239–240°. $\lambda_{max.}$ (pH 1) 234, 263 mμ (*A* 260/280, 1.80); (pH 13) 235, 278 mμ (*A* 260/280, 1.04).

Anal.—Calcd. for $C_{17}H_{20}N_4O_4$: C, 59.3; H, 5.85; N, 16.3. Found: C, 59.0; H, 5.98; N, 16.0.

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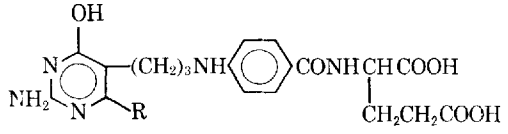
Analogs of Tetrahydrofolic Acid XXXIV

Hydrophobic Bonding to Dihydrofolic Reductase VI. Mode of Phenyl Binding of Some 6-Arylpyrimidines

By B. R. BAKER* and HOWARD S. SHAPIRO

New data are presented which strongly support the concept that the increment in better binding observed by substituting a phenyl group on the 6-position of 4-pyrimidinol is due to hydrophobic bonding of the phenyl group. Furthermore, 11 6-phenylpyrimidines with various substituents at the 2,4, and 5-positions were compared with 18 5-aryl and 5-arylalkylpyrimidines as inhibitors of dihydrofolic reductase. The results cannot be explained by a single conformation of the pyrimidine being complexed to the enzyme; therefore, a number of rotational conformers for the pyrimidine ring are proposed where the strong hydrophobic bonding by the phenyl or phenylalkyl substituent is the determining factor for the particular preferred conformation of a given inhibitor. Such a hypothesis has previously been invoked to explain the inhibitor and substrate binding to chymotrypsin by Niemann *et al.*

THE 6-PHENYLPYRIMIDINE analog (I) (1) of tetrahydrofolic acid was observed to be a twelvefold better inhibitor of dihydrofolic reductase at pH 7.4 than the prototype 6-methylpyrimidine analog (II) (2, 3). When assayed



I, R = C_6H_5
II, R = CH_3

with folic acid as substrate at pH 6.1, I was found to be a twentyfold better inhibitor of the reductase than II (1, 4). Three possible explanations for this increased binding were proposed (1)—namely, (*a*) a charge-transfer complex, (*b*) the phenyl influences the binding of the pyrimidine ring, and (*c*) hydrophobic bonding. Since inductive effects by substitutents on the phenyl ring could not be completely correlated (5), explanations (*a*) and (*b*) can now be considered unlikely. The emergence of strong hydrophobic bonding by 5-alkylpyrimidines (6) and the further experimental evidence (7, 8) for hydrophobic bonding by the aryl group of 1-aryl-1,2-dihydro-*s*-triazines, 2-aryl-*s*-triazines, and 5-arylpyrimidines suggested that the mode of phenyl binding of 6-phenylpyrimidines be further investigated from the standpoint of hydrophobic bonding.

Rather than proceeding chronologically on the development of the possible hydrophobic bonding of the phenyl group of 6-phenylpyrimidines—as is customarily done with papers from this laboratory—it is somewhat easier to follow the evidence if the assumptions on binding are presented in logical order rather than by order arrived at from the tortuous trail of chronology.

DISCUSSION

The following recent developments on binding of inhibitors to dihydrofolic reductase are pertinent to the arguments to follow.

Received November 8, 1965, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo.
Accepted for publication December 22, 1965.
This work was generously supported by grants CA-05867 and CA-06624 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Previous paper: Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *J. Pharm. Sci.*, **55**, 302(1966).
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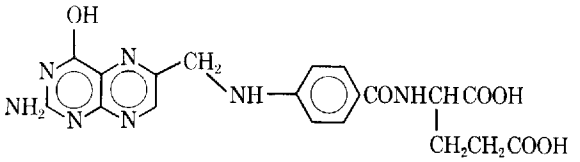


III

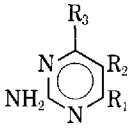
(*a*) The pH profiles for some strongly basic and some weakly basic pyrimidine-type inhibitors were determined (9, 10). These results gave experimental evidence to support the hypothesis that the pyrimidines had only 2 binding points to the enzyme and that a variety of conformations of the pyrimidines were possible for complexing to the enzyme; the points of binding of the pyrimidine proposed were: first, a general bonding to the electron-rich π-cloud of the pyrimidine ring by a weakly acidic group on the enzyme, and second, 1 additional hydrogen bond.

(*b*) Experimental evidence has been presented (11, 12) that the hydrophobic bonding region most probably is not between the binding regions for the pyrimidyl and *p*-aminobenzoyl moieties of inhibitors such as II or folic acid.

Since folic acid (III) and aminopterin are good inhibitors of dihydrofolic reductase (9, 13), both inhibitors and the substrate, dihydrofolate, must have a definite conformation when complexed to the enzyme. The conformation of the pteridin moiety of folic acid (III) and aminopterin can be assigned as in III and the enzyme can then take a conformation in space that will fit the pteridine as written. The conformation of the various pyrimidines will then be written with relationship to the assigned conformation of the pteridine moiety of III. The assumption has already been made that pyrimidines and pteridines have 2 binding points to the enzyme and that the pyrimidines may have any one of a number of conformations when complexed to the enzyme, depending upon the substituents attached to the pyrimidine (9, 10).

A single conformation for pyrimidine binding cannot explain all the inconsistencies on binding

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



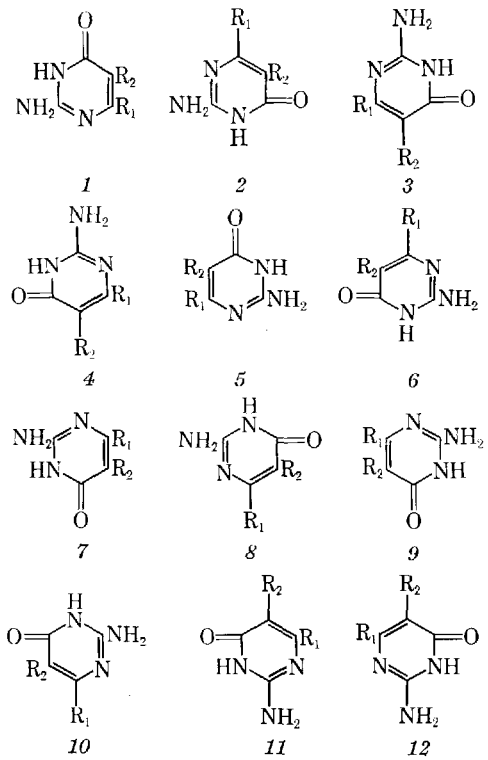
Compd.	R_3	R_2	R_1	μM Concn. for 50% Inhibition[a]	Suggested Conformation for Binding[b]
IV	OH	$C_6H_5NH(CH_2)_3$—	CH_3	800[c,d]	*11*
V	SH	$C_6H_5NH(CH_2)_3$—	CH_3	44	*11*
VI	OH	$C_6H_5NH(CH_2)_3$—	C_6H_5	Insol.[e]	*2*
VII	SH	$C_6H_5NH(CH_2)_3$—	C_6H_5	>1000[d,e,f]	*2*
VIII	H	$C_6H_5NH(CH_2)_3$—	CH_3	480[c]	*11*
IX	H	$C_6H_5NH(CH_2)_3$—	C_6H_5	>3000[d,f]	*2*
X	NH_2	$C_6H_5(CH_2)_4$—	CH_3	0.027[g]	*11, 12*
XI	NH_2	$C_6H_5NH(CH_2)_3$—	CH_3	2.2[c]	*11, 12*
XII	NH_2	$C_6H_5(CH_2)_4$—	C_6H_5	1.1	*2*
XIII	NH_2	$C_6H_5NH(CH_2)_3$—	C_6H_5	0.88	*2*
XIV	NH_2	H	CH_3	1100[h]	*2, 3, 5, 6, 9–12*
XV	NH_2	H	C_6H_5	160[d,i]	*2*
XVI	NH_2	n-C_4H_9	CH_3	2.0[d,h]	*11, 12*
XVII	NH_2	n-C_4H_9	C_6H_5	29[d]	*2*
XVIII	NH_2	H	p-$C_6H_5C_6H_4$	29[d]	*2*
XIX	NH_2	$C_6H_5(CH_2)_4$—	n-C_3H_7—	0.021	*12*
XX	OH	$C_6H_5(CH_2)_4$—	n-C_3H_7—	900[d]	*11*
XXI	OH	$C_6H_5(CH_2)_4$—	CH_3	30[d,j,k]	*11*
XXII	SH	$C_6H_5(CH_2)_4$—	C_6H_5	320[d]	*2*
XXIII	H	$C_6H_5(CH_2)_3$—	C_6H_5	3800[d,l]	*2*
XXIV	NH_2	$C_6H_5NH(CH_2)_3$—	$C_6H_5CH_2$—	3.8	*12*
XXV	NH_2	$C_6H_5(CH_2)_4$—	$C_6H_5CH_2$—	0.34	*12*
XXVI	OH	$C_6H_5(CH_2)_4$—	C_6H_5—	Insol.[e]	*2*

