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Analogs of Tetrahydrofolic Acid. XXXI. Hydrophobic Bonding to Dihydrofolic Reductase. III. Further Observations on Conformational Aspects of Hydrophobic Bonding With Some 5-Alkyl-2,4-diamino-6-pyrimidinols

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Thirteen 5-alkyl-2,4-diamino-6-pyrimidinols have been compared as inhibitors of dihydrofolic reductase in an attempt to delineate some of the conformational requirements for hydrophobic bonding to the enzyme; definite conformational limitations were observed. For example, cyclohexyl and *trans*-crotyl side-chains gave inhibitors that were as effective as the *n*-butyl-6-pyrimidinol, whereas, isoamyl was better than all three; these results indicate that the *n*-butyl group complexes in a near-staggered conformation and additional hydrophobic bonding can be obtained by branching at C_3 of the butyl group. Methyl branching at C_2 of the *n*-butyl group also gave a better inhibitor than *n*-butyl, but the 2-methylbutyl side-chain was only half as effective as isoamyl; in contrast, the 1-methylbutyl side-chain gave an inhibitor only one-half as effective as *n*-butyl. That the amount of hydrophobic bonding was simply an "extraction process" dependent upon the size of the hydrocarbon group was unequivocally eliminated.

That strong hydrophobic bonding to dihydrofolic reductase by alkyl and aralkyl groups attached to the 5-position of pyrimidines or the 1-position of 1,2-dihydro-*s*-triazines has been recently discovered (3); it was also proposed (3) that an aryl group in these positions also complexed to dihydrofolic reductase by hydrophobic bonding, a proposal that has received further strong experimental support (2b). The strength that this hydrophobic bonding can have is notable; the phenylbutyl group contributes a free energy of binding of about 6 kilocal./mole compared to the substrate (K_m about 1×10^{-6}) which has a free energy of binding of about 8 kilocal./mole (3).

Since an *n*-butyl group can approach phenyl in hydrophobic bonding ability and since isoamyl is as good or better than phenyl, it was proposed that the *n*-butyl group might complex to the hydrophobic region in a skew conformation (IIB) (Chart I) (3). This skew conformation would require about 800 cal./mole from the normal ground state staggered conformation of *n*-butyl (IIA), this energy being obtained at the expense of the binding energy released; since the isoamyl group (IV) (Chart I) already has the butyl part in a skewed conformation it was expected and found to be a better inhibitor of the enzyme (3). This latter result could also be explained on the basis that the *n*-butyl group actually complexed in a near staggered conformation (IIA) and the extra methyl of the isoamyl group (IV) merely gave additional hydrophobic bonding. In order to differentiate between these two modes of binding and to obtain additional support for the resultant interpretation, a selected series of 5-alkyl-2,4-diamino-6-pyrimidinols were synthesized and evaluated; the results are the subject of this paper.

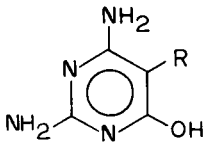
The key compound for differentiating which of the two *n*-butyl conformations, IIA and IIB, is complexed to dihydrofolic reductase is the *trans*-crotyl pyrimidine, XII (Chart I). Since the *trans*-crotyl has a fixed staggered conformation, XII, then it will bind as well as *n*-butyl if the latter has the staggered conformation, IIA; however, if the *n*-butyl complexes in the skewed conformation, IIB, then the *trans*-crotyl group (XII) should bind no better than the 5-*n*-propylpyrimidine (III). In Table I it can be seen that the *trans*-crotylpyrimidine (XII) was equally as effective as the *n*-butylpyrimidine, II; both II and XII were better than 10-times more effective than the *n*-propylpyrimidine (III), thus giving strong experimental support that the *n*-butyl group complexes to dihydrofolic reductase in a near staggered conformation, IIA (Chart I).

