Irreversible Enzyme Inhibitors LXIX

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase III. Bromoacyl Derivatives of 5-(*p*-Aminophenoxypropyl)-2,4,6-triaminopyrimidines

By B. R. BAKER and DANIEL V. SANTI

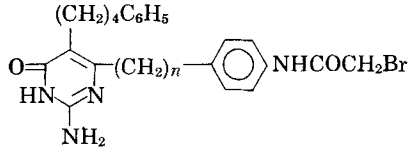
5-(*p*-Aminophenoxypropyl)-2,4,6-triaminopyrimidine (XIV*d*) was synthesized by alkylation of malononitrile by 3-bromopropyl *p*-nitrophenyl ether (X*b*), followed by ring closure with guanidine and catalytic reduction of the nitro group. Selective haloacylation of the aromatic amino group of XIV*b* was accomplished by protonation of the triaminopyrimidine moiety of XIV*d* with acetic acid. Treatment with the anhydrides of bromoacetic acid, *p*-bromoacetamidophenylbutyric acid, and *N*-bromoacetyl-β-alanine gave the pure bromoacyl derivatives, XIV*e*, *i*, and *j*. These three compounds were good reversible inhibitors of dihydrofolic reductase, but failed to show irreversible inhibition; these failures are attributed to the phenoxypropyl group of the inhibitors (XIV) being complexed with a hydrophobic region on the enzyme—a region not apt to have groups that could form a covalent bond.

PRIOR TO THE discovery of a strong hydrophobic bonding region on dihydrofolic reductase (1) with its conformational requirements (2–6) and the possible pyrimidine rotomers that could bind to the enzyme (5–9), more than 30 candidate compounds were synthesized and evaluated as active-site-directed irreversible inhibitors (5, 10) of this enzyme with negative results. By proper utilization of the hydrophobic region, successful active-site-directed irreversible inhibitors (I–III) were found (11, 12). By complexing the hydrophobic region by a 5-phenylbutyl group—as in the pyrimidines, I and II (11)—or by 6-phenyl as in III (12), the remaining aralkyl group then projected into a hydrophilic region on the enzyme and could covalently link a nucleophilic group on the enzyme.

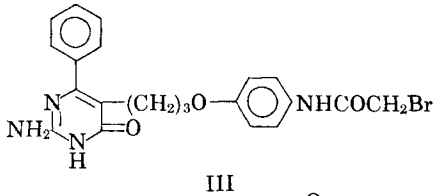
DISCUSSION

The first candidate active-site-directed irreversible inhibitor successfully synthesized in this laboratory was IV; the latter was a good reversible inhibitor of dihydrofolic reductase, but gave no inactivation of the enzyme. Subsequent work on hydrophobic bonding to dihydrofolic reductase (1–9) allowed a suitable rationalization for this failure; the 5-side chain of IV was most probably complexed in a hydrophobic region and, therefore, was not likely to be juxtaposed to a nucleophilic region on the enzyme. The synthesis of inhibitors such as IV was accomplished only after a number of false starts; this successful synthesis of IV paved the way for the ultimate synthesis and enzymic evaluation of the successful candidate inhibitors (I–III) (11, 12).

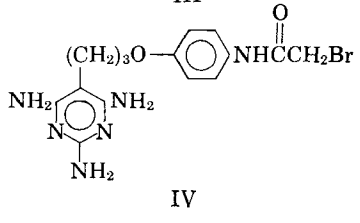
Since the anilinopropyl pyrimidinol (VII*a*) was a reasonably good inhibitor of dihydrofolic reductase (13, 14), the related *p*-bromoacetamidoanilinopropyl pyrimidinol (VII*e*) was selected as a candidate active-site-directed irreversible inhibitor (5, 10) for dihydrofolic reductase, and its synthesis was undertaken. The various reactions following are outlined in Scheme I. The first general route developed to structures of type VII was the reductive condensation of an arylamine (VI) with the pyrimidine-5-propionaldehyde (V) (13, 15). The condensation failed to give VII*b* with the more weakly basic *p*-nitroaniline (VI*b*). The required intermediate (VII*b*) was synthesized by the second general route starting the *N*-(*p*-nitrophenyl)-*p*-toluenesulfonamide *via* VIII*b* and IX*b* (16). Re-