[a] Dihydrofolic reductase was a 45–95% saturated ammonium sulfate fraction prepared from pigeon liver acetone powder and assayed with 6 μM dihydrofolate, and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM Versene as previously described (14). The 50% inhibition points were determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration; the 50% inhibition point occurs at the intercept of $V_0/V_I = 2$ (35, 36). The technical assistance of Miss Maureen Baker, Mrs. Shirley Humphrey, Mrs. Gail Salomon, and Miss Karen Smith is acknowledged. [b] See Table II. [c] Data from *Reference 14*. [d] Cell contained 10% *N,N*-dimethylformamide. [e] Too insoluble to determine 50% inhibition point with this assay. However, with folic acid as a substrate at pH 6.0, VI had $Ki = 1.8 \times 10^{-6}$, XXVI had $Ki = 0.58 \times 10^{-6}$, VII had $Ki = 9.8 \times 10^{-6}$, IV had $Ki = 63 \times 10^{-6}$, and V had $Ki = 4.5 \times 10^{-6}$ (5). [f] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is at least 4 times greater than the concentration measured. [g] Data from *Reference 15*. [h] Data from *Reference 3*. [i] Data from *Reference 8*. [j] Same result obtained with or without *N,N*-dimethylformamide present. [k] Data from *Reference 37*. [l] Estimated from $V_0/V_I = 1.30$, and the error is larger than when $V_0/V_I = 2$.

previously observed, such as (*a*) 2-amino-5-(anilinopropyl)-6-methyl-4-pyrimidinethiol (V) is a better inhibitor than the 4-pyrimidinol (IV) (14), but in the 6-phenyl series (VI and VII) the reverse is true (5). (*b*) 2-Amino-5-(anilinopropyl)-6-methyl-4-pyrimidinol (IV) still binds as well to the enzyme when the 4-oxo group is removed (VIII) (14), but in the 6-phenyl series, removal of the oxo group of VI gives an inhibitor (IX) that has a large loss in binding compared to IV (Table I). (*c*) 2,4-Diamino-6-methyl-5-(phenylbutyl)pyrimidine (X) is a considerably better inhibitor than the corresponding 5-anilinopropyl pyrimidine (XI) (15) (Table I), but in the 6-phenyl series, the 2 compounds (XII and XIII) give about the same inhibition (5). (*d*) In contrast to (*b*), 2,4-diamino-6-phenylpyrimidine (XV) is a better inhibitor than 2,4-diamino-6-methylpyrimidine (XIV) (8), but when an *n*-butyl or a phenylbutyl side chain is introduced, the 6-methyl series (XVI and X) is better than the 6-phenyl series (XVII and XII) (Table I). There are numerous other examples. By the use of several conformations for pyrimidine binding, but using only a 2-point attachment of the pyrimidine ring, all of these inconsistencies can be suitably rationalized.

If no assumptions on binding are made, then there are 12 major possible conformations for pyrimidine binding (Table II) including the *1* conformation given for folic acid and aminopterin.

TABLE II.—POSSIBLE BINDING CONFORMATIONS FOR 2-AMINO-5,6-DISUBSTITUTED-4-PYRIMIDINOLS TO DIHYDROFOLIC REDUCTASE



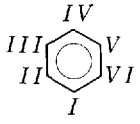
1 *2* *3*

4 *5* *6*

7 *8* *9*

10 *11* *12*

If one considers that 60°-twists between the 12 conformations could be further split into fraction of degrees of rotation, there are an infinite number of conformations; for example, the difference between *conformation 1* and *conformation 11* is a 60° twist. It might be argued that other conformers with smaller rotations between *1* and *11* should be considered, but as a first approximation, these 60° *rotational conformers* listed in Table II can be used to rationalize most of the inhibitor data. The designation of pyrimidine numbering cannot be used to signify the position of a group with respect to the given conformation of folic acid, *1*, with other conformers unless the particular conformer is also named; therefore the binding areas in Table II are numbered, based on conformer *1*, as follows:



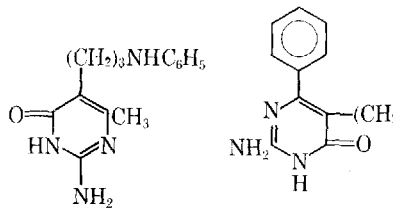
As pointed out previously, the pyrimidine probably has 2 binding points: (*a*) between a weakly acidic group on the enzyme to the electron-rich π-cloud of the pyrimidine ring and (*b*) a hydrogen bond to the enzyme where the pyrimidine group is an electron donor to the enzyme (9). The first bond (*a*) would be fairly independent of conformation since it is somewhat centralized, but the second bond must be in one of the areas *I* to *VI*.

Assume that the hydrogen bond (*b*) is at area *VI*. It should be noted among the 12 conformers that an NH_2, ring N, or C=O can be an electron donor for a hydrogen bond, but the acidic ring NH cannot and a C=S can donate for a hydrogen bond only poorly. On this basis the following conformers are possible for the 2-aminopyrimidines depending upon the 4-substituent. (*a*) 2-Amino-4-oxo: *2, 3, 5, 6, 10, 11*. (*b*) 2,4-Diamino: *2, 3, 5, 6, 9, 10, 11, 12*. (*c*) Among the conformations possible for (*a*) and (*b*), the 2-amino-4-H and 2-amino-4-thione can have conformations *5, 6, 9, 10, 11*, if binding is as good as 2-amino-4-oxo, but *2*, or *3*, if binding is poorer than 2-amino-4-oxo.

