylating group into a nucleophilic region of the enzyme (10). In contrast, the candidate irreversible inhibitor (II*b*) with a 6-methyl group failed to inactivate the enzyme (12), since the 5-side-chain bearing the alkylating group was probably complexed to the hydrophobic region of the enzyme where nucleophilic groups are not apt to be present (2, 8, 11). Thus, over 30 candidate irreversible inhibitors failed to inactivate dihydrofolic reductase for the same reasons that III failed; some of these unsuccessful attempts—prior to knowledge of hydrophobic bonding to dihydrofolic reductase—have been described (11-15).

DISCUSSION

During the time that synthetic methods for structures such as I (16-19) and II*a* (10, 19) were being devised, a parallel investigation of candidate irreversible inhibitors of dihydrofolic reductase was embarked upon that utilized hydrophobic bonding in a different way. One of the studies on dihydrophobic bonding (6) sought to answer the question of where the hydrophobic bonding region on the enzyme was located with respect to the binding area for the substrate, dihydrofolic acid, or its inhibitor, folic acid (III). The results of this study suggested that the hydrophobic bonding region was near either the 4-position or 8-position of folic acid when it was complexed to the enzyme; furthermore, the hydrophobic region was not in between the pyrimidyl and *p*-aminobenzoyl-L-
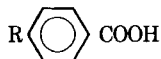

III
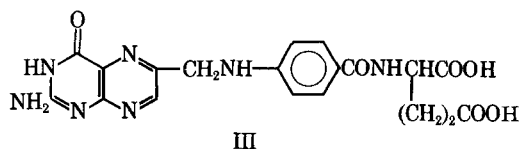
glutamate moieties when folic acid (III) was complexed to the enzyme. As part of the evidence for these deductions, it was observed that *p*-alkyl, *p*-aralkyl, or *p*-aryl groups on benzoic acid, such as IV–VII, could hydrophobically bind to dihydrofolic reductase. It was therefore reasoned that if a

IV

V

VI   VII
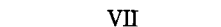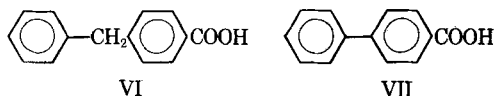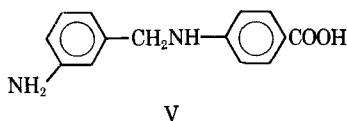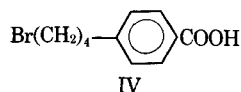
terminal chloromethyl ketone or bromoacetamido group were attached to molecules such as IV–VII these alkylating functions might project into a hydrophilic area on the enzyme when the inhibitors were complexed to the hydrophobic region of the dihydrofolic reductase. In Table I are summarized the enzyme data on inhibitors of this type.

Since the *p*-bromobutyl group of IV showed hydrophobic bonding, the corresponding *p*-(4-bromoacetamidobutyl)benzoic acid (IX) was synthesized as a candidate irreversible inhibitor. Unfortunately, IX showed no reversible inhibition,

TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY

Compd.	R	Reversible[a] I_{50}[c]	Reversible[a] Estimated[d] $K_i \times 10^4$ M	Irreversible[b] mM Concn.	Irreversible[b] EI,[e] %	Irreversible[b] Time, min.	Irreversible[b] Inactivation, %
VIII	H	>300[f]	>500				
IV	Br(CH$_2$)$_4$	6.2[f]	10				
IX	BrCH$_2$CONH(CH$_2$)$_4$—	>24	>40				
V	*m*-NH$_2$C$_6$H$_4$CH$_2$NH—	0.76[f]	1.3				
VI	*p*-C$_6$H$_5$CH$_2$—	10[f]	17				
VII	*p*-C$_6$H$_5$	6.7[f]	11				
X	*m*-BrCH$_2$CONHC$_6$H$_4$CH$_2$NH—	>20	>33				
XI	*p*-ClCH$_2$COC$_6$H$_4$NHCH$_2$—	0.5[g]	0.83	0.20	70	120	35
XII	*p*-ClCH$_2$COCH=CHC$_6$H$_4$NHCH$_2$—	1.2[h]	2.0	0.20	50	120[i]	95
XIII	Chloroacetone			0.10		60	0

