Specific Enzyme Inhibitors in Vitamin Biosynthesis. Part II.[1] Revised Structures for Some 8-Substituted Pyrido[2,3-*d*]Pyrimidines

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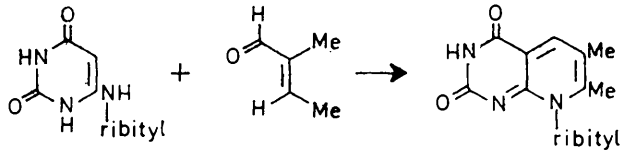
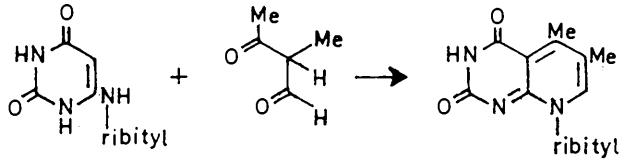
The structures assigned to some 8-substituted pyrido[2,3-*d*]pyrimidines in an earlier paper have been re-examined. These compounds were synthesised as potential competitive inhibitors of the enzyme riboflavin synthetase. Data from enzyme inhibition studies suggested that the original structures had been wrongly assigned. This was confirmed by studies of nuclear Overhauser effects and by photochemical degradation of the compounds.

In Part I [1] an approach to the rational design of specific inhibitors of vitamin biosynthesis was presented. In particular, several 8-substituted pyrido[2,3-*d*]pyrimidines were synthesised with the hope that these close structural analogues of the pteridine precursor (1) involved in the biosynthesis of riboflavin [2] would be specific inhibitors of the enzyme riboflavin synthetase. The two synthetic methods used are illustrated by the examples given in Scheme 1.

(a) 'Doebner-Miller-type' synthesis



(b) Synthesis from β-dicarbonyl derivatives



Scheme 1

The orientation of addition of the αβ-unsaturated carbonyl component to the 6-substituted aminopyrimidine in reaction (a) was determined [1] by analogy with the Doebner–Miller synthesis of quinolines,[3] and by spectroscopic studies on a tricyclic intermediate isolated in this reaction. The orientation of the addition in reaction (b) was then determined by comparison with the products formed in (a). This latter assignment led to a discrepancy with similar condensations of 6-(unsubstituted amino)pyrimidines, where condensation of the aldehyde function of a β-oxo-aldehyde invariably occurs at position 5 of the pyrimidine ring.[4]

Three independent lines of evidence have now been obtained which indicate that the original structural assignments as shown in Scheme 1 are incorrect. This evidence is discussed below.

Data from Enzyme Inhibition Studies.—The Doebner–Miller-type condensation of 6-D-ribitylaminouracil (2) with 2-methylbut-2-enal (5) gave an 8-substituted pyridopyrimidine reported [1] to be 6,7-dimethyl-8-D-ribitylpyrido[2,3-*d*]pyrimidine-2(8*H*),4(3*H*)-dione, and now formulated as the 5,6-dimethyl isomer (9).

Conversely, condensation of (2) with 2-methyl-3-oxobutanal (18), gave a pyridopyrimidine which was identified [1] as 5,6-dimethyl-8-D-ribitylpyrido[2,3-*d*]-pyrimidine-2(8*H*),4(3*H*)-dione and is now formulated as the 6,7-dimethyl isomer (13).

These compounds were synthesised as structural analogues of the pteridine precursor (1) involved in the biosynthesis of riboflavin,[2] with the hope that they would act as competitive inhibitors of the substrate (1) for the enzyme riboflavin synthetase and therefore would selectively inhibit the biosynthesis of the vitamin. This hypothesis has now been tested. Riboflavin synthetase was isolated from dried baker's yeast and was partially purified by ammonium sulphate precipitation and dialysis. Measurement of the inhibition constants gave the results recorded in Table 1. The fact that compound (13) is a better competitive inhibitor of riboflavin

Table 1
Enzyme inhibition data

Inhibitor	Inhibition constant K_I/mol l^{-1}
(9)	$>3 \times 10^{-4}$
(13)	2×10^{-5}
(10)	$3{\cdot}5 \times 10^{-4}$
(11)	$1{\cdot}2 \times 10^{-4}$
(14)	$1{\cdot}7 \times 10^{-5}$

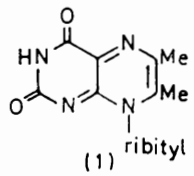
synthetase than is compound (9) confirms the structural assignments given in this paper: the pyridopyrimidine (13) is a closer structural analogue of the pteridine substrate (1) than is the isomeric compound (9).