If *additional binding* occurs at the *p*-aminobenzoyl locus (area *V*) by R_1 or R_2, then the following conformations of those cited still remain possible. (*a*) 2-Amino-4-oxo: *2, 11*. (*b*) 2,4-Diamino: *2, 11*. (*c*) 2-Amino-4-H or 2-amino-4-thione: *11* if binding is as good as 2-amino-4-oxo, but *2*, if binding is poorer than 2-amino-4-oxo.

Assume that additional hydrophobic bonding occurs in area *IV*. Then the following conformations are still possible if either R_1 or R_2 bonds to the hydrophobic region, regardless of whether there is binding at the *p*-aminobenzoyl locus. (*a*) 2-Amino-4-oxo: *2, 6, 11*. (*b*) 2,4-Diamino: *2, 6, 11, 12*. (*c*) Among the conformations for (*a*) and (*b*), the 2-amino-4-H and 2-amino-4-thione can have conformations *6* and *11* if binding is as good as 2-amino-4-oxo, but only *2* if binding is poorer than 4-oxo.

Consider the specific case of 2-amino-5-(anilinopropyl)-6-methylpyrimidine with a 4-substituent. Since R_2 = anilinopropyl is the only group that can hydrophobically bond at region *IV* or complex to the *p*-aminobenzoyl locus (region *V*), and since both the 4-mercapto and 4-H compounds bind better than the 4-oxo compound, the following conforma-



IV–*11* VI–*2*

tions are possible if the anilinopropyl group is complexed to the hydrophobic region. (*a*) 2-Amino-4-oxo: *2* and *11*, but not *6*. (*b*) 2,4-Diamino: *2*, *11*, and *12*, but not *6*. (*c*) 2-Amino-4-H and 2-amino-4-SH: *11* since these compounds bind better than the 2-amino-4-oxo, but not conformation *2*.

The binding of the anilinopropyl group to the *p*-aminobenzoyl locus can only be by conformation *2* since *11* has the R_2 group placed incorrectly. Since the replacement of the 4-oxo group by 4-thione or 4-H gave better inhibitors, conformation *2* is not possible. Therefore, the anilinopropyl group cannot complex with the *p*-aminobenzoyl region *V*, but can only be hydrophobically bonded to the *IV* region. Either conformation *11* or *12* is satisfactory for 2,4-diamino and 2-amino-4-H, but only *11* is satisfactory for the 2-amino-4-oxo and 4-thione derivatives. Again it should be pointed out that *12* is not satisfactory when an acidic NH is present at the *VI* region needed for hydrogen bonding, but *12* is satisfactory with the 4-amino or 4-H since the acidic hydrogen of *12* has been removed. Evidence that *12* is the preferred conformation for the 2,4-diamino compound will be presented later in the fine points on interpretation.

Now consider the specific case of 2-amino-5-(anilinopropyl)-6-phenylpyrimidine with various 4-substituents. This case differs from the 6-methyl case in that either the 5- or 6-side chain of the pyrimidine could be complexed to the hydrophobic region. The following conformations are possible. (*a*) 2-amino-4-oxo: *2*, *6*, *11*. (*b*) 2,4-Diamino: *2*, *6*, *11*, *12*. (*c*) Of the conformations possible in (*a*) and (*b*), the 2-amino-4-H gives poorer binding than 2-amino-4-oxo only in conformation *2*.

Therefore, conformation *2* will explain all the binding of the 2-amino-5-(anilinopropyl)-6-phenylpyrimidines with 4-substituents. Conformation *2* places the anilinopropyl group at the *p*-aminobenzoyl locus and the 6-phenyl group in the hydrophobic region *IV*. There is additional information which supports the suggestion that the 6-phenyl group is hydrophobically bonded and that will be discussed later.

Although all of the data can be explained by the assumptions that there are 2 bonds from the enzyme to a pyrimidine—one which is complexed with the electron-rich π-cloud of the pyrimidine and the other is at the *VI*-region—are there other sets of assumptions which can or cannot explain the observed inhibition data?

First, is it necessary to assume that one of the 2 bonds is to the electron-rich π-cloud of the pyrimidine? Cannot there be 2 bonds to 2 definite groups in regions *I–VI*? In such a consideration, one can eliminate immediately any bonding by the hydrogen of an acidic NH, otherwise 2-amino-4-pyrimidinols would be better inhibitors than 2,4-diaminopyrimidines rather than vice versa. Assume that there are 2 hydrogen bonds to the *II* and *VI* regions; then the following conformations in Table II are possible. (*a*) 2-Amino-4-oxo: *2*, *6*. (*b*) 2,4-Diamino: *2*, *6*, *11*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformation *11* or *12* if it binds as good as 2-amino-4-oxo.

Therefore, the assumption of 2 hydrogen bonds to areas *II* and *VI* is invalid, since the 4-oxo derivative cannot bind in conformation *11* or *12* and the 4-H derivative cannot bind in conformation *2* or *6*. Similarly, 2 hydrogen bonds to areas *II* and *IV*, or areas *IV* and *VI*, or areas *I* and *IV* can be eliminated.

Assume that there are 2 hydrogen bonds to areas *I* and *II*, then the following conformations in Table II are possible. (*a*) 2-Amino-4-oxo: *1*, *12*. (*b*) 2,4-Diamino: *1*, *2*, *6*, *7*, *11*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformations *1* and *12* if it binds as good as 2-amino-4-oxo.

With these 2 particular hydrogen bonds it is not possible to account for 2-amino-5-(3-anilinopropyl)-6-phenylpyrimidine (IX) being a poorer inhibitor than the corresponding 4-oxopyrimidine (VI), since both allowable 4-oxo conformations, *11* and *12*, would not lose a binding group if the 4-oxo group were removed. Therefore, the assumption that pyrimidine binding occurs through 2 hydrogen bonds at the *I* and *II* regions is invalid. Similarly, hydrogen bonds to areas *I* and *VI* can be eliminated. Thus, all 10 combinations of 2 hydrogen bonds to discrete areas are eliminated. Similarly, 3 or 4 hydrogen bonds can be eliminated, the 4 hydrogen bond theory (16) having been eliminated by other means (9, 10).

With the assumption that 1 of the bonds to the pyrimidine is a general one depending on the electron-rich π-cloud (general basicity) of the pyrimidine, how much can the other two assumptions made earlier be varied and explain the data? These other 2 assumptions agreeing with the data were that (*a*) a hydrogen bond to the *VI* region was present, and (*b*) hydrophobic bonding was in the *IV* region.

First, keep (*a*) constant and vary (*b*). If the hydrophobic bonding region were at the *III* area, then the following conformations are possible. (*a*) 2-Amino-4-oxo: *5*, *6*. (*b*) 2,4-Diamino: *5*, *6*, *9*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformations *5*, *6*, *9*, and *12* if binding is as good as 4-oxo, but there is no possible conformation for the 4-H being poorer than 4-oxo.

Therefore, these parameters will not allow for a conformation that will bind 2-amino-5-(anilinopropyl)-6-phenylpyrimidine with a 4-oxo group (VI), but that will not bind to the corresponding 4-H pyrimidine (IX). Thus, the assumption of hydrophobic bonding at the *III* region is invalid. By similar arguments, hydrophobic bonding at regions *I*, *II*, and *V* can be eliminated.