[a] The dihydrofolic reductase was a 45–90% ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described (20). [b] Dihydrofolic reductase was incubated at 37° with the inhibitor in Tris buffer (pH 7.4) as previously described; in each case an enzyme control showed 0–4% inactivation. [c] I_{50} = millimolar concentration necessary for 50% inhibition. [d] Estimated from $K_i = K_m (I/S)$ where $K_m = 1 \times 10^{-6}$ M, I = inhibitor concentration, and S = dihydrofolate concentration; this equation is usually valid when $S > 4$ K_m, since $S = 6$ K_m (1, 21). [e] Calculated from $[EI] = [E_t]/(1 + K_i/[I])$ where E_t = total enzyme, $[EI]$ = fraction of enzyme reversibly complexed, and I = inhibitor concentration (2, 22). [f] Data from *Reference 6*. [g] Estimated from 17% inhibition observed at 0.1 mM, the highest concentration allowing full light transmission. [h] Estimated from 34% inhibition observed at 0.6 mM, the highest concentration allowing full light transmission. [i] Intermediate times were not run to determine if 95% inactivation was reached sooner.

greater than fourfold loss in reversible binding occurring compared to IV; therefore, IX was not further investigated. One of the best benzoic acid derivations showing hydrophobic bonding was the *m*-aminobenzylamino derivative, V (6); when V was converted to its bromoacetyl derivative (X), reversible binding was again lost. Apparently the planar amide group of IX and X are not tolerated within the enzyme–inhibitor complex; furthermore, the amide group is quite polar and may still be in the vicinity of the hydrophobic region of dihydrofolic reductase. Therefore, the chloromethyl ketones (XI and XII) were synthesized and evaluated.

The α-chloroacetophenone derivative (XI) was one of the best reversible inhibitors of the *p*-substituted benzoic acid type with $K_i = 8 \times 10^{-5}$ M. When 0.2 mM of XI was incubated with dihydrofolic reductase at 37°, 35% inactivation occurred in 2 hr.; this concentration of XI is sufficient to convert 70% of the dihydrofolic reductase to a reversible complex.

Although XII with its longer *p*-group was not so good a reversible inhibitor as XI, XII was a better irreversible inhibitor; at a concentration of 0.2 mM, XII converts 50% of the available enzyme to a reversible complex, and then showed 95% inactivation in 2 hr. at 37°. Note that chloroacetone at 0.1 mM failed to show any inactivation in 1 hr. at 37°; if XI and XII had inactivated dihydrofolic reductase by a random bimolecular mechanism (2, 22), then XI, XII, and chloroacetone should have inactivated the enzyme at near the same rate; these differences in rates are taken as strong evidence that the inactivation of XI and XII occurs through a complex by the active-site-directed mechanism (2, 22).

Whether XI and XII alkylate the same amino acid on dihydrofolic reductase that is attacked by the pyrimidines (I and II*a*) must await experiments on a purified enzyme—a problem for protein structure chemists.

CHEMISTRY

Methods

When *p*-bromopropylbenzoic acid (XIV) (6, 23) was treated with 2.5 mole ratio of sodium cyanide in *N,N*-dimethylformamide, considerable polymeric material was found; presumably, the carboxylate anion of XIV displaced the bromine atom by intermolecular attack. When the ratio of sodium cyanide was increased to 6:1, the reaction proceeded more smoothly to give *p*-cyanobutylbenzoic acid (XV) in 60% yield with considerably less formation of polymer. Hydrogenation of the cyano group in alcohol containing a 2:1 ratio of hydrochloric acid (24) in the presence of platinum oxide proceeded smoothly to the hydrochloride of XVI in quantitative yield which was suitable for further transformation, but was characterized as the free base. Reaction of XVI hydrochloride as its anion in aqueous acetone containing two equivalents of sodium hydroxide with *p*-nitrophenyl bromoacetate (19) gave the candidate irreversible inhibitor (IX) in 38% yield; the purified IX moved as a single spot on TLC and gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (19).