The 3-butenylpyrimidine (XIII) was one-half as good an inhibitor as the *n*-butylpyrimidine (II), but XIII was a 5-fold better inhibitor than the *n*-propylpyrimidine (III); these results also confirm that the *n*-butyl group is complexed in a near-staggered conformation, but not fully staggered.

If the *n*-butyl group is complexed to the enzyme in a near-staggered conformation, IIA, then the extra methyl group (IV) of isoamyl - which is skewed

TABLE I

Inhibition of Dihydrofolic Reductase by, and Physical Properties of



Compound Number	R (a)	μM Conc. for 50% Inhibition	% Yield (b)	m.p. °C dec.	Analyses Calcd. C	H	N	Found C	H	N
I	C_6H_5	2.1 (c)								
II	n-C_4H_9-	40 (c)								
III	n-C_3H_7-	450 (c)								
IV	i-C_5H_{11}-	4.0 (c)								
V	$(CH_3)_2C{=}CHCH_2$-	17 (c)								
VI	$CH_3CH_2CH_2CH(CH_3)$-	82	11 (d)	249-251 (f)	55.1	8.21	28.5	55.1	8.24	28.4
			40 (e)	254-256						
VII	$CH_3CH_2CH(CH_3)$-	620	44 (g)	275-277 (h)	52.7	7.80	30.8	52.6	7.74	30.6
VIII	$(CH_3)_2CHCH_2CH(CH_3)$-	8.1	25 (e)	173-175 (f, i)	55.9 (j)	8.68	26.1	56.1	8.73	25.5
IX	$CH_3CH_2CH(CH_3)CH_2$-	14	10 (d)	261-263 (h)	55.1	8.21	28.5	55.1	8.29	28.3
X	cyclohexyl-	35	12 (e)	307-310 (h)	57.7	7.74	26.9	57.6	7.75	26.3 (k)
XI	cyclopentyl-	450	18 (d)	291-293 (h)	55.7	7.27	28.8	55.6	7.41	28.3 (k)
XII	$CH_3CH{=}CHCH_2$-	35	14 (d)	245-247 (f)	53.3	6.71	31.1	53.5	6.65	31.2
XIII	$CH_2{=}CHCH_2CH_2$-	89	12 (d)	247-249 (f)	53.3	6.71	31.1	53.1	6.75	30.8