There is 1 other set of assumptions which will explain all the data—namely, (*a*) 1 general bond due to the electron-rich π-cloud of the pyrimidine, (*b*) a hydrogen bond to the *IV* region, and (*c*) hydrophobic bonding at the *VI* region. Conformation *1* will accommodate the 6-phenyl series and conformation *7* will accommodate the 6-methyl series; note that *1* is a "flipped-over" conformation of *2*, and *7* is a "flipped-over" conformation of *11*. To dis-

tinguish this mode of binding from a hydrogen bond to the *VI* area and hydrophobic bonding to the *IV* area is not possible with the compounds in Table I; such a useful differentiation will require, and is worthy of, further study. Whether there are other combinations of these 3 basic assumptions that will explain all the data would probably require computer techniques to make sure that no possibilities have been overlooked.

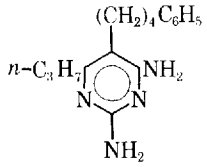
Additional evidence that the 6-phenyl group of 6-phenylpyrimidines is hydrophobically bonded to dihydrofolic reductase can be gleaned from Table I. (*a*) 2,4-Diamino-6-phenylpyrimidine (XV) is a sevenfold better inhibitor than 2,4-diamino-6-methylpyrimidine (XIV) (Table I). (*b*) The hydrophobic region is already complexed by the 6-phenyl of XV, since introduction of a *n*-butyl group on XV gives an inhibitor (XVII) that is only fivefold better. In contrast, the 5-*n*-butyl-6-methylpyrimidine (XVI) is a 550-fold better inhibitor than the corresponding 5-H pyrimidine (XIV). (*c*) Further change of the *n*-butyl group of the 6-phenylpyrimidine (XVII) to anilinopropyl (XIII) or phenylbutyl (XII) gives about the same thirtyfold increment in binding indicating that the aryl 5-side-chain group of XII and XIII is not complexed in the hydrophobic region but more likely is complexed to the *p*-aminobenzoyl locus. In contrast, change of the butyl group of the 6-methylpyrimidine (XVI) to anilinopropyl (XI) gives no increment, but change to phenylbutyl (X) gives an 85-fold increment in binding, indicating that this terminal aryl group in the 6-methyl series is complexed to the hydrophobic region where phenylbutyl would be expected to bind better than anilinopropyl.

These 3 basic assumptions explain the following points.

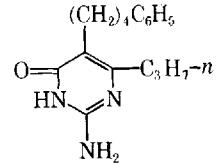
(*a*) In the 6-phenyl series with binding in conformation *2*, replacement of the 4-oxo group (VI) by 4-H (IX) or 4-thione (VII) decreases the binding, whereas in the 6-methyl series with conformation *11*, replacement of the 4-oxo group (IV) by 4-H (VIII) or 4-thione (V) gave better binding.

(*b*) The 4-thione group of V in conformation *11* is adjacent to the hydrophobic region and could lead to better binding than the 4-oxo group of IV, since the former is better tolerated in a hydrophobic region. In the 4-H series (VIII), one might expect VIII to be even less repulsed in the *III* region; however, it is possible that the highly polar 3-N of VIII is water solvated which could then make VIII about the same as IV as an inhibitor.

(*c*) A new point that is now explainable is in the comparison of the 6-methyl series (X, XXI) and 6-propyl series (XIX, XX). Note that with 2,4-diamino substituents, X and XIX are equal in effectiveness, but the 2-amino-4-oxo derivative in the 6-methyl series (XXI) is a thirtyfold better inhibitor than in the 6-propyl series (XX). The conformational binding assignments for 2,4-diamino-6-methylpyrimidines with a 5-side chain were *11* and *12*, whereas the 2-amino-4-oxo-6-methyl pyrimidines could only have conformation *11*. Conformation *11* forces the *n*-propyl group into region *V*, a probable hydrophilic region, whereas conformation *12* allows the propyl group to be in a hydrophobic region *III*. Thus, some repulsion of a propyl group in conformation *11* could occur which would explain the difference between XX and XXI. The opposite type of repulsion has been



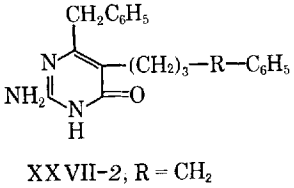
XIX-*12*



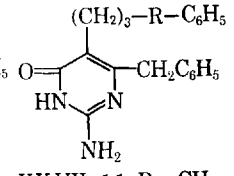
XX-*11*

observed with 5-phenylbutyl-2,4,6-triaminopyrimidine (6, 17); since conformations *11* and *12* are equivalent with the 4,6-diaminopyrimidines, 1 amino group must project into the hydrophobic *III* region with resultant repulsion of the polar amino group. One would expect the order of repulsion in the *III* region to be $NH_2 > =O > =S$.

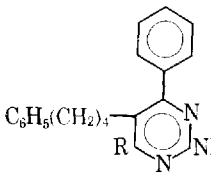
(*d*) The fact that 2,4-diamino-5-phenylbutyl-6-benzyl pyrimidine (XXV) is an elevenfold better inhibitor than the corresponding 5-anilinopropyl pyrimidine (XXIV) indicates that XXIV and XXV have conformation *12*, where the 5-side chain is hydrophobically bonded to area *IV*; conformation *2* is unlikely with the 6-benzyl hydrophobically bonded since one would expect less difference between the anilinopropyl and phenylbutyl side chains as previously noted with the comparison of XII and XIII in the 6-phenyl series. Note, however, that in the 2-amino-4-oxo-6-benzyl series, conformation *12* is not allowable due to the acidic NH in the *VI* region. Therefore, in this series, conformations *2* and *11* must be considered. It was previously reported (5) that in the 6-benzyl series the 5-anilinopropylpyrimidine (XXVIII) was about twice as effective as the corresponding 5-phenylbutylpyrimidine (XXVII). Since there is so little difference in binding between these two 5-side chains, conformation *2* is preferred which places the 5-side chain at the *p*-aminobenzoyl locus where the phenylbutyl and anilinopropyl side chains could be expected to have a similar amount of binding (note the similar comparison of XII and XIII in Table I where conformation *2* is favored). Even though the phenylbutyl side chain could be expected to give much stronger hydrophobic bonding than a benzyl side chain (19), the combination of probable hydrophilic repulsion of the benzyl group in conformation *11* could be sufficient to give the best net binding in conformation *2*. Where the balance between hydrophobic bonding in region *IV* and *p*-aminobenzoyl locus binding in region *V* is close, it is quite difficult to differentiate the preferred conformations. For example, these results could also be explained by XXVIII having conformation *2* and XXVII conformation *11*; if such were the case, then the 6-methyl 5-(phenylbutyl)-4-pyrimidinol (XXI) should bind the same as XXVII-*11*—since there is no hydrophobic bonding by the benzyl group in XXVII-*11*. With the anilinopropyl side chain, conformation XXVIII-*2* could bind better in the 6-benzyl series—with its benzyl hydropho-



XXVII-*2*, R = CH_2
XXVIII-*2*, R = NH

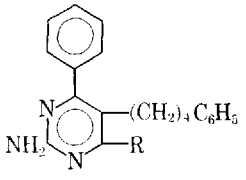


XXVII-*11*, R = CH_2
XXVIII-*11*, R = NH



XII–*6*, R = NH_2

XXIII–*6*, R = H



XII–*2*, R = NH_2

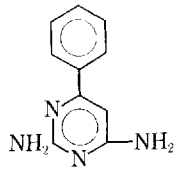
XXIII–*2*, R = H

bically bonded and the anilino bonded at the *p*-aminobenzoyl locus in region *V*—than can the 6-methyl pyrimidine (IV) which can have hydrophobic bonding in conformation *11*, but with no additional binding at the *p*-aminobenzoyl locus.