In order to synthesize X, a number of blocking

groups for the secondary amine group of XVII—such as tosyl—were investigated. Since these blocked derivatives gave a number of unexpected problems, the selective bromoacylation of XVII was investigated; a primary amino group should be more reactive than a secondary amino group and in addition this secondary amino group of XVII should be further deactivated by the electron-withdrawing *p*-carboxyl group (19). Reaction of XVII (6) with one equivalent of bromoacetic anhydride in acetone at 0° (19) gave the desired candidate irreversible inhibitor, X, in 71% yield; further purification gave a compound that moved as a single spot on TLC and gave a negative Bratton-Marshall test for primary aromatic amines, but gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (19)—thus showing that the bromoacetyl group had reacted with the primary amino group of XVII to give X.

Since the aminophenyl dioxolanes (XVIII and XIX) were readily available from another study (12), their reductive condensation with *p*-formylbenzoic acid was investigated. Reductive condensation of XVIII in methanol with the aid of sodium borohydride afforded XX in 69% yield; similarly, the styryl dioxolane (XIX) afforded XXI in 48% yield. Hydrolysis of the dioxolane blocking group of XX with boiling 65% ethanol containing 0.05 N hydrochloric acid afforded the active-site-directed irreversible inhibitor (XI) in 67% yield; similarly, hydrolysis of XXI gave XII in 34% yield (Scheme I).

Synthesis

Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in Nujol mull, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in 10% alcohol with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by visual examination under ultraviolet light and by the 4-(*p*-nitrobenzyl)pyridine spray for active halogen (19).

p-Cyanopropylbenzoic Acid (XV).—A magnetically stirred mixture of 972 mg. (4 mmoles) of XIV (6, 23), 1.17 Gm. (23.9 mmoles) of sodium cyanide, and 40 ml. of *N,N*-dimethylformamide was heated in a bath at 100–110° for 18 hr. The cooled mixture was poured into 200 Gm. of ice water and the pH was adjusted to about 1 with dilute hydrochloric acid. The polymeric material was separated by filtration and had $\lambda_{max.}$ 5.88 (ester C=O); 5.95 (acid C=O); 9.08 μ (aromatic ester C—O—C).

The filtrate was extracted with chloroform (5 × 50 ml.). The combined, dried extracts were spin evaporated *in vacuo*. Recrystallization of the residue from benzene–petroleum ether (b.p. 60–110°) gave 451 mg. (60%) of product, m.p. 150–157°, suitable for further transformation. Recrystallization from the same solvent pair gave white crystals, m.p. 158–160°; $\lambda_{max.}$ (pH 1): 244; (pH 7): 235 mμ; $\lambda_{max.}$ 4.45 (C≡N); 5.94 (C=O); 6.24 μ (C=C).

Anal.—Calcd. for $C_{11}H_{11}NO_2$: C, 69.8; H, 5.86; N, 7.40. Found: C, 70.1; H, 5.83; N, 7.70.