In order to check the validity of these values the three isomeric monomethylpyridopyrimidines (10), (11), and (14) were synthesised. Thus, condensation of 6-D-ribitylaminouracil (2) with crotonaldehyde (6) gave the 5-monomethylpyridopyrimidine (10). Similar condensation of the pyrimidine (2) with methacrylaldehyde (7) gave the 6-monomethyl derivative (11), and reaction with the dimethyl acetal of acetoacetaldehyde (19) gave the 7-monomethylpyridopyrimidine (14).

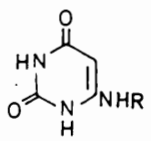
The inhibition constants for the monomethylpyridopyrimidines are given in Table 1 and follow the pattern

[1] Part I, T. Paterson and H. C. S. Wood, *J.C.S. Perkin I*, 1972, 1041.
[2] G. W. E. Plaut, *J. Biol. Chem.*, 1960, **235**, PC41; 1963, **238**, 2225; H. Wacker, R. A. Harvey, C. H. Winestock, and G. W. E. Plaut, *ibid.*, 1964, **239**, 3493.
[3] R. C. Elderfield in 'Heterocyclic Compounds,' Wiley, New York, 1961, vol. 4, p. 1.
[4] R. K. Robins and G. H. Hitchings, *J. Amer. Chem. Soc.*, 1958, **80**, 3449.

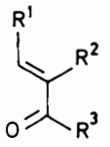
observed with the dimethyl derivatives (9) and (13), *i.e.* compound (14) is a better competitive inhibitor of riboflavin synthetase than (10).



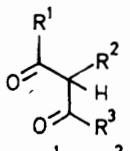
(1)



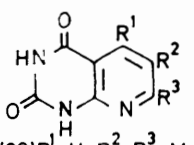
(2) R=D-ribityl
(3) R=CH_2·CH_2OH
(4) R=H



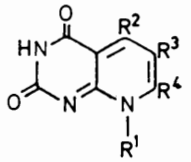
(5) R^1=R^2=Me, R^3=H
(6) R^1=Me, R^2=R^3=H
(7) R^1=R^3=H, R^2=Me
(8) R^1=R^2=H, R^3=Me



(18) R^1=H, R^2=R^3=Me
(19) R^1=R^2=H, R^3=Me
(dimethyl acetal)



(20) R^1=H, R^2=R^3=Me
(21) R^1=R^2=Me, R^3=H
(22) R^1=Me, R^2=R^3=H
(23) R^1=R^2=H, R^3=Me

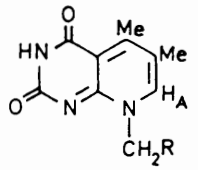


(9) R^1=D-ribityl, R^2=R^3=Me, R^4=H
(10) R^1=D-ribityl, R^2=Me, R^3=R^4=H
(11) R^1=D-ribityl, R^2=R^4=H, R^3=Me
(12) R^1=CH_2·CH_2·OH, R^2=Me, R^3=R^4=H
(13) R^1=D-ribityl, R^2=H, R^3=R^4=Me
(14) R^1=D-ribityl R^2=R^3=H, R^4=Me
(15) R^1=CH_2·CH_2·OH, R^2=R^3=H, R^4=Me
(16) R^1=CH_2·CH_2·OH, R^2=R^3=Me, R^4=H
(17) R^1=CH_2·CH_2·OH R^2=H, R^3=R^4=Me