I, $n=2$
II, $n=4$



III



IV

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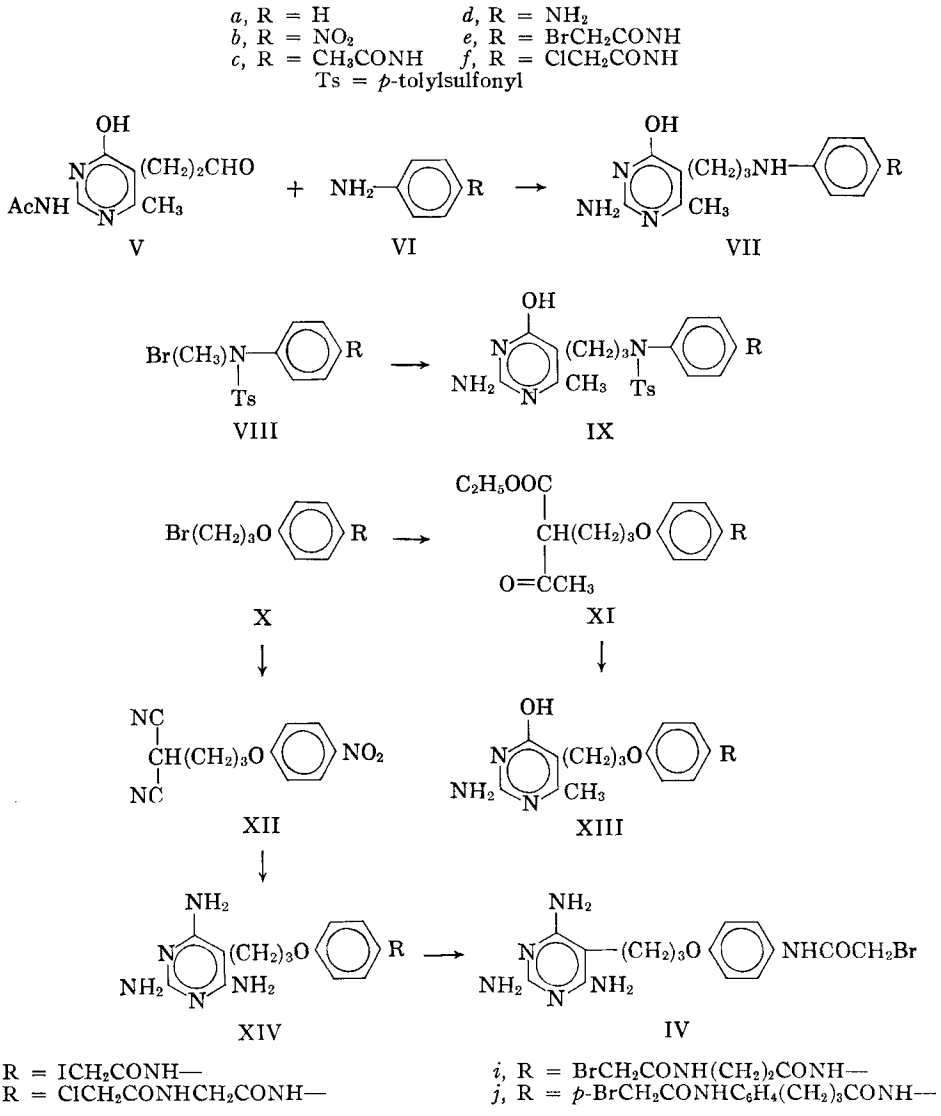


Scheme I

duction of the nitro group of VII*b* to the substituted *p*-phenylenediamine (VII*d*) or preparation of VII*d* from the *p*-acetamidoanilinopropyl pyrimidinol (VII*c*) in this and the corresponding 6-phenyl series (17) appeared to be intractable due to the instability of the *p*-phenylenediamine moiety.

