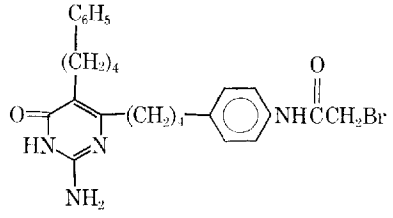
Irreversible Enzyme Inhibitors LXVIII

2-Amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

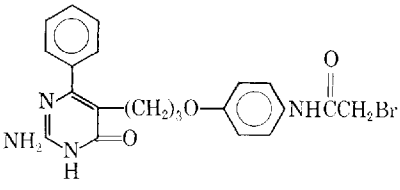
By B. R. BAKER and HOWARD S. SHAPIRO

2-Amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol (II), when incubated with dihydrofolic reductase at 37°, inactivated the enzyme with a half-life of about 45 min. In contrast, iodoacetamide and *p*-bromoacetamidophenylbutyric acid at the same concentration showed no inactivation of the enzyme in the same time. An interesting contrast to II was the 6-methyl analog (III) of II which inactivated the enzyme at about one-seventh the rate of II. This result gives unequivocal support for a previous suggestion that 6-methylpyrimidines and 6-phenylpyrimidines do not reversibly complex with dihydrofolic reductase in the same manner, else II and III should have inactivated the enzyme at the same rate at equal concentrations of reversible complex. These experiments are best explained on the basis of active-site-directed irreversible inhibition of dihydrofolic reductase.

OVER THIRTY attempts[1] to create an active-site-directed irreversible inhibitor (1) of dihydrofolic reductase were aborted prior to the discovery of the strong hydrophobic region on the enzyme adjacent to the active-site (2–8) and the realization that the pyrimidine type of inhibitor may have any one of several possible rotational conformers when complexed to the enzyme (8–10). Attention was then turned to the synthesis of candidate active-site-directed irreversible inhibitors that were substituted with one group on the 5- or 6-position that could complex to the hydrophobic region but also had a 6- or 5-substituent that could project into the hydrophilic region of the enzyme such as I and II. Efforts to synthesize the 5-phenylbutyl pyrimidine (I) and the 6-phenyl pyrimidine (II) started about the same time; I was found to be the first example of the long-sought active-site-directed irreversible inhibitors of dihydrofolic reductase, and II was found to be an irreversible inhibitor shortly thereafter. The synthesis and enzymic evaluation of I was presented in the previous paper of this series (11); the synthesis and enzymic evaluation of II is the subject of this paper.



I



II

DISCUSSION

Due to the lack of solubility, the maximum concentration in 10% *N,N*-dimethylformamide that could be reached with the 6-phenylpyrimidine (II) was 200 μM; at this concentration II showed 20% reversible inhibition in the presence of 6 μM dihydrofolate (12). From these data the 50% inhibition concentration was estimated to be 800 μM, and the K_i was estimated (3) to be $1 \times 10^{-4} M$. From Eq. 1, it could be estimated that at a concentration of 40 μM, 25% of the enzyme was in the form of an enzyme–II complex. It is the concentration of this complex that determines the rate of irreversible inhibition with an active-site-directed irreversible inhibitor (13–15) where [EI] = the enzyme–inhibitor reversible complex, E_t = the total active enzyme, [I] = the inhibitor concentration, and K_i = the reversible dissociation constant of EI (14, 15).

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

When dihydrofolic reductase was incubated in the absence of TPNH at 37° with 40 μM II at pH 7.4 in 10% *N,N*-dimethylformamide by the procedure previously described (9, 11), the enzyme was inactivated with a half-life of about 50 min.; thus, cor-

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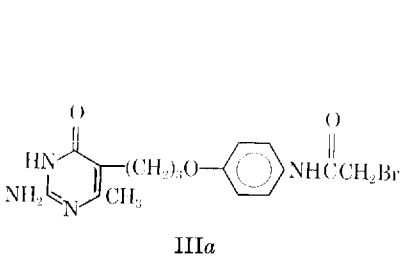
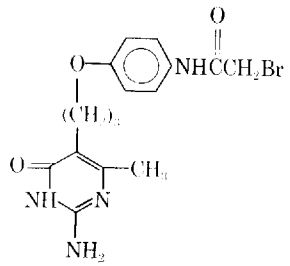
For a preliminary announcement of this work see *Reference 8*.