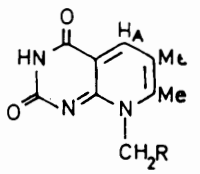
Studies of Nuclear Overhauser Effects.—The problem of deciding the orientation in the foregoing condensation reactions resolves itself into one of measuring the distance between the 'aromatic' proton H_A and the methylene group attached to N-8 of the pyrido[2,3-*d*]-pyrimidine ring (see Scheme 2). A measure of this distance can be obtained from the size of the nuclear Overhauser effect (N.O.E.).[5,6]

Since the n.m.r. line assignments were unambiguous in the spectra of the 8-(2-hydroxyethyl) analogues, the pyridopyrimidines (16) and (17) were used in the preliminary N.O.E. experiment. Thus, condensation of 6-(2-hydroxyethylamino)uracil (3) with 2-methylbut-2-enal (5) gave the 5,6-dimethylpyridopyrimidine (16), and condensation with 2-methyl-3-oxobutanal (18) gave the 6,7-dimethyl isomer (17). These assignments are the reverse of those given in our earlier paper.[1]

The four structures shown in Scheme 2 were thus available. For the compounds (16) and (17) the Overhauser effects were determined by recording the integral



(9) R=[CH(OH)]$_3$·CH_2·OH
(16) R=CH_2·OH



(13) R=[CH(OH)]$_3$·CH_2·OH
(17) R=CH_2·OH

SCHEME 2

of the peak due to H_A while a second (decoupling) field was applied at the frequency of the N-8 methylene group. As the increase in the integral of the observed nucleus (H_A) is a function of its proximity to the irradiated nucleus [5] (8-CH_2), the measurement of the integral increases for (16) and (17) should allow unambiguous structural assignments to be made for these compounds.

The assignment of the 8-CH_2 signal for the ribityl compounds (9) and (13) was more difficult, and since this is crucial to the experiment a plot was made of Overhauser effect (increase in integral of H_A) *versus* decoupling frequency, at high power, over the entire aliphatic proton range for each of the compounds.

The appearance of maxima in this plot indicated the frequencies at which groups close to H_A occurred. Having determined the frequencies of these groups from the maxima in the plot at high power, we repeated the N.O.E. experiment at low power in the same way as for the 8-(2-hydroxyethyl) compounds (16) and (17).

Further details of the N.O.E. technique are given in the Experimental section and the results are shown in Table 2. Consideration of these effects in relation to

TABLE 2

Overhauser effect on 'aromatic' proton, H_A

Compound	N.O.E.[a] (8-CH_2)	$\tau_{irr.}$(max.)	N.O.E.[a] (6-CH_3)	$\tau_{irr.}$(max.)
(16)	33 ± 4%	5·65	12 ± 3%	7·82
(17)	0	5·43	35 ± 2%	7·72
(9)	21 ± 3%	5·91	13 ± 2%	7·78
(13)	0	5·90—5·30	36 ± 2%	7·67

[a] Reported as the percent increase in the integral of resonance signal when another resonance [$\tau_{irr.}$(max.)] is irradiated by a second r.f. field.

the structures shown in Scheme 2 confirms that compounds (9) and (16) are 5,6-dimethylpyridopyrimidines and that (13) and (17) are the 6,7-dimethyl analogues.

Photolytic Degradation Experiments.—The possibility of degrading pyridopyrimidines of the type (9) and (13) by selective photolytic removal of the N-8 side chain was suggested by analogy with the photolysis of isoalloxazine[7] and of 8-D-ribityl-lumazines.[8] The products of this

[5] J. H. Noggle and R. E. Schirmer, 'The Nuclear Overhauser Effect: Chemical Applications,' Academic Press, New York, 1971.
[6] W. Egan, S. Forsén, and J. Jacobus, *J.C.S. Chem. Comm.*, 1973, 42; A. Garbesi, G. Barbarella, and A. Fava, *ibid.*, p. 155.
[7] W. M. Moore, J. T. Spence, F. A. Raymond, and S. D. Colson, *J. Amer. Chem. Soc.*, 1963, **85**, 3367; E. C. Smith and D. E. Metzler, *ibid.*, p. 3285.
[8] T. Masuda, *Chem. and Pharm. Bull.* (*Japan*), 1956, **4**, 375.

degradation would be ' aromatic ' pyridopyrimidines of a type synthesised previously by Robins and Hitchings,[4] who were able to assign structures unambiguously to their products by comparison with compounds synthesised both from pyrimidine [4] and from pyridine [9,10] precursors. This would therefore constitute a means of relating the isomeric 8-substituted pyridopyrimidines to compounds of unequivocal structure.