Since the anilinopropyl group of VII*a* can be replaced with phenoxypropyl (XIII*a*) with no change in binding to the enzyme (18) and since the *p*-anisidine is much more stable than *p*-phenylenediamine, the synthesis of XIII*b* was investigated. The latter was readily synthesized from *p*-nitrophenyl 3-bromopropyl ether (X*b*) by alkylation of ethyl acetoacetate to XI*b* followed by ring closure with guanidine to XIII*b*. Such a synthesis has considerable latitude in variation of the 4- and 6-substituents of the pyrimidine ring. Therefore, the 2,4,6-triaminopyrimidine ring system bearing a 5-(*p*-nitrophenoxypropyl) group (XIV*b*) was selected for study with the extra consideration that 2,4,6-triaminopyrimidines are better reversible inhibitors of dihydrofolic reductase than the corresponding 2-amino-6-methyl-4-pyrimidinols (1, 5, 19).

Alkylation of malononitrile with the bromopropyl phenyl ether (X*b*) gave a crude product (XII) which was condensed with guanidine in ethanol to give the triaminopyrimidine (XIV*b*) in 51% over-all yield; this product contained some minor impurities (by TLC) that could not be removed. Since XIV*b* was rather intractable, it was reduced catalytically to XIV*d* with a Raney nickel catalyst; the anisidine derivative (XIV*d*) was readily purified in 56% yield.

Methods were then developed for selective halo-

acylation of the polyfunctional XIV*d* to give derivatives of type XIV*e–j* where only the aromatic amino group has become acylated. The first studies in this laboratory on selective acylation of aminophenyl groups attached to an aminopyrimidine were performed on XIV*d*. Although the methods developed for selective bromoacylation of these aminopyrimidines have been collected in one paper (20) to indicate the scope and limitations of the reaction, the background work was performed on XIV*d* and will be reported here.

It was reasoned that the greatest selectivity in acylation of the aromatic amino group of XIV*d* would occur if the more strongly basic triaminopyrimidine moiety could be protonated throughout the experiment. Such a procedure would be more difficult with an acid chloride than with an anhydride, since the evolved hydrogen chloride would be more difficult to keep neutralized at acid pH than the organic acid evolved from an anhydride. The first acylation was performed with chloroacetic anhydride on XIV*d* in acetone containing excess aqueous 5% acetic acid; within 10 min. precipitation occurred. By use of excess chloroacetic anhydride to overcome partial aqueous hydrolysis of the anhydride, the reaction was carried to completion. The salt that separated appeared to be a mixture of monoacetate and monochloroacetate. Upon warming the mixture, the salt redissolved; the solution was added to excess aqueous potassium carbonate, then the isolated free base of XIV*f* was converted to the acetate salt; when dried without heating, this material gave combustion values for a diacetate salt. Extended drying at 100° gave varying amounts of acetate in the salt. Attempts to convert XIV*f* to the iodo derivative (XIVg) with sodium iodide in acetone containing glacial acetic acid were unsuccessful, the products seeming to be unstable.

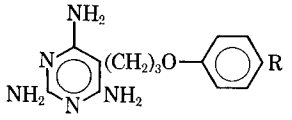
Similarly, the anhydride of chloroacetylglycine could be used to form XIV*h*—isolated as an acetate salt. However, with bromoacetic anhydride, the product (XIV*e* = IV) could not be treated with aqueous potassium carbonate since some of the bromoacetyl group hydrolyzed to hydroxyacetyl. Therefore, a different set of criteria and a different isolation had to be devised, namely (*a*) the Bratton-Marshall test (13, 15, 21) for aromatic amines was negative; (*b*) thin-layer chromatography showed the presence of a single spot that gave a positive *p*-nitrobenzylpyridine test for active halogen (22, 23); (*c*) ultraviolet spectra were essentially unchanged showing that an amino group on the pyrimidine had not become acylated (24).