Previous paper: Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **55**, 1417(1966).

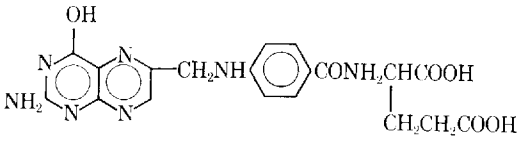
[1] For a listing of some of these unsuccessful candidate irreversible inhibitors see *References 11* and *15*.



IIIa



IIIb



IV

rected for equal concentrations of reversible E · · · I complex, II inactivates dihydrofolic reductase about one-half the rate of I. When 40 μM each of II, iodoacetamide, and *p*-bromoacetamido phenylbutyric acid (16) were incubated with dihydrofolic reductase simultaneously for 60 min. at 37°, only II showed irreversible inhibition; thus, the bimolecular mechanism of inactivation was readily eliminated (14, 15). Protection against inactivation was shown by the reversible inhibitors, folic acid (IV) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine, as noted with the inactivation of dihydrofolic reductase by I (11).

An interesting comparison as irreversible inhibitors are the 6-phenylpyrimidine (II) and the 6-methylpyrimidine (III), both having the identical *p*-bromoacetamidophenoxypropyl side chain at the 5-position. From previous studies on hydrophobic bonding (8), it was suggested that the data were best rationalized if a 6-phenylpyrimidine, such as II, has a conformation as depicted in II when compared to an assigned conformation of the pteridine ring in folic acid indicated in IV. In contrast, it was suggested that a 6-methylpyrimidine, such as III, would have its large 5-group complexing with the hydrophobic region as in conformation III*b*, but III would not complex in conformation III*a* or the conformation taken by II (8, 11). If II and III assumed identical conformations when complexed to the enzyme, then the alkylating function of both would be positioned in the enzyme complex in an identical fashion and both should attack an enzymic nucleophilic group at the same rate. The 6-methylpyrimidine (III) slowly inactivated dihydrofolic reductase with a half-life of about 220 min. at 37°, compared to II with a half-life of 45 min. Since III has K_i about $8 \times 10^{-5} M$, 40 μM III would convert 33% of the total enzyme to a reversible E · · · I complex; thus compared to 40 μM II—which gives 25% E · · · I complex—II inactivates dihydrofolic reductase about seven times the rate of III. This sevenfold difference in rate proves unequivocally that the main mode of reversible complexing to dihydrofolic reductase by II and III is different as previously suggested for 6-phenylpyrimidines (8). This slow inactivation by III is probably not due to a random bimolecular reaction (1, 14, 15); a more plausible explanation is that one out of seven times III complexes in the same conformation as II—then inactivates through the reversible complex—although this mechanism is not certain.