p-(4-Aminobutyl)benzoic Acid (XVI).—A solution of 859 mg. (4.55 mmoles) of XV in 100 ml. of ethanol and 0.75 ml. of 12 N aqueous hydrochloric
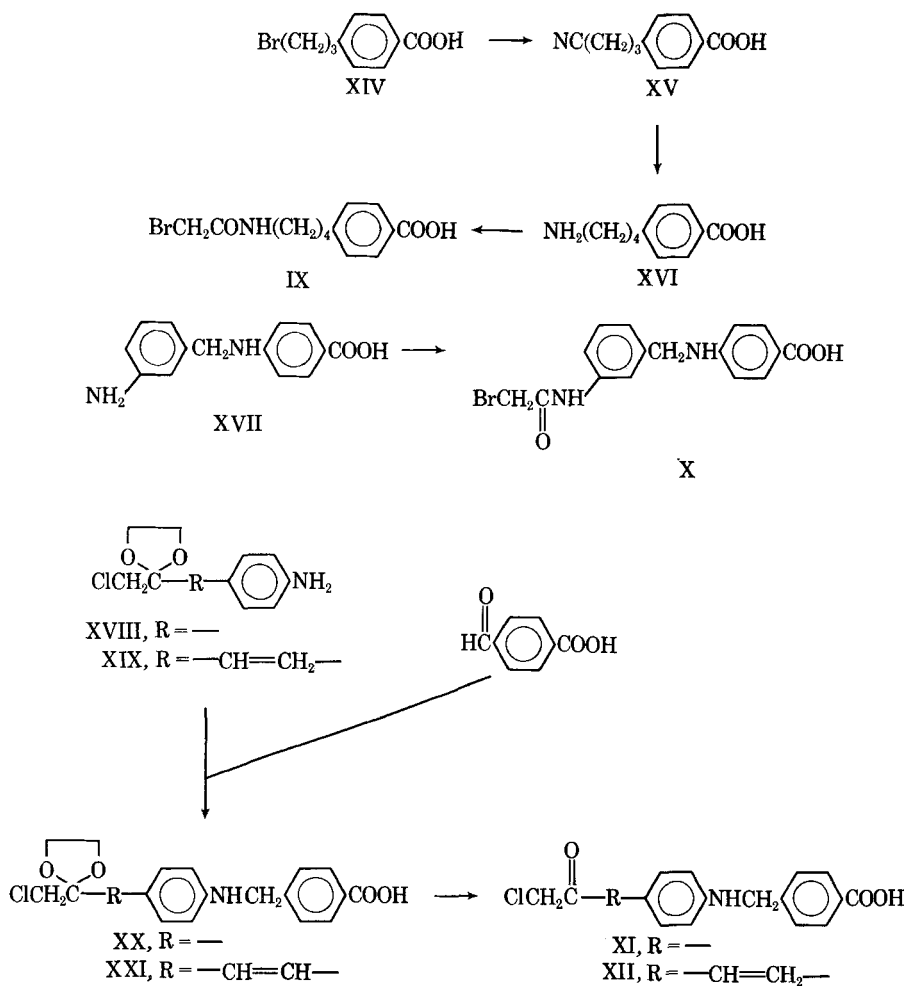
Scheme I

acid was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide until 3 mole equivalents of hydrogen were absorbed. The filtered solution was spin evaporated to dryness *in vacuo* to give 1086 mg. (>100%) of crude XVI hydrochloride, m.p. 230–240° dec., that was suitable for the next step; this material contained some ammonium chloride after recrystallization from methanol–ether, as shown by combustion analysis.

A portion of the hydrochloride was dissolved in 10 ml. of water, then the solution was clarified with decolorizing carbon. The solution was made basic with ammonia water, then spin evaporated *in vacuo*. Recrystallization from the minimum of hot water gave white crystals of XVI as a zwitterion, m.p. 259–262°; $\lambda_{max.}^{H_2O}$ (pH 1): 244; (pH 7): 234; (pH 13): 239 mμ; $\lambda_{max.}$ 2.90, 3.10 (NH); 4.74 (NH$^+$); 6.32 (C=C); 6.55–6.65 (COO$^-$); 11.80 μ (*p*-C$_6$H$_4$).

Anal.—Calcd. for C$_{11}$H$_{15}$NO$_2$: C, 68.4; H, 7.82; N, 7.25. Found: C, 68.1; H, 7.98; N, 7.36.

p - [4 - (Bromoacetamido)butyl]benzoic Acid (IX).—To a solution of 230 mg. (1 mmole) of XVI hydrochloride in 30 ml. of water was added 0.67 ml. (2 mmoles) of 3 N aqueous sodium hydroxide

followed by 5 ml. of acetone. After addition of 291 mg. (1.1 mmoles) of *p*-nitrophenyl bromoacetate, the mixture was magnetically stirred at ambient temperature for about 1 hr. The solution was spin evaporated *in vacuo* to about 20 ml. when an oil separated. The supernatant liquid was decanted and the oil rinsed with two 10-ml. portions of water. The combined aqueous solutions were acidified to about pH 1 with dilute hydrochloric acid. The product was collected on a filter and washed with water; yield, 120 mg. (38%), m.p. 160–168°. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°), then toluene gave white crystals, m.p. 175–176°; $\lambda_{max.}$ (pH 1): 247; (pH 7): 238 mμ; $\lambda_{max.}$ 3.04 (NH); 5.98 (carboxyl C=O); 6.09 (amide I); 6.24 (C=C); 6.50 μ (amide II). Thin-layer chromatography with benzene–methanol (3:1) showed a single spot under ultraviolet light; this spot gave a positive 4-(*p*-nitrobenzyl)pyridine test (19) for active halogen. Even though TLC indicated only one component, combustion analysis indicated the compound was only 90% pure.