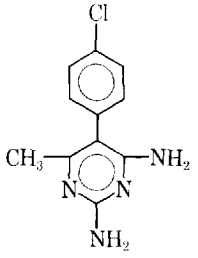
(*e*) It might be possible that 2,4-diamino-5-phenylbutyl-6-phenylpyrimidine (XII) is complexed in the *6*-conformation if the added increment in binding between XII and the *n*-butylpyrimidine (XVII) is due to hydrophobic bonding by the ω-phenyl in the *III–IV* region. If such were the case, then the corresponding 4-H pyrimidine (XXIII) should be as good an inhibitor, complexed in the conformation *6*, as the 4-oxopyrimidine (XXVI). Unfortunately, XXVI was too insoluble to determine the concentration necessary for 50% inhibition of dihydrofolic reductase; therefore, a less direct comparison was made. In the 6-methyl series, removal of the 4-amino group of XI to give VIII led to a 220-fold decrease in binding—due primarily to a loss of basicity (9, 10)—but VIII and IV were similar in binding. In the 6-phenyl series, removal of the 4-amino group of XII to give XXIII led to a 3500-fold decrease in binding. The sixteen-fold difference in these 2 increments is about what could be expected if 1 binding point were missing in XXIII, the remaining 220-fold decrease between XII and XXIII being presumably due to decreased basicity (9, 10). Therefore, this quite tenuous interpretation indicates that XII and XXIII bind in conformation *2*, and not in conformation *6*.

An important difference remains in rationalizing why a 6-phenylpyrimidine such as XV with conformation *2* gives only about a sixfold increment by hydrophobic bonding, but a 5-phenyl pyrimidine such as XXIX or a 1-phenyl-dihydro-*s*-triazine (XXX) in conformation *12* gives about a 1000-fold increment in binding (6). There are some obvious possibilities, such as (*a*) the 6-phenyl pyrimidine (XV) may be a weaker base than XXIX (pKa 7.7) or XXX (pKa 11.2) (21); (*b*) the 3-point attachment of pyrimidine π-cloud, hydrogen bond, and hydrophobic region are juxtapositioned somewhat differently in the case of XV *versus* the case XXIX or XXX, that is, a lateral movement of XV-*2* to the left by 1 atom distance compared to XXIX or XXX would be necessary for the same hydrogen bonding in the *VI* region; (*c*) the π-cloud overlaps between the phenyl and pyrimidine rings may be more favorable for π-cloud and hydrophobic interaction for XXIX with the enzyme than XV; (*d*) if the 4-NH_2 group of XV-*2* is less basic than the 3-N of XXIX-*12* or the 5-N of XXX-*12*, then XV-*2* will bind less effectively, but not so much as the 150-fold less effectiveness noted.

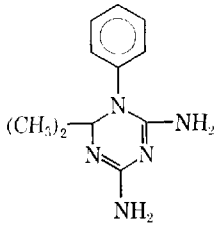
Spectrophotometric determination of the pKa of XV afforded a value of 6.23. Thus, XV is 6.4% protonated at the pH 7.4 of the assay, XXIX is 67% protonated, and XXXII is 99.9%.



XV-*2*



XXIX-*12*

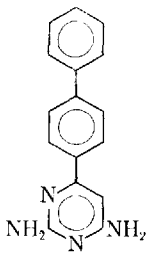


XXX-*12*

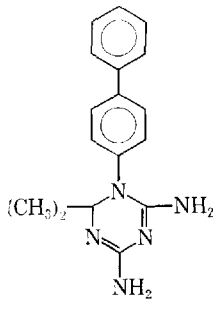
The difference in binding observed between XXIX and the totally unprotonated 6-trifluoromethyl analog of XXIX was only 250-fold (10). Thus, the 800-fold difference in binding between XV and XXIX is only partially accounted for by the difference in their respective basicities.

That the aryl group of XV-*2* was not placed in the hydrophobic region in exactly the same manner as the aryl group of XXX-*12* was clearly shown by comparison of the *p*-biphenyl analog (XVIII-*2*) with the *p*-biphenyl-*s*-triazine (XXXI-*12*). The 6-(*p*-biphenyl)pyrimidine (XVIII-*2*) was a five-fold better inhibitor than the corresponding 6-phenylpyrimidine (XV-*2*), indicating some additional hydrophobic bonding compared to XV-*2*; in contrast, the *p*-biphenyl-*s*-triazine (XXXI-*12*) was 1400-fold less effective than the corresponding phenyl-*s*-triazine (XXX-*12*) (7). It should be noted that the steric interference of the *p*-phenyl group of XXXI-*12* with enzyme binding was greatly reduced in the corresponding *m*-biphenyl-*s*-triazine (XXXII-*12*) which was only elevenfold less effective than XXX-*12* (7).

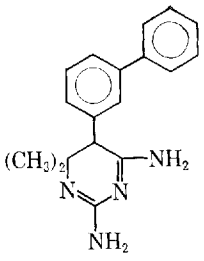
Another important difference exists between 2,4-diamino-6-phenyl-5-(phenylbutyl)pyrimidine (XII) and the corresponding 6-methyl (X) and 6-propyl analogs (XIX). If a 5-phenylbutyl group in conformation *11* or *12* can give so much stronger hydrophobic bonding than 6-phenyl in conformation *2*, why does not the 6-phenyl analog (XII) assume



XVIII-*2*



XXXI-*12*



XXXII-*12*

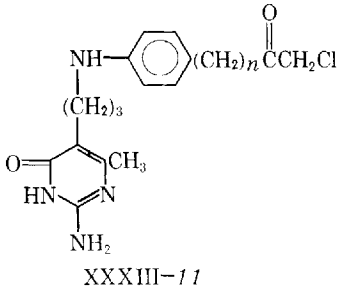
conformation *11* or *12* in order to get maximum hydrophobic bonding from the phenylbutyl group? If such were the case, then X, XII, and XIX would be expected to be equally effective. Since XII is so much less effective, it can only be concluded that XII cannot complex to the enzyme in conformation *12* due to steric interaction with the enzyme when the flat coplanar benzene ring is placed in the *III* region. If XII assumed conformation *11*, this would place the 4-amino group in the *III* region, a position believed to be the cause of a 130-fold repulsion as noted previously with 2,4,6-triamino-5-(phenylbutyl)pyrimidine compared to X (6, 17). Since this 130-fold repulsion is even larger than the 41-fold observed difference between the 6-methyl (X) and 6-phenyl pyrimidine (XII), conformation *2* might be preferred for XII. Although conformation *11* could be a possibility, conformation *11* would predict that XII would be a ten to thirty-fold better inhibitor than the anilinopropyl pyrimidine (XIII) which it is not. It is also possible that the preferred conformation for XII is *11*, but for XIII it is *2*. Ultimately, it should be possible to differentiate the conformational preference for *2* or *11* with a particular 5-side chain by comparison of active-site-directed irreversible inhibitors (18) which vary only in the hydrophobic or hydrophilic nature of the bridge between the 5-position of the pyrimidine and the terminal aryl group.