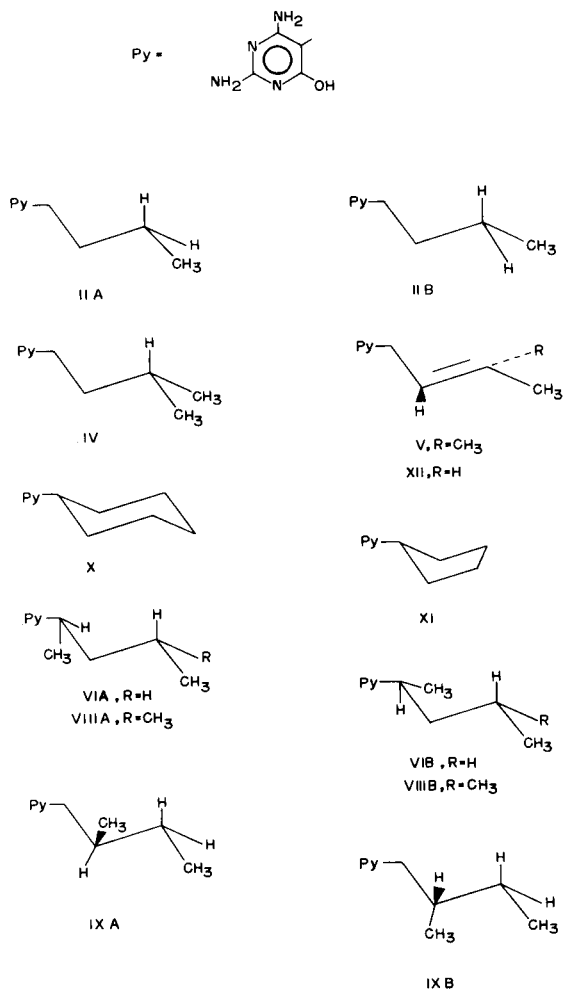
Dihydrofolic reductase was a 45-90% of saturated ammonium sulfate fraction isolated and assayed with 6 μM dihydrofolate and 12 μM TPNH in 9:1 0.05 M (pH 7.4) aqueous Tris buffer: N,N-dimethylformamide as previously described (7). The technical assistance of Maureen Baker, Shirley Humphrey, and Karen Smith with these assays is acknowledged. (a) All compounds were prepared by condensation of the appropriate alkylated ethyl cyanoacetate with guanidine as previously described for preparation of I-V (3, 8). (b) The yields are overall from the requisite alkyl bromide or alkanone (unless otherwise indicated) and are minimum values for analytically pure material since no effort was made to rework the mother liquors. (c) Data from reference 3. (d) The intermediate alkylated ethyl cyanoacetate was prepared by alkylation of ethyl cyanoacetate with sodium hydride in dimethyl sulfoxide with the requisite alkyl bromide, except with XII where crotyl chloride was employed (3, 8); the dimethyl sulfoxide was not removed by high vacuum distillation, but by thorough water washing of a benzene solution of the product. (e) The intermediate alkyl ethyl cyanoacetate was prepared by reductive condensation of the requisite ketone with ethyl cyanoacetate (9). (f) Recrystallized from aqueous acetone. (g) The intermediate alkylated ethyl cyanoacetate was purchased from Aldrich Chemical Co. (h) Recrystallized from aqueous ethanol. (i) The crude oily product was crystallized by solution in warm 2 N aqueous hydrochloride, removal of an oily insoluble by-product by benzene extraction, then adjustment of the pH to 7. (j) Calcd. for one-quarter mole of water. (k) Correct nitrogens could not be obtained, most probably because of combustion difficulties with this high melting compound leading to nitrogenous ash.

with respect to the staggered butyl chain, IIA - presumably can give additional hydrophobic bonding. Support for such a hypothesis might be obtainable with a *cis*-crotyl group or a cyclohexyl group. The cyclohexyl group (X) (Chart I) was chosen since it has skewed carbons between C_1 and C_4 in the ground-state; in addition to the *cis*-crotyl group having an eclipsed conformation, it is more difficult to synthesize than the cyclohexylpyrimidine. Note in Table I that cyclohexyl (X) was equivalent in binding to n-butyl (II), thus confirming that a skewed C_4-methyl group can hydrophobically bind to the enzyme.

That there are some conformational restrictions to optimum hydrophobic bonding and that the bonding is not due simply to an extraction phenomenon related to the hydrocarbon content (4) was shown in several ways:

(a) comparison of 1-methylbutyl (VI) with 2-methylbutyl (IX) and 3-methylbutyl (isoamyl, IV)

CHART I



(Table I) shows considerable difference in the order of bindings, IV being 20-times more effective than VI;

(b) comparison of 3,3-dimethylallyl (V), which has an eclipsed conformation, with the skewed isoamyl group (IV) (Chart I) shows that IV is a 4-fold better inhibitor than V;

(c) the cyclopentyl group (XI) (Chart I) is only one-hundredth as effective as isoamyl.

Note that the cyclohexyl group (X) is 13-times more effective than the cyclopentyl group (XI) (Table I), but that the cyclopentyl is equivalent to *n*-propyl (III) in binding; this comparison gives strong evidence that only C_1, C_2, and C_3 of the cyclopentyl is hydrophobically bonded, and C_4 and C_5 neither complex nor interfere with complex formation. Furthermore, about the same increment in increased binding occurs from *n*-propyl to *n*-butyl as from cyclopentyl to cyclohexyl.