Treatment of XIV*d* in 2:1 acetone–10% acetic acid at 0° with 2–3 equivalents of bromoacetic anhydride resulted in a salt of the product (XIV*e* = IV) separating from solution. Since this salt was probably a mixture of acetate salt and bromoacetate salt and since IV could not be converted to the free base without decomposition, the acetate salt of IV was directly converted to the salt of a stronger acid such as hydrobromic or sulfuric acid; either a monohydrobromide or dihydrobromide of IV could be prepared, depending upon the conditions (20).

Similarly, the use of the anhydrides of *N*-bromoacetyl-β-alanine and *p*-bromoacetamidophenylbutyric acid (20) gave XIV*i* and *j*, respectively, isolated as their acetate and sulfate salts, respectively.

Enzymatic Evaluation—Table I lists the results on reversible and irreversible inhibition of dihydrofolic reductase with the bromoacyl derivatives of structure IV or XIV. All three compounds are better reversible inhibitors than the 4-pyrimidinol (II). However, when incubated at a concentration sufficient to convert 50% of the enzyme to the reversible complex (E · · · I), the bromoacyl derivatives of structure IV or XIV failed to show any inactivation in 1 hr. at 37°. In contrast, the 5-phenylbutyl-6-(*p*-bromoacetamidophenylbutyl)-4-pyrimidinol (II) showed 50% inactivation in 18 min. (11). These results are believed to be due to the 5-phenoxypropyl group of XIV being complexed to a hydrophobic region on the enzyme where there are not apt to be any polar nucleophilic groups.

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
XIV*d*	NH_2	57	9				
IV	$BrCH_2CONH$	28	5	5	50	60	0
XIV*i*	$BrCH_2CONH(CH_2)_2CONH$	100	16	16	50	60	0
XIV*j*	p-$BrCH_2CONHC_6H_4(CH_2)_3CONH$	13	2	2	50	120	0
II[e]		240	40	40	50	18	50
				10	20	25	43

[a] The dihydrofolic reductase was a 45–90% ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μ*M* dihydrofolate and 12 μ*M* TPNH in 0.05 *M* Tris buffer (pH 7.4) containing 10 m*M* mercaptoethanol, as previously described (14). [b] Dihydrofolic reductase was incubated with the inhibitor at 37° in 0.05 *M* Tris buffer (pH 7.4) containing *no* mercaptoethanol and *no* TPNH, but diluted with 10% of its volume with *N*,*N*-dimethylformamide in which the inhibitor had been dissolved; in each case an enzyme control was run that showed 0–4% inactivation (22). [c] Estimated from $K_i = I \times Km/S$ where I = inhibitor concentration giving 50% inhibition (5, 25); this equation is valid since $S = 6\ Km > 4\ Km$. [d] Calculated from $[EI] = [Et]/[1 + Ki/I]$, where $[Et]$ = the concentration of total active enzyme and $[EI]$ = fraction of Et reversibly complexed by I (5, 26). [e] Data from *Reference 11*.

EXPERIMENTAL

Melting points were taken 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 137B recording spectrophotometer. Ultraviolet spectra were taken in water, unless otherwise indicated, 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.

3-Bromopropyl p-Nitrophenyl Ether (Xb)—To a well stirred solution of 120 Gm. (0.60 mole) of 1,3-dibromopropane in 75 ml. of dimethylsulfoxide was added 29.4 Gm. (0.15 mole) of sodium *p*-nitrophenolate dihydrate. After 1 hr. the mixture had formed a jell; it was heated on a steam bath for 30 min., then spin-evaporated *in vacuo* at 100°. The residue was partitioned between 750 ml. of benzene and 500 ml. of water. The organic layer was washed successively with five 500-ml. portions of water, two 500-ml. portions of 0.5 *N* aqueous sodium hydroxide, then once more with water. The benzene layer was spin-evaporated *in vacuo;* the last traces of higher boiling materials were removed at 100° at 1 mm. The oily residue was crystallized from ethanol. The product was collected on a filter and washed with petroleum ether (b.p. 30–60°) until the washings were colorless; yield, 26.0 Gm. (67%), m.p. 52–54°; this product was suitable for further transformations even though TLC with petroleum ether (b.p. 60–110°)–benzene (1:1) showed it was only about 90% pure. Recrystallization from petroleum ether (b.p. 60–110°) gave yellow crystals, m.p. 57–58°. [Lit. m.p. 58–59° (27).]