In summary, the inhibition of dihydrofolic reductase observed with a variety of phenyl and phenylalkyl pyrimidines cannot be rationalized with a single rotational conformer; therefore, a number of rotational conformers are proposed and the strong hydrophobic bonding is the determining factor for the particular preferred conformation of a given inhibitor. Such a concept for enzyme binding is not new. Complexing to chromotrypsin can occur in a number of rotational conformations where an aryl or large alkyl group on the substrate or inhibitor is determinant (22, 23); this bonding by aryl or alkyl groups to chymotrypsin is probably of a hydrophobic nature (24, 25). Similarly, 3-(β-D-ribofuranosyl)adenine and its nucleotide analogs can bind to some enzymes normally requiring adenosine or its corresponding nucleotide derivatives. Since the strong binding by the sugar moiety is apparently determinant, it has been proposed (26, 27) that a "flip" conformation for the adenine of the 3-ribosyl derivatives can account for the binding data.

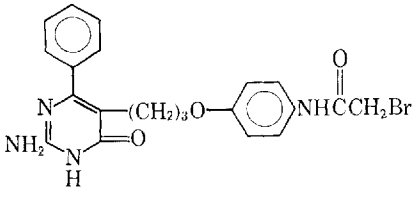
The most important deduction that arises from the derivations presented here is that if only 1 hydrophobic group is present on an inhibitor of dihydrofolic reductase, then this group will be complexed in a particular conformer that allows the hydrophobic bonding to be determinant. Therefore, a potential active-site-directed irreversible inhibitor (18) of dihydrofolic reductase such as XXIIII (3) would have its alkylating function in the hydrophobic area; by definition, the hydrophobic area of the enzyme has no polar groups that could be attacked by XXXIII in its favored conformation *11*. However, if 2 side chains are present, at least 1 of which is hydrophobic, then the hydrophobic group will complex in the hydrophobic region in a conformation that will project the second side chain in a hydrophilic region of the enzyme. Thus, XXXIV in conformation *2* is an active-site-directed irreversible inhibitor (18) of dihydrofolic reductase (28). By similar reasoning, XXXV, which should complex in conformation *11*, was synthesized and was also an active-site-directed irreversible inhibitor (29). These results will be presented in future papers.

EXPERIMENTAL

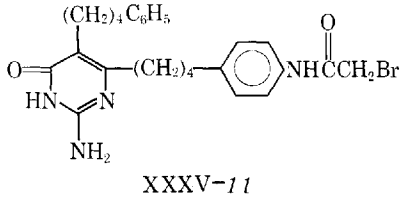
Methods.—The required 2-amino-5,6-disubstituted-4-pyrimidinols (XX, XXIX, and XL) were synthesized by alkylation of the appropriate β-keto ester followed by condensation with guanidine (30). The 2,4-diaminopyrimidines (XVII–XIX) were synthesized from the 4-pyrimidinols *via* the 4-chloro-



XXXIII-*11*



XXXIV-*2*



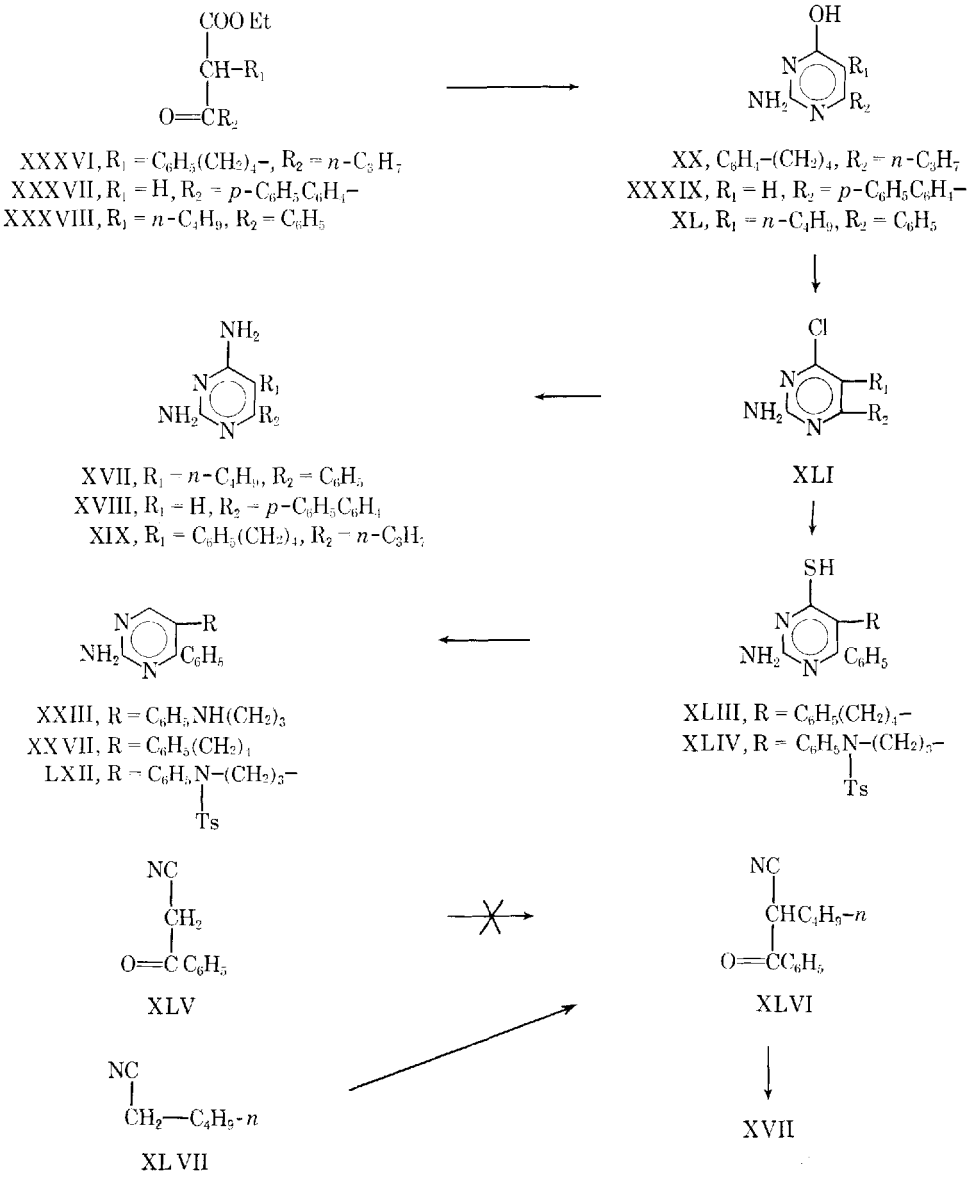
XXXV-*11*

pyrimidines (XLI) by treatment with ammonia (31). Reaction of the appropriate 4-chloro-6-phenylpyrimidine (XLI) with thiourea (5) afforded the 4-mercaptopyrimidines (XLIII and XLIV) which were desulfurized with Raney nickel (31) to the 2-amino-4-H-pyrimidines (XXVII and LXII). Treatment of LXII with hydrogen bromide in acetic acid (30, 32) removed the *N*-tosyl blocking group to give the required XXIII. (Scheme I.)