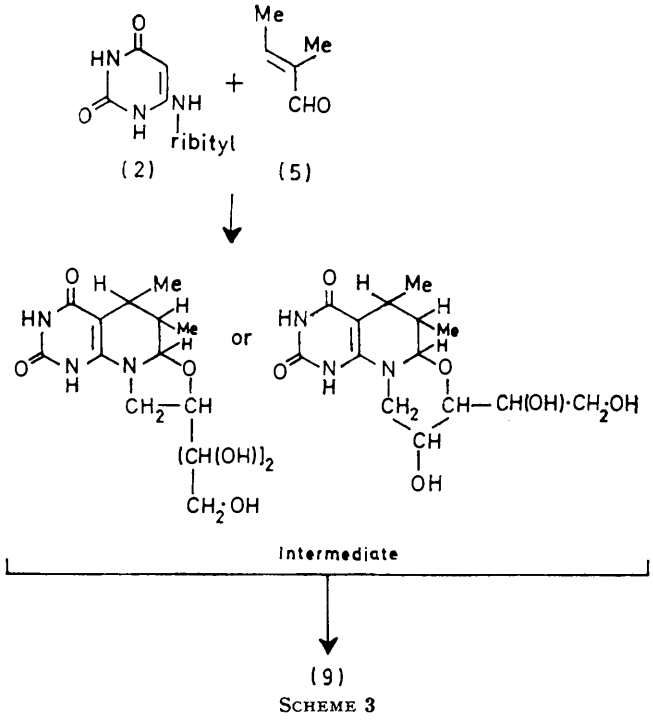
Irradiation of an aqueous acidic (pH 3) solution of the 8-D-ribitylpyrido[2,3-*d*]pyrimidine (13) gave a white precipitate after 1 h. This was identical with an authentic sample of 6,7-dimethylpyrido[2,3-*d*]pyrimidine-2(1*H*),4(3*H*)-dione (20) (kindly provided by Dr. Hitchings) as judged by paper chromatography, u.v. and i.r. spectra, and m.p. The same product (20) was obtained from photolysis of the 8-(2-hydroxyethyl) analogue (17) in acidic solution. This evidence supports the foregoing findings that (13) and (17) are 6,7-dimethylpyridopyrimidines.

This result was corroborated by further photolysis experiments. Irradiation of compounds (9) and (16) in a similar manner gave the same product, which was different from the 6,7-dimethylpyridopyrimidine (20). As the 5,6-dimethyl isomer of (20) was unavailable, it was synthesised by an adaptation of the ' Doebner–Miller-type ' synthesis. Thus condensation of 6-aminouracil (4) with 2-methylbut-2-enal (5) in strongly acidic solution gave a product having spectroscopic properties in accord with a dimethylpyridopyrimidine which was different from (20) and was thus identified as 5,6-dimethylpyrido[2,3-*d*]pyrimidine-2(1*H*),4(3*H*)-dione (21). Further elaboration of this synthesis is discussed later. Compound (21) proved to be identical with the material obtained from the photolysis reactions of (9) and (16), showing that the latter must be 5,6-dimethyl derivatives.

Photolytic degradation of the 8-(2-hydroxyethyl)-monomethylpyridopyrimidines (12) and (15) gave the products (22) and (23), respectively. The structures of the latter were determined by independent synthesis by the extension of the ' Doebner–Miller-type ' route just described. Thus the product (22) from photolysis of (12) is also obtained by condensation of 6-aminouracil (4) with crotonaldehyde (6). Similarly the photolysis product (23) from (15) can be synthesised from 6-aminouracil (4) and either methyl vinyl ketone (8) or acetoacetaldehyde dimethyl acetal [1] (19). The identification of compounds (12) and (15) [and hence of the ribityl derivatives (10) and (14)] as the 5-methyl- and 7-methylpyrido[2,3-*d*]pyrimidine-2(8*H*),4(3*H*)-diones, respectively, follows.