2-Amino-6-methyl-5-(p-nitrophenoxypropyl)-4-pyrimidinol (XIIIb)—A mixture of 70 ml. of *tert*-butyl alcohol, 3.90 Gm. (0.03 mole) of ethyl acetoacetate, and 0.72 Gm. (0.03 mole) of sodium hydride as a 56.5% suspension in mineral oil was magnetically stirred until hydrogen evolution was complete. After the addition of 5.2 Gm. (0.02 mole) of X*b*, the mixture was gently refluxed for 24 hr. protected from moisture. Acidified with glacial acetic acid, the mixture was spin-evaporated *in vacuo* finally at about 1 mm. to remove ethyl acetoacetate. The oily residue was dissolved in about 50 ml. of chloroform and washed with water. After being dried with magnesium sulfate, the solution was evaporated *in vacuo* to give crude XI*b* as an oil; yield, 5.7 Gm. of an oily residue containing some mineral oil.

A part (3.12 Gm., 10 mmoles) of the residual XI*b* and 0.99 Gm. (5 mmoles) of guanidine carbonate in 20 ml. of *tert*-butyl alcohol was gently refluxed with magnetic stirring for 48 hr. The cooled mixture was filtered and the product washed with 25 ml. of hot ethanol. The crude product was dissolved in about 15 ml. of hot 1 *N* aqueous sodium hydroxide. The hot solution was clarified by filtration, then acidified to about pH 5 with 3 *N* aqueous hydrochloric acid. After being cooled to 0°, the mixture was filtered and the product was recrystallized from 2-methoxyethanol–water; yield, 1.26 Gm. (42% based on X*b*) of light yellow crystals, m.p. 260–261° dec. $\nu_{max.}$ 3400–3100 (broad, NH); 1640, 1600, 1590 (NH, C=O, C=C, C=N); 1525, 1320 (NO_2); 1255 (C—O—C); 750, 720, 690 cm.$^{-1}$ (p-C_6H_4).

Anal.—Calcd. for $C_{14}H_{16}N_4O_4$: C, 55.3; H, 5.30; N, 18.4. Found: C, 55.1; H, 5.43; N, 18.6.

5-(p-Nitrophenoxypropyl)-2,4,6-triaminopyrimidine (XIVb)—To a magnetically stirred mixture of 5 ml. of dimethylsulfoxide and 0.24 Gm. (10 mmoles) of sodium hydride as a 56.5% dispersion in mineral oil was added 0.72 Gm. (11 mmoles) of malononitrile in 2 ml. of dimethylsulfoxide over a period of about 5 min. with cooling in a water bath. When hydrogen evolution was complete, a solution of 2.6 Gm. (10 mmoles) of X*b* in 4 ml. of dimethylsulfoxide was added. After 10 min. at ambient temperature, the mixture was heated at 75° for 30 min., then poured into 50 ml. of benzene. The mixture was washed with three 50-ml. portions of water, then spin-evaporated *in vacuo*. To the residual XII*b* was added a filtered solution of 1.05 Gm. (11 mmoles) of guanidine hydrochloride and 0.595 Gm. (11 mmoles) of sodium methoxide in 10 ml. of absolute ethanol. After being refluxed with magnetic stirring for 3 hr. protected from moisture, the mixture was cooled. The amorphous product was collected on a filter and washed with 100 ml. of hot ethanol in portions; yield, 1.57 Gm. (51%), m.p. >250°. $\nu_{max.}$ 3450, 3390, 3200 (NH); 1660, 1620–1575 (broad) (NH, C=C, C=N, NO_2); 1335 (NO_2); 757, 720, 690 cm.$^{-1}$ (p-C_6H_4). $\lambda_{max.}$ (pH 1): 284, 320 mμ; (pH 13): 278, 320 mμ.

TLC in ethanol showed one major spot and several minor spots; no suitable purification method could be found, but the reduction product (XIV*d*) could be purified.