An alternate route to 5-alkyl-2,4-diamino-6-phenylpyrimidines such as XVII *via* α-butylbenzoylacetonitrile (XLVI) was investigated since it would be of general utility. Attempts to prepare XLVI by alkylation of benzoylacetonitrile (XLV) with sodium hydride and *n*-butyl bromide in such diverse solvents as dimethylsulfoxide or benzene gave only *O*-alkylation. However, XLVI could be prepared by Claisen condensation of hexanonitrile (XLVII) with ethyl benzoate (33). Condensation of XLVI with guanidine carbonate by fusion at 180° gave the 2,4-diaminopyrimidine (XVII) directly in 21% 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 KBr disk (unless otherwise indicated) with a Perkin-Elmer model 137B spectrophotometer; ultraviolet spectra were determined with a Perkin-Elmer model 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on silica gel GF_{254} (Brinkmann), and spots were detected under ultraviolet light.

2 - Amino - 5 - phenylbutyl - 6 - n - propyl - 4-pyrimidinol (XX).—To a magnetically stirred solution of 3.1 Gm. (20 mmoles) of ethyl *n*-butyroylacetate in 10 ml. of reagent dimethylsulfoxide pro-



Scheme I

tected from moisture was added portionwise 0.867 Gm. (20 mmoles) of 55.6% dispersion of sodium hydride in mineral oil. When the evolution of hydrogen had ceased, 3.85 Gm. (18 mmoles) of 4-phenylbutyl bromide (34) was added. After being stirred for about 18 hr., the mixture was warmed on a steam bath for 30 min. and then neutralized with acetic acid. The warm mixture was poured into a stirred mixture of 50 ml. of benzene and 30 ml. of water. The separated aqueous phase was extracted once more with benzene. The combined benzene solutions were washed with several portions of ice cold 3% aqueous sodium hydroxide, then water. Dried with magnesium sulfate, the benzene solution was spin-evaporated *in vacuo* leaving 5 Gm. of crude XXXVI as an oil.

The crude XXXVI was dissolved in 25 ml. of absolute ethanol, then refluxed with 1.62 Gm. (9 mmoles) of guanidine carbonate for 19 hr. The cooled reaction mixture was neutralized to near pH 7 with 3 *N* aqueous hydrochloric acid. The product was collected on a filter and washed with ethanol; yield, 1.54 Gm., m.p. 209–215°. By concentration of the filtrate an additional 1.17 Gm. (total 53%) was obtained, m.p. 209–215°. Recrystallization of a portion from aqueous ethanol gave white crystals, m.p. 209–215°, which moved as one spot on TLC in 3:1 benzene–methanol. The compound had $\lambda_{max.}$ 2.98 (NH); 6.05, 6.10, 6.50, 6.70, (NH, C=O, C=C, C=N); 13.5, 14.3 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 269 mμ (ϵ 7800); (pH 7): 275 mμ (ϵ 4400); (pH 13): 282 mμ (ϵ 9800).

Anal.—Calcd. for $C_{17}H_{23}N_3O$: C, 71.6; H, 8.07; N, 14.7. Found: C, 71.6; H, 8.20; N, 14.7.

2 - Amino - 5 - n - butyl - 6 - phenyl - 4 - pyrimidinol (XL).—Alkylation of 5 Gm. of ethyl benzoylacetate with *n*-butyl bromide in *tert*-butyl alcohol followed by condensation with guanidine carbonate in the same solvent, as described for the preparation of a related 6-phenylpyrimidinol (30), gave 2.24 Gm. (39%) of product, m.p. 309–314°. Recrystallization from ethanol–toluene gave white crystals, m.p. 310–315° dec. $\lambda_{max.}$ 2.83 (NH); 6.05, 6.12, 6.35 (NH, C=O, C=N, C=C); 14.3 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 233 (ϵ 14,400), 280 mμ (ϵ 10,900); (pH 7): 235 (ϵ 17,400), 300 mμ (ϵ 8100); (pH 13): 289 mμ (ϵ 9600).

Anal.—Calcd. for $C_{14}H_{17}N_3O$: C, 69.1; H, 6.99; N, 17.3. Found: C, 69.4; H, 7.18; N, 16.9.

2 - Amino - 6 - (4 - biphenylyl) - 4 - pyrimidinol (XXXIX).—To a solution of 5.3 Gm. (23.4 mmoles) of ethyl 4-biphenylylcarboxylate in 7.2 Gm. (82 mmoles) of reagent ethyl acetate was added 3.16 Gm. (58.5 mmoles) of sodium methoxide. The mixture was heated in a bath at 80° under a condenser with stirring and protected from moisture for 20 hr. The cooled reaction mixture was diluted with several volumes of benzene, then neutralized with glacial acetic acid. The sodium acetate was removed by filtration and washed with benzene. The combined filtrate and washings were spin-evaporated *in vacuo;* ethyl acetoacetate was then removed by continued spin-evaporation in a hot water bath in high vacuum leaving 6.23 Gm. of crude XXXVII as an oil.

A solution of 4.82 Gm. (18 mmoles) of crude XXXVII in 40 ml. of absolute ethanol was refluxed with 1.8 Gm. (10 mmoles) of guanidine carbonate with magnetic stirring for 17 hr. The cooled reaction mixture was filtered and the product washed with alcohol, then water; yield, 1.57 Gm., m.p. 370–372° dec. From the filtrate was isolated an additional 0.87 Gm. (total, 52% over-all), m.p. 373–376° dec. Two recrystallizations from aqueous 2-methoxyethanol afforded white crystals, m.p. 378–380° dec. $\lambda_{max.}$ 2.95 (NH); 5.85, 6.02, 6.08 (NH, C=O, C=C, C=N); 12.2, 14.5 μ (phenyl); $\lambda_{max.}$ (pH 1): 308 mμ (ϵ 25,300); (pH 7): 285 mμ (ϵ 32,100); (pH 13): 295 mμ (ϵ 16,700).

Anal.—Calcd. for $C_{16}H_{13}N_3O$: C, 73.0; H, 4.94; N, 15.9. Found: C, 72.7; H, 5.07; N, 15.6.

2,4 - Diamino - 5 - phenylbutyl - 6 - n - propylpyrimidine (XIX).—A mixture of 500 mg. (1.76 mmoles) of XX and 4 ml. of phosphorus oxychloride was heated for 45 min. under a reflux condenser in a bath preheated and maintained at 110°. The cooled mixture was poured into 35 Gm. of ice and 40 ml. of ether with stirring. After 15 min., the layers were separated. The ether layer was washed with 5% aqueous sodium bicarbonate (2 × 30 ml.) and water (2 × 30 ml.), then dried with magnesium sulfate. Spin-evaporation *in vacuo* left XLI (R_1 = C_6H_5—$(CH_2)_4$—, R_2 = *n*-C_3H_7) as a gum which could not be crystallized, but had $\lambda_{max.}$ (pH 1): 315 mμ (*cf. Reference 31*).

The crude gum was dissolved in 40 ml. of methanol saturated with ammonia, then heated in a steel bomb at 150° for 24 hr. The solution was clarified by filtration through a Celite pad, then spin-evaporated *in vacuo* to a small volume. The solution was made strongly alkaline with 10% aqueous sodium hydroxide, then diluted with water to turbidity and cooled at −4°. The crude product was collected on a filter and recrystallized twice from aqueous methanol; yield, 115 mg. (23%), m.p. 121–127°. For analysis, the material was dissolved in warm 10% aqueous acetic acid. The solution was clarified by filtration, then the product was precipitated by addition of excess 10% aqueous sodium hydroxide. Recrystallization from aqueous ethanol gave 70 mg. (14%) of analytically pure product, m.p. 126–128°. $\lambda_{max.}$ 2.80, 3.00 (NH); 6.05, 6.39 (NH, C=C, C=N); 13.03, 14.43 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 280 mμ (ϵ 7400); (pH 7): 289 mμ (ϵ 7300); (pH 13): 301 mμ (ϵ 7900).