Anal.—Calcd. for C$_{13}$H$_{16}$BrNO$_3$: C, 49.7; H,

5.12; N, 25.4. Found: C, 50.2; H, 5.10, Br, 23.1, 23.4.

N - (m - Bromoacetamidobenzyl) - p - aminobenzoic Acid (X).—To a solution of 484 mg. (2 mmoles) of XVII (6) in 4 ml. of acetone in an ice bath was added 520 mg. (2 mmoles) of bromoacetic anhydride (19). During 30 min. at 0°, the solution deposited tan crystals; 5 ml. of ether was added and the product was collected by centrifugation and washed with ether (5 × 8 ml.); yield, 518 mg. (71%), m.p. 192–195° dec. Recrystallization from aqueous ethanol with the aid of decolorizing carbon gave 355 mg. (49%) of nearly white crystals, m.p. 183–186°; λ_{max}^{KBr} 2.94, 3.03 (NH); 3.78–3.96 (broad, acidic OH); 6.05, 6.25, 6.43, 6.76 μ (C=O, NH, C=C). The compound gave a negative Bratton-Marshall test for primary aromatic amines (19) and a positive test for active halogen with 4-(p-nitrobenzyl)pyridine (19); it moved as a single spot on TLC in benzene–dioxane–glacial acetic acid (90/25/4).

Anal.—Calcd. for $C_{16}H_{15}BrN_2O_3$: C, 52.9; H, 4.16; Br, 22.0. Found: C, 53.1; H, 4.28; Br, 21.8.

2 - {p - [N - (p - Carboxybenzyl)amino]phenyl}-2 - chloromethyl - 1,3 - dioxolane (XX).—To a magnetically stirred mixture of 1.285 Gm. (6 mmoles) of XVIII (12) and 0.903 Gm. (6 mmoles) of p-formylbenzoic acid in 150 ml. of methanol was added 3.0 Gm. of sodium borohydride in portions over about 30 min. with cooling in a water bath; at the end of the addition, the mixture was homogeneous. After being stirred for 18 hr. at ambient temperature, the solution was spin evaporated *in vacuo.* The residue was leached with 50 ml. of boiling benzene to remove any unreacted XVIII. The residue was dissolved in water, a little insoluble material was removed by filtration, then the filtrate was acidified to about pH 1 with dilute aqueous hydrochloric acid with cooling. After the addition of 2 Gm. of Celite, the mixture was filtered and the filter cake washed with water, then dried. The cake was extracted with boiling acetone (3 × 50 ml.). The combined, filtered extracts were spin evaporated *in vacuo.* The residue was triturated with petroleum ether; yield, 1.446 Gm. (69%), m.p. 206–208°. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°) gave white crystals, m.p. 212–213°; λ_{max} (pH 1): 236; (pH 7): 259; (pH 13): 254 mμ; λ_{max} 2.95 (NH); 5.98 (carboxyl C=O); 6.21 (C=C); 9.65, 9.81 (ether C—O—C); 12.16 μ (p-C$_6$H$_4$).

Anal.—Calcd. for $C_{18}H_{18}ClNO_4$: C, 62.2; H, 5.22; N, 4.03. Found: C, 62.0; H, 5.26; N, 3.94.

2 - {p - [N - (p - Carboxybenzyl)amino]styryl}-2 - chloromethyl - 1,3 - dioxolane (XXI).—This was prepared from 482 mg. (2 mmoles) of XIX as described for the preparation of XX. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–110°) gave 358 mg. (48%) of product, m.p. 181–183°. Recrystallization from the same solvent pair gave nearly white crystals, m.p. 183–185°; λ_{max} (pH 1): 251; (pH 7): 232, 299; (pH 13): 291 mμ; λ_{max} 2.99 (NH); 6.00 (carboxyl C=O); 6.25 (C=C); 9.73, 9.85 (ether C—O—C); 12.25 μ (p-C$_6$H$_4$).

Anal.—Calcd. for $C_{20}H_{20}ClNO_4$: C, 64.3; H, 5.39; Cl, 9.48. Found: C, 64.0; H, 5.51; N, 9.62.