The ' Doebner–Miller-type ' syntheses with the 6-unsubstituted aminouracil are the first recorded syntheses of this type, it being thought [1] previously that *substituted* amino-derivatives were essential for reaction to occur.

The foregoing evidence is overwhelmingly in favour of reversing the original structural assignments for the 8-substituted pyrido[2,3-*d*]pyrimidines. The tricyclic intermediate formed in the ' Doebner–Miller type ' reaction can now be formulated as a tetrahydropyrido-[2,3-*d*]pyrimidine with either a five- or a six-membered ring between C-7 and N-8. This is formed by an intramolecular nucleophilic displacement reaction involving the hydroxy-group attached to C-2′ or C-3′ of the N-8 side chain as exemplified in Scheme 3. The spectroscopic data [1] for this compound are in accord with either structure.



SCHEME 3

The mechanistic implications of this reversal of the original structures [1] are now in line with other work. In the ' Doebner–Miller-type ' reaction attack by the most nucleophilic position in the pyrimidine (C-5) occurs at the carbon atom β to the carbonyl function. In the original quinoline synthesis [3] the most nucleophilic position is the amino-group in the aromatic amine and it is this group which attacks at the β-position of the unsaturated carbonyl derivative. In the β-oxo-aldehyde synthesis attack of C-5 of the pyrimidine occurs at the aldehyde carbonyl group as was found by Robins and Hitchings [4] in their related synthesis.

The *raison d'être* for the foregoing syntheses was to produce specific enzyme inhibitors of structures analogous to those of enzymic substrates. The first clue that the structures of these compounds had been wrongly assigned came from enzyme inhibition studies. This reversal of the role of the specificity built into these compounds, where it acts in a diagnostic fashion relating activity to structure (and not *vice versa*), provides good evidence for the essential validity of this approach to the inhibition of vitamin biosynthesis.

EXPERIMENTAL

U.v. spectra were determined with a Unicam SP 8000A spectrophotometer for aqueous solutions of standard pH.

[9] A. Dornow and E. Neuse, *Chem. Ber.*, 1951, **84**, 296.
[10] A. H. Tracy and R. C. Elderfield, *J. Org. Chem.*, 1941, **6**, 63.

N.m.r. spectra were determined with either a Perkin-Elmer R14 (100 MHz) or a Varian XL-100 spectrometer (100 MHz) (tetramethylsilane as standard). I.r. spectra were run with a Perkin-Elmer 257 grating spectrophotometer. Mass spectra were run with an A.E.I. MS 902 instrument.

Paper chromatograms (Whatman No. I paper) were developed by the ascending technique with (A) butan-1-ol–glacial acetic acid–water (5 : 2 : 3), (B) propan-1-ol–ammonia–water (6 : 3 : 1), and (C) 3% ammonium chloride as solvents. Spots were located by illumination with filtered u.v. light (λ 254 and 365 nm).

5-Methyl-8-D-ribitylpyrido[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (10).—6-D-Ribitylaminouracil (2) (800 mg) and crotonaldehyde (6) (1·0 ml) in 20% hydrochloric acid (10 ml) were stirred at room temperature in air for 12 h. The red solution was evaporated to dryness, the gum was dissolved in the minimum of ethanol, and then ether was added to precipitate a gummy solid. The supernatant liquid was decanted and the solid dissolved in 2-ethoxyethanol (30 ml). This solution was heated on a water-bath for 4 h while oxygen was bubbled through. Addition of ether (200 ml) after cooling gave a brown precipitate. This was filtered off, redissolved in water (2 ml), and chromatographed on a column of Amberlite CG 50 resin (elution with water). The elution of the pyridopyrimidine was followed by monitoring the u.v. absorption of the eluate at 357 nm (pH 13). Evaporation and recrystallisation from water gave the *pyridopyrimidine* (10) (49 mg), m.p. 114—130° (decomp.) (Found: C, 49·85; H, 5·25; N, 13·45. $C_{13}H_{17}N_3O_6$ requires C, 50·2; H, 5·45; N, 13·5%), $\lambda_{max.}$ (pH 1) 346, 311, and 275 nm, (pH 13) 357 and 256 nm, τ ($CF_3{\cdot}CO_2H$) 6·88 (3H, s, 5-CH_3), 5·93—4·73 (7H, m, ribityl), 2·41 (1H, d, *J* 8 Hz, 6-H), and 1·37 (1H, d, *J* 8 Hz, 7-H).