5-(p-Aminophenoxypropyl)-2,4,6-triaminopyrimidine (XIVd)—A mixture of 7.6 Gm. (0.025 mole) of crude XIV*b*, 200 ml. of 70% aqueous ethanol, and about 15 Gm. of Raney nickel was shaken with hydrogen at 2–3 Atm. until reduction was complete (4 hr.). The mixture was filtered through a pad of diatomaceous earth[1] then spin-evaporated *in vacuo*. The oily residue readily crystallized on trituration with ethanol. Two recrystallizations from ethanol afforded 3.9 Gm. (56%) of buff-colored crystals, m.p. 179–180°; the compound moved as a single spot on TLC in ethanol. $\lambda_{max.}$ (pH 1): 283 mμ (ε 18,400); (H_2O): 279 mμ (ε 12,600); (pH 13): 277 mμ (ε 12,400). $\nu_{max.}$ 3500, 3400, 3150 (NH); 1650–1575 (broad) (NH, C=C, C=N); 1240 (C—O—C); no nitro band near 1340 cm.$^{-1}$.

Anal.—Calcd. for $C_{13}H_{18}N_6O$: C, 56.9; H, 6.62; N, 30.7. Found: C, 56.7; H, 6.62; N, 30.9.

5-(p-Chloroacetamidophenoxypropyl)-2,4,6-triaminopyrimidine (XIVf)—To a magnetically stirred solution of 822 mg. (3 mmoles) of XIV*d* in 12 ml. of acetone and 12 ml. of 5% aqueous acetic acid cooled in an ice bath was added a solution of 1.026 Gm. (6 mmoles) of chloroacetic anhydride in 6 ml. of acetone. The mixture was stirred at ambient temperature for 2 hr., then the separated solid was redissolved by warming. The solution was poured into 60 ml. of 5% potassium carbonate previously cooled to 0°. The product was collected on a filter and washed with 25 ml. of cold water, then dried. The dry XIV*f* free base was dissolved in a small volume of glacial acetic acid, then slowly precipitated by dropwise addition of ethyl acetate.

[1] Marketed as Celite by Johns-Manville Corp., New York, N. Y.

The *diacetate* salt was collected, washed with ethyl acetate and ether, then dried at room temperature *in vacuo;* yield, 1004 mg. (71%), m.p. 144–146° dec. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500, 3400, 3300–3150 (NH); 1670 (amide I); 1640–1610 (C=C, C=N); 1570 cm.$^{-1}$ (amide II). TLC in chloroform–ethanol (5:3) showed one spot.

Anal.—Calcd. for $C_{15}H_{19}ClN_6O_2 \cdot 2CH_3COOH$: C, 48.5; H, 5.78; N, 17.8. Found: C, 48.7; H, 5.94; N, 17.6.

When dried at 100° in high vacuum to constant weight, the analysis indicated that most of the diacetate had been converted to the free base; the m.p. had now changed to >200° dec.

Anal.—Calcd. for $C_{15}H_{19}ClN_6O_2$: C, 51.6; H, 5.43; O, 10.8. Found: C, 50.9; H, 5.64; O, 10.8.

N-Bromoacetyl-β-alanine—To a magnetically stirred solution of 4.56 Gm. (0.05 mole) of β-alanine and 2.81 Gm. (0.05 mole) of potassium hydroxide in 15 ml. of water cooled in an ice–salt bath was added, in five portions (5 min. apart), 5.25 ml. (0.06 mole) of bromoacetyl bromide; immediately after each portion of bromoacetyl bromide was added in five portions 25 ml. of 2.8 *N* aqueous potassium carbonate. After being stirred an additional 1 hr. at −5 to 0°, the solution was acidified to about pH 3 with 12 *N* aqueous hydrochloric acid, then extracted with ethyl acetate (3 × 75 ml.). The combined extracts were washed with 75 ml. of water, dried with magnesium sulfate, then spin-evaporated *in vacuo*. The residue was leached with hot petroleum ether (b.p. 60–110°) (2 × 50 ml.), then crystallized from chloroform at −20°; yield, 4.07 Gm. (40%), m.p. 71–77°. Two more recrystallizations from chloroform at −20° gave 2.44 Gm. (23%) of white crystals, m.p. 83–84°. $\nu_{max.}^{Nujol}$ 3300 (amide NH); 1700 (carboxyl C=O); 1640 (amide I); 1550 cm.$^{-1}$ (amide II).