Anal.—Calcd. for $C_{17}H_{24}N_3$: C, 71.8; H, 8.45; N, 19.7. Found: C, 71.8; H, 8.60; N, 19.4.

5 - n - Butyl - 2,4 - diamino - 6 - phenylpyrimidine (XVII).—*Preparation A.*—Conversion of 500 mg. (2.06 mmoles) of XL to XVII *via* LXI, as described for the preparation of XIX, gave 143 mg. (29%) of analytically pure product, m.p. 149–152°. $\lambda_{max.}$ 2.80, 2.95 (NH); 6.05, 6.20, 6.40 (NH, C=C, C=N); 13.05, 14.25 μ (C_6H_5).

Anal.—Calcd. for $C_{14}H_{18}N_4$: C, 69.4; H, 7.44; N, 23.1. Found: C, 69.6; H, 7.57; N, 23.3.

Preparation B.—α-Benzoylhexanonitrile (XLVI) was prepared by Claisen condensation of hexanonitrile and ethyl benzoate; a yield of 60% has been recorded (33). A mixture of 1 Gm. (5 mmoles) of XLVI and 450 mg. (2.5 mmoles) of guanidine carbonate was placed in a bath preheated to 120°, then the temperature was raised to 180° over a period of 30 min. After an additional 10 min. at 180°, gas evolution was complete. The cooled residue was extracted with hot ethanol. The filtered solution was spin-evaporated *in vacuo*. The residue was extracted with hot 10% aqueous acetic acid

and separated from some insoluble gum. The cooled extract was poured into an excess of cold 10% aqueous sodium hydroxide. The product was collected and recrystallized from aqueous ethanol with the aid of charcoal; yield, 256 mg. (21%) of white crystals, m.p. 151–152°, that were identical with *Preparation A* as shown by mixed melting point, infrared spectra, and TLC in 3:1 benzene–methanol.

6 - (4 - Biphenylyl) - 2,4 - diaminopyrimidine (XVIII).—A mixture of 1.00 Gm. (3.8 mmoles) of XXXIX, 10 ml. of phosphorus oxychloride, and 1 ml. of triethylamine was refluxed for 75 min., then processed and treated with ammonia as described for the preparation of XIX; the yield of analytically pure white crystals after recrystallization from 2-methoxyethanol by addition of water was 145 mg. (15%), m.p. 273–275°. $\lambda_{max.}$ 2.92, 3.00 (NH); 6.25, 6.35, 6.50 (NH, C=C, C=N); 12.3, 13.71, 14.50 μ (phenyl); $\lambda_{max.}$ (pH 1): 313 mμ (ϵ 25,000); (pH 7, 13): 278 mμ (ϵ 28,000).

Anal.—Calcd. for $C_{16}H_{14}N_4$: C, 73.3; H, 5.34; N, 21.4. Found: C, 73.7; H, 5.43; N, 21.2.

2 - Amino - 6 - phenyl - 5 - phenylbutyl - 4 - pyrimidinethiol (XLIII).—To a solution of 675 mg. (2 mmoles) of XLI [R_1 = $C_6H_5(CH_2)_4$—, R_2 = C_6H_5] (5) in 20 ml. of *tert*-butyl alcohol was added 160 mg. (2.1 mmoles) of thiourea. After being refluxed for 2 hr., the solution was treated with 12 ml. of 10% aqueous sodium hydroxide, then was refluxed for 15 min. more. The mixture was diluted with 20 ml. of water and then acidified to about pH 5 with 3 *N* hydrochloric acid. The yellow precipitate was collected on a filter and washed with water; yield, 448 mg., m.p. 186–198°. The solid was dissolved in hot ethanol, filtered from some insoluble material, then the solution was diluted to turbidity with water; yield, 239 mg. (36%) of analytical sample, m.p. 225–227°, which moved as one spot on TLC in 3:1 benzene–methanol. $\lambda_{max.}$ 2.90, 2.98 (NH); 6.08, 6.40, 6.49 (NH, C=C, C=N); 13.3, 14.4 μ (C_6H_5).

Anal.—Calcd. for $C_{20}H_{21}N_3S$: C, 71.6; H, 6.27; N, 12.5. Found: C, 71.6; H, 6.32; N, 12.3.

2 - Amino - 6 - phenyl - 5 - phenylbutylpyrimidine (XXVII) Hydrochloride.—To a solution of 100 mg. (0.30 mmole) of XLIII in 10 ml. of ethanol was added about 200 mg. of Raney nickel. The mixture was refluxed with magnetic stirring for 1 hr., then filtered through a Celite pad. The ethanol solution was spin-evaporated *in vacuo* leaving 50 mg. of a glass. A solution of this glass in ether was treated with excess hydrogen chloride gas. The gummy hydrochloride was crystallized from ethyl acetate–petroleum ether (b.p. 60–110°); yield, 30 mg. (30%) of white crystals, m.p. 147–149°, that moved as a single spot on TLC in 3:1 benzene–methanol. $\lambda_{max.}$ 5.88 ($C=NH^+$), 5.99, 6.25 (NH, C=C, C=N); 13.5, 14.1, 14.4 μ (C_6H_5).

Anal.—Calcd. for $C_{20}H_{21}N_3 \cdot HCl$: C, 70.5; H, 6.47; N, 12.4. Found: C, 70.4; H, 6.60; N, 12.5.

2 - Amino - 5 - (3 - anilinopropyl) - 6 - phenylpyrimidine (XXIII).—A solution of 500 mg. of XLIV (5) in 20 ml. of ethanol was refluxed with about 600 mg. of Raney nickel for 2 hr. The clarified solution was evaporated *in vacuo*. The residue was dissolved in chloroform; the solution was clarified by filtration, then spin-evaporated *in vacuo* leaving 200 mg. (43%) of crude LXII as a glass.

To the crude LXII was added 86 mg. of phenol and 4 ml. of 30% hydrogen bromide in acetic acid. The mixture was magnetically stirred for 18 hr. protected from moisture, then poured into several volumes of ether. The solid which separated was triturated with fresh ether, then collected on a filter. The hydrobromide salt was dissolved in 25 ml. of water, then the solution was made strongly alkaline with 10% sodium hydroxide. The product was collected on a filter, washed with water, then recrystallized from aqueous ethanol with the aid of charcoal; yield, 86 mg. (64%) of analytically pure material, m.p. 131–132°. $\lambda_{max.}$ 2.85 (NH); 6.20, 6.25, 6.39 (NH, C=C, C=N); 13.41, 14.45 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 233 (ϵ 17,400), 285 (ϵ 6400), 325 mμ (ϵ 6900); (pH 7, 13): 313 mμ (ϵ 6600).

Anal.—Calcd. for $C_{19}H_{20}N_4$: C, 75.0; H, 6.56; N, 18.4. Found: C, 74.7; H, 6.53; N, 18.4.

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