α - (p - Chloroacetylanilino) - p - toluic Acid (XI).—A solution of 363 mg. (1.04 mmoles) of XX in 54 ml. of absolute ethanol and 18 ml. of 0.2 N hydrochloric acid was refluxed for 30 min., then cooled overnight at 3°. The product (283 mg.) was collected on a filter and recrystallized from absolute ethanol to give 212 mg. (67%) of yellow crystals, m.p. 240° dec.; λ_{max} (pH 1): 242, 343; (pH 7): 238, 339; (pH 13): 235 (inflection), 343 mμ; λ_{max} 2.99 (NH); 5.82 (sh, ketone C=O); 6.03 (carboxyl C=O); 6.31 (C=C); 12.31 (p-C$_6$H$_4$); no C—O—C bands near 9–10 μ. The compound gave a positive 4-(p-nitrobenzyl)pyridine test (19) for active halogen and moved as a single spot on TLC in benzene–methanol (3:1).

Anal.—Calcd. for $C_{16}H_{14}ClNO_3$: C, 63.3; H, 4.65; Cl, 11.7. Found: C, 63.1; H, 4.79; Cl, 11.8.

α - [p - (4 - Chloro - 1 - butene - 3 - one - 1 - yl) anilino] - p - toluic Acid (XII).—This was prepared from XXI as described for XI. Recrystallization from absolute ethanol gave 88 mg. (34%) of yellow-orange crystals which blackened at 200° and did not melt to 250°; λ_{max} (pH 1): 292; (pH 7, 13): 240, 410 mμ; λ_{max} 3.00 (NH); 5.90 (sh, ketone C=O); 6.00 (carboxyl C=O); 6.38 (C=C); 12.45 (p-C$_6$H$_4$); no C—O—C bands near 9.5 μ. The product moved as a single spot on TLC in benzene–methanol (3:1) and gave a positive 4-(p-nitrobenzyl)pyridine test for active halogen (19).

Anal.—Calcd. for $C_{18}H_{16}ClNO_3$: C, 65.6; H, 4.89; Cl, 10.8. Found: C, 65.4; H, 4.82; Cl, 11.0.

REFERENCES

(1) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.,* **54,** 1415(1965).
(2) 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.
(3) Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.,* **54,** 1740(1965).
(4) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.,* **2,** 335(1965).
(5) Baker, B. R., and Lourens, G. J., *ibid.,* **2,** 344(1965)·
(6) Baker, B. R., Schwan, T. J., Novotny, J., and Ho, B.-T., *J. Pharm. Sci.,* **55,** 295(1966).
(7) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *ibid.,* **55,** 302(1966).
(8) Baker, B. R., and Shapiro, H. S., *ibid.,* **55,** 308(1966).
(9) Baker, B. R., and Jordaan, J. H., *ibid.,* **55,** 1417 (1966).
(10) Baker, B. R., and Shapiro, H. S., *ibid.,* **55,** 1422 (1966).
(11) *Ibid.,* **56,** 33(1967).
(12) Baker, B. R., and Jordaan, J. H., *J. Med. Chem.,* **8,** 35(1965).
(13) Baker, B. R., and Santi, D. V., *J. Pharm. Sci.,* unpublished data.
(14) Baker, B. R., and Ho, B.-T., *ibid.,* **56,** 28(1967).
(15) Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.,* **2,** 21(1965).
(16) *Ibid.,* **3,** 315(1966).
(17) *Ibid.,* **3,** 319(1966).
(18) *Ibid.,* **3,** 324(1966).
(19) Baker, B. R., Santi, D. V., Coward, J.K., Shapiro, H. S., and Jordaan, J. H., *ibid.,* **3,** December(1966).
(20) Baker, B. R., Ho, B.-T., and Neilson, T., *ibid.,* **1,** 79(1964).
(21) Baker, B. R., and Patel, R. P., *J. Pharm. Sci.,* **53,** 714(1964).
(22) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.,* **3,** 459(1962).
(23) Blicke, F. F., and Lilienfeld, W. M., *J. Am. Chem. Soc.,* **65,** 2281(1943),
(24) Baker, B. R., and Coward, J. K., *J. Pharm. Sci.,* **54,** 714(1965).