Anal.—Calcd. for $C_5H_8BrNO_3$: C, 28.6; H, 3.82; N, 6.70. Found: C, 28.4; H, 3.84; N, 6.55.

No effort was made to obtain optimum yields.

5-[(p-Chloroacetylglycylamido)phenoxypropyl]-2,-4,6-triaminopyrimidine (XIVh) Acetate—To a magnetically stirred solution of 1.516 Gm. (10 mmoles) of *N*-chloroacetylglycine in 30 ml. of reagent ethyl acetate cooled in an ice bath was added 1.236 Gm. (6 mmoles) of *N,N*-dicyclohexylcarbodiimide. After being stirred 2 hr. at ambient temperature, the mixture was filtered and the dicyclohexylurea was washed with two 10-ml. portions of ethyl acetate. The combined filtrate and washings were spin-evaporated *in vacuo* (bath 25°) to about 10 ml. Addition of about 35 ml. of petroleum ether (b.p. 30–60°) gave 0.896 Gm. (67%) of *N*-chloroacetylglycine anhydride that was not further purified and had $\nu_{max.}$ 3300 (NH); 1825, 1760 (anhydride C=O); 1675 (amide I); 1550 cm.$^{-1}$ (amide II).

Reaction of XIV*d* with an excess of this anhydride as described for the preparation of XIV*f* gave 56% of product after recrystallization from glacial acetic acid–ethyl acetate and drying at 100° *in vacuo* for 24 hr. After being dried at 100° in high vacuum, it showed no definite m.p., but gradually decomposed over 200°. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500–3200 (NH); 1780 (amide I); 1660–1600 (NH, C=C, C=N); 1560 cm.$^{-1}$ (amide II). TLC in chloroform–ethanol (5:3) showed a single spot.

Anal.—Calcd. for $C_{17}H_{22}ClN_7O_3 \cdot {}^1/_2CH_3COOH$: C, 49.3; H, 5.50; N, 22.4; O, 14.6. Found: C, 49.2; H, 5.54; N, 22.4; O, 13.7.

5-[(p-Bromoacetyl-β-alaninamido)phenoxypropyl]-2,4,6-triaminopyrimidine (XIVi) Diacetate—To a magnetically stirred solution of 274 mg. (1 mmole) of XIV*d* in 6 ml. of acetone and 2 ml. of 10% aqueous acetic acid cooled in an ice bath was added 643 mg. (1.6 mmoles) of *N*-bromoacetyl-β-alanine anhydride (prepared as described for the anhydride of chloroacetylglycine in tetrahydrofuran as solvent). After 1 hr. at 0°, during which time a salt of XIV*i* separated, the mixture was filtered, and the product was washed with acetone. The product was recrystallized from glacial acetic acid by addition of ethyl acetate; yield, 485 mg. (83%), m.p. 158–161°. A second recrystallization gave 395 mg. (68%), m.p. 160–162°. $\lambda_{max.}$ (pH 1): 284 mμ; (pH 13): 276 mμ. $\nu_{max.}$ 3500–3200 (NH); 1680–1600 (amide I, C=C, C=N); 1570 cm.$^{-1}$ (amide II). The compound traveled as a single spot on TLC in chloroform–ethanol (5:3); the spot gave a negative Bratton-Marshall test and a positive *p*-nitrobenzylpyridine test (20). For analysis the compound was dried at room temperature in high vacuum.

Anal.—Calcd. for $C_{18}H_{24}BrN_7O_3 \cdot 2CH_3COOH$: C, 45.1; H, 5.50; Br, 13.6. Found: C, 45.1; H, 5.65; Br, 13.9.

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