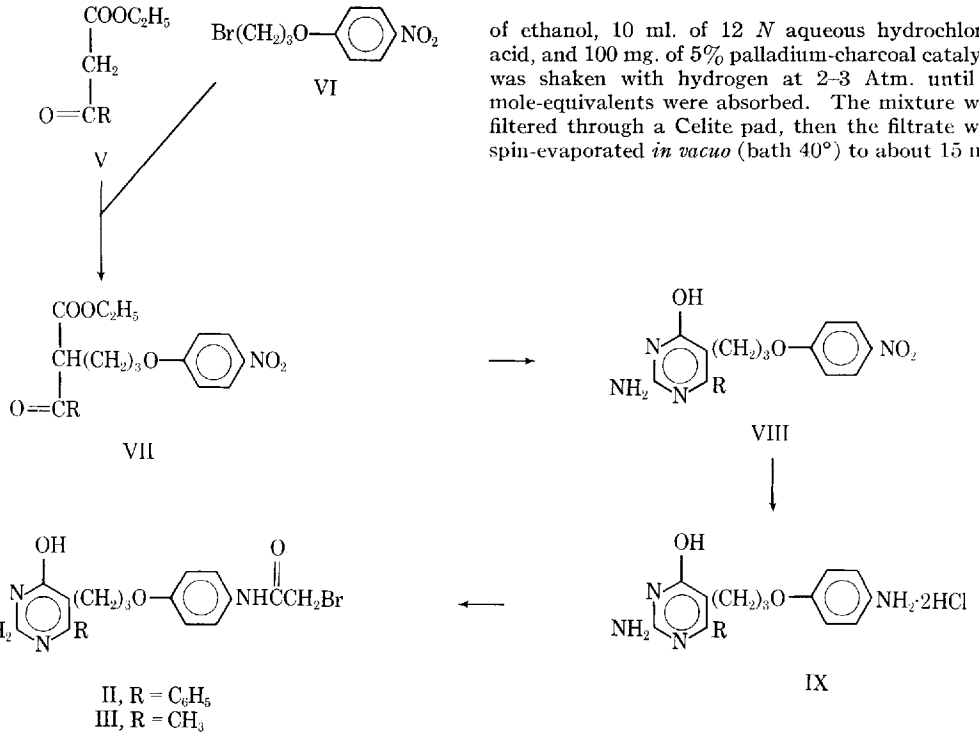
CHEMISTRY

Methods.—The general method of synthesis of 2-amino-6-R-4-pyrimidinols bearing a functionalized phenyl in a 5-side-chain developed earlier in this program (17) worked smoothly for the synthesis, the requisite precursors bearing a terminal nitrophenoxy group on the 5-side-chain (VIII). (Scheme I.) Alkylation of ethyl benzoylacetate (V*a*) with *p*-nitrophenyl-3-bromopropyl ether (VI) (18) in dimethylsulfoxide in the presence of sodium hydride gave the crude β-keto ester (VII*a*); condensation of VII*a* with guanidine carbonate in *tert*-butyl alcohol (19) gave the 4-pyrimidinol in 31% over-all yield of pure material. Earlier ethyl acetoacetate (V*b*) had been converted to VIII*b* *via* VII*b* (20).

Hydrogenation of the 5-nitrophenoxypropyl pyrimidinols (VIII) in ethanolic hydrochloric acid with a palladium-charcoal catalyst afforded pure IX*a* and IX*b* in 69 and 64% yields, respectively. The selective bromoacetylation of IX with bromoacetic anhydride in *N*,*N*-dimethylformamide has been previously described in a paper from this laboratory on selective bromoacylation methods for a variety of types of side-chain amino groups on a variety of 2-aminopyrimidines (16).

Synthesis.—Melting points were determined in capillary tubes on a Mel-Temp block, and those below 230° are corrected. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 recording spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B recording spectrophotometer.

2 - Amino - 5 - (p - nitrophenoxypropyl) - 6-phenyl-4-pyrimidinol (VIIIa).—To a magnetically stirred solution of 3.84 Gm. (20 mmoles) of ethyl benzoylacetate in 10 ml. of reagent dimethylsulfoxide protected from moisture was added 0.866 Gm. of 55.6% sodium hydride dispersed in mineral oil. When hydrogen evolution had ceased, 4.70 Gm. (18 mmoles) of VI (18) was added in one portion. After being stirred at ambient temperature for 18 hr., the



a series, R = C_6H_5
b series, R = CH_3

Scheme I

mixture was heated on a steam bath for 1 hr., then acidified with acetic acid, and poured into 100 ml. of 1:1 benzene–water with good stirring. The separated aqueous phase was extracted with 50 ml. of benzene. The combined benzene solutions were washed with two 50-ml. portions of ice cold 3% aqueous sodium hydroxide to remove unchanged V*a*, then washed with water. Dried with magnesium sulfate, the solution was spin-evaporated *in vacuo* leaving 6.3 Gm. of crude VII*a* as an oil.