The relative contribution to binding - positive or negative - by methyl substitution on the *n*-butyl group at C_1, C_2, or C_3 was studied for two purposes; first, it would be of interest to see if there were increased hydrophobic bonding and secondly, if decreased binding due to a steric interaction of the methyl group to binding could occur. If substitution at any of these positions does not give decreased binding, then it might be possible to place a longer branch at that position; this branch might be able to bridge to a hydrophilic area on the enzyme surface; this hydrophilic area might then be subject to attack by a properly positioned covalent forming group on the inhibitor leading to an active-site-directed irreversibel inhibitor (5) of dihydrofolic reductase.

Substitution of a methyl group at the C_1-position (VI) of *n*-butyl (II) or at the C_1-position (VIII) of isoamyl (IV) led to a consistent two-fold loss in binding (Table I). A similar, but slightly lesser effect was observed with VII and III. These results would appear to be best rationalized by considering the *d*- and *l*-forms of VI-VIII (Chart I). One of the enantiomers would have the methyl group below the plane of the pyrimidine ring (VIA and VIIIA) when the *n*-butyl chain is in the staggered conformation. With the reasonable assumption that there is a relatively flat enzyme interaction with the bottom of the pyrimidylbutyl moiety as depicted in VIB, then one of the two enantiomorphs (VIA and VIIIA) will have its methyl group projecting into the enzyme surface, thus interfering with binding; the other enantiomer (VIB and VIIIB) has its methyl group in the plane of C_1-C_2 bond which should not interfere with binding as noted with cyclohexyl (X) and cyclopentyl (XI). It follows that since one enantiomer could have about the same activity as the alkylpyrimidine unsubstituted at C_1 and the other enantiomer would be considerably less effective, one could expect the *dl*-pairs VI and VIII to be one-half as effective as II and IV, respectively. It would be of interest to resolve either VI or VIII and compare the relative ability of the enantiomers to inhibit.

Substitution of a methyl group at C_2 (IX) of the *n*-butylpyrimidine (II) gave a 3-fold better inhibitor (Table I); however, this increment was less than the 10-fold increment observed by C_3-substitution to give the isoamylpyrimidine (IV). Again one should consider that one (IXB) (Chart I) of the two enantiomers could have a methyl group projecting into the enzyme surface whereas the other enantiomer (IXA) has its methyl group nearly in the plane of the pyrimidine ring; thus if the enzyme is complexed to the butylpyrimidine below the plane of the ring, one might see additional hydrophobic binding from enantiomer IXA, but a steric interaction to binding with enantiomer IXB. Such interactions would then predict that the 2-methylbutyl group would be more effective than butyl, but one-half or less as effective as the isoamyl group, depending upon whether the 2-methyl of IXA gives as much or less hydrophobic bonding than the 3-methyl of IV.

EXPERIMENTAL

Methods.

Melting points were determined in capillary tubes on a Mel-temp block and those below 230° were corrected. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer and all compounds in Table I had λ max (*p*H 1) 276 mμ; (*p*H 13) 272 mμ. Thin layer chromatograms were run with ethanol on Brinkmann silica gel G and all compounds in Table I moved as single spots when detected under ultraviolet light. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer and all compounds had the spectra in agreement with their assigned structures.

The evidence that crotyl pyrimidine, XII, had the *trans*-configuration and not *cis* is worthy of special note since the main conformational argument for the binding of the *n*-butyl group of II is dependent upon this configurational assignment. The starting material, *trans*-crotyl chloride, showed a strong band at 963 cm^{-1}, due to the CH out-of-plane deformation of a *trans*-vinyl group; similarly, XII showed a band at 963 cm^{-1} of medium intensity. Neither the crotyl chloride nor XII had absorption in the 670-740 region; the *cis*-vinyl CH out-of-plane deformation is reported to occur between 675 and 730 cm^{-1} whereas the *trans*-vinyl CH is reported to occur at 965-990 cm^{-1}, usually at 965 cm^{-1} (6). Since no *cis*-vinyl compound has as yet ever had a 965 cm^{-1} band (6), it is clear that XII has the *trans*-crotyl structure.

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(1) This work was supported by Grants CA-05867 and CA-06624 from the National Cancer Institute, U. S. Public Health Service.

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