The oil was dissolved in 50 ml. of *tert*-butyl alcohol, 1.8 Gm. (10 mmoles) of guanidine carbonate was added, then the mixture was gently refluxed with magnetic stirring for 48 hr. The cooled mixture was filtered and the product was washed with cold ethanol; yield, 2.65 Gm. By dilution of the filtrate with water an additional 0.90 Gm. (total 54%) of crude product was obtained. Two recrystallizations of the combined crude products from aqueous 2-methoxyethanol gave 2.05 Gm. (31%) of pure product as white crystals, m.p. 277–278°; $\lambda_{max.}$ (pH 1): 242 (ϵ 17,200), 290 mμ (ϵ 16,800); (pH 7): 242 (ϵ 17,800), 310 mμ (ϵ 21,900); (pH 13): 242 (ϵ 15,800), 310 mμ (ϵ 16,800); $\lambda_{max.}$ 2.92 (NH); 6.08, 6.31, 6.67 (NH, C=O, C=C, C=N); 6.72, 7.50 (NO_2); 7.93 (ether C—O—C); 11.88 (*p*-C_6H_4); 13.33, 14.40 μ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{18}N_4O_4$: C, 62.3; H, 4.92; N, 15.3. Found: C, 62.3; H, 5.12; N, 15.1.

2 - Amino - 5 - (p - aminophenoxypropyl) - 6-phenyl-4-pyrimidinol Dihydrochloride (IXa).—A mixture of 500 mg. (1.37 mmoles) of VIII*a*, 90 ml. of ethanol, 10 ml. of 12 *N* aqueous hydrochloric acid, and 100 mg. of 5% palladium-charcoal catalyst was shaken with hydrogen at 2–3 Atm. until 3 mole-equivalents were absorbed. The mixture was filtered through a Celite pad, then the filtrate was spin-evaporated *in vacuo* (bath 40°) to about 15 ml. The solution was diluted with ether to turbidity, then chilled at 3° for about 18 hr. The product was collected on a filter and washed with ether; yield, 473 mg. (93%), m.p. 185–190°. Recrystallization from absolute ethanol–ether with the aid of decolorizing carbon gave 315 mg. (69%) of pure product as nearly white crystals, m.p. 187–190°; $\lambda_{max.}$ 2.99 (NH); 5.98 (C=NH^+), 6.15, 6.50 (NH, C=O, C=C, C=N); 8.04 (ether C—O—C); 12.23 (*p*-C_6H_4); 13.35, 14.29 μ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{20}N_4O_2 \cdot 2HCl \cdot H_2O$: C, 53.5; H, 5.63; N, 13.3. Found: C, 53.7; H, 5.77; N, 13.3.

2 - Amino - 5 - (p - aminophenoxypropyl) - 6-methyl-4-pyrimidinol Dihydrochloride (IXb).—Reduction of 304 mg. (1 mmole) of VIII*b* (20), as described for the preparation of IX*a*, gave 288 mg. (83%) of product, m.p. 265–268°. Recrystallization from 2-methoxyethanol–ether with the aid of decolorizing carbon afforded 222 mg. (64%) of pure product as white crystals, m.p. 271–272°; $\lambda_{max.}$ 2.98, 3.10 (NH); 5.94 (C=NH^+); 6.03, 6.31, 6.66 (NH, C=O, C=N, C=C); 8.00 (ether C—O—C), 11.85, 12.05 μ (*p*-C_6H_4).

Anal.—Calcd. for $C_{14}H_{18}N_4O_2 \cdot 2HCl$: C, 48.4; H, 5.76; N, 16.1. Found: C, 48.3; H, 5.88; N, 16.0.

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Chemistry and Pharmacology of a Glycoside of *Vallaris solanacea*

By M. M. VOHRA, G. K. PATNAIK, R. S. KAPIL, and N. ANAND

One of the glycosides of *Vallaris solanacea* has been identified as *O*-acetyl-solanoside (*O*-acetyl acofreosyl-digitoxigenin). It possesses potent cardiotonic activity. Its pharmacological properties have been compared with those of lanatoside C and digoxin.

In a previous communication (1) it was reported that the glycoside mixture obtained from the leaves of *Vallaris solanacea* Kuntze (N.O. *Apocynaceae*) possesses powerful digitalis-like activity. Two recent publications by Kaufmann *et al.* (2, 3), describing the isolation and determination of the structure of six new glycosides from the seeds of *V. solanacea*, have prompted the authors to report the observation of glycoside B, one of the glycosides obtained from the leaves.

EXPERIMENTAL

Chemistry

Shade-dried leaves of *V. solanacea*, collected from Kashmir, India, were percolated with ethanol and the percolate was concentrated to about one-fifth its volume. The concentrate was diluted with an equal volume of water and extracted with benzene. The aqueous phase was concentrated *in vacuo* and extracted with chloroform. The residue from the chloroform extract was taken up in chloroform and chromatographed on a column of silica gel (E. Merck, fine grade), and the column developed with chloroform containing increasing proportions of methanol when three major glycoside fractions A, B, and C were obtained. On thin-layer chromatography [Silica Gel G; solvent system, ethyl methyl ketone–cyclohexane (1:1)] fractions A and C showed up as two spots, while fraction B gave only one spot (spots detected by spraying with water). Preparative thin-layer chromatography of fraction B [Silica Gel G; solvent system, chloroform–isopropyl alcohol (19:1)] gave glycoside B, which was crystallized from chloroform–hexane and ethanol–water mixture, m.p. 137–139° (Kofler block). The angle of rotation was $[\alpha]_D^{20} - 16$ (±2) (c, 1 in methanol). The homogeneity of the glycoside was established by thin-layer chromatography (Silica Gel G) in four solvent systems: ethyl acetate; chloroform–isopropyl alcohol (19:1); ethyl methyl ketone–cyclohexane (1:1); benzene methanol (3:1).

Anal.—Calcd. for $C_{32}H_{48}O_9$: C, 66.6; H, 8.39. Found: C, 67.05; H, 8.75.

Mannich hydrolysis of glycoside B gave a single sugar which was identified as acofreose by paper chromatographic comparison with an authentic sample [solvent systems: methyl ethyl ketone–*n*-butanol (1:1)/borate buffer; toluene–*n*-butanol (1:1)/water], while Killiani hydrolysis yielded digitoxigenin. The highly nonpolar character of glycoside B suggested the possibility of one of its hydroxyl groups being blocked. Its I.R. absorption spectrum and color reactions indicated its identity with *O*-acetyl-acofreoside of digitoxigenin (*O*-acetyl-solanoside) (3). This was confirmed by comparison with an authentic sample of *O*-acetyl-solanoside using thin-layer chromatography as described above.

A thin-layer chromatographic comparison kindly carried out by Reichstein of fractions A and C with the glycosides obtained from the seeds (2, 3), showed that the glycosides contained in the leaves are very similar to those present in the seeds.

Pharmacological Activity

Materials and Methods.—A stock solution of glycoside B containing 500 mcg./ml. was prepared

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The authors are grateful to Professor T. Reichstein for direct comparison of the glycosides obtained by us with those isolated in their laboratory, to Mr. P. A. George for statistical analysis, to Dr. (Mrs.) S. Nitya Nand for help in ECG studies, and to Dr. C. Ray for his interest in the work.