6-Methyl-8-D-ribitylpyrido[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (11).—6-D-Ribitylaminouracil (2) (800 mg) and methacrylaldehyde (7) (1·0 ml) in 20% hydrochloric acid (10 ml) were stirred at room temperature for 12 h. The mixture was evaporated to a gum which was redissolved in ethanol, then ether was added to precipitate a white solid. On filtration this solid became gummy and was dissolved in ethoxyethanol (30 ml). This solution was heated on a water-bath while oxygen was bubbled through. After cooling, ether (200 ml) was added to this solution to give a tan solid which was collected. The solid was dissolved in water (2 ml) and chromatographed on Amberlite CG 50 resin as described before. Evaporation of the aqueous eluate gave a yellow solid which was recrystallised from aqueous ethanol to give the *pyridopyrimidine* (11) (143 mg), m.p. 227—228° (Found: C, 50·5; H, 5·6; N, 13·8. $C_{13}H_{17}N_3O_6$ requires C, 50·2; H, 5·45; N, 13·5%). $\lambda_{max.}$ (pH 1) 356, 325, and 275 nm, (pH 13) 373 and 257·5 nm, τ (D_2O) 7·63 (3H, s, 6-CH_3), 6·33—4·95 (broad envelope + HOD peak, ribityl), 1·68 (1H, d, *J* 2 Hz), and 1·56 (1H, d, *J* 2 Hz).

7-Methyl-8-D-ribitylpyrido[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (14).—6-D-Ribitylaminouracil (2) (520 mg) and acetoacetaldehyde dimethyl acetal (19) (1·0 ml) in 0·5M-hydrochloric acid (10 ml) were refluxed for 2 h. The deep red solution was neutralised with 0·1M-sodium hydroxide and evaporated at 40° to 2 ml. This solution was chromatographed on Amberlite CG 50 resin (elution with water). Evaporation of the aqueous eluate (700 ml) gave a brown oil which solidified on treatment with ethanol. Recrystallisation from ethanol–water gave pale yellow needles (304 mg) of the *pyridopyrimidine* (14), m.p. 170° (decomp.) (Found: C, 50·6; H, 5·4; N, 13·55. $C_{13}H_{17}N_3O_6$ requires C, 50·2; H, 5·45; N, 13·5%), $\lambda_{max.}$ (pH 1) 356, 323, and 276 nm, (pH 13) 367 and 262 nm, τ (D_2O) 7·18 (3H, s, 7-CH_3), 6·40—4·97 (broad multiplet + HOD peak, ribityl), 2·86 (1H, d, *J* 9 Hz, 6-H), and 1·54 (1H, d, *J* 9 Hz, 5-H).

Enzyme Inhibition Studies.—Riboflavin synthetase was isolated from dried baker's yeast (kindly supplied by The Distillers Co. Ltd.) by a modification of the method of Plaut [11] to give an active freeze-dried extract which was used for the inhibition studies. Thus dried baker's yeast (300 g) and toluene (19 ml) in water (1 l) were stirred for 12 h at room temperature. The supernatant (820 ml) obtained after centrifugation of this material was treated with ammonium sulphate (267 g) at room temperature to precipitate protein. The residue obtained after centrifugation was dissolved in 0·1M-ammonium acetate–0·01M-sodium sulphite buffer (200 ml) and was treated again with ammonium sulphate (59 g). The residue obtained after centrifugation was dissolved in the buffer (100 ml) and dialysed overnight at 4°. The dialysate, after adjustment to pH 8·3 with ammonium hydroxide and addition of 2-mercaptoethanol to a final concentration of 0·2 mol l^{-1}, was treated with cold acetone (111 ml) at 0—5°. Centrifugation removed the precipitated protein and a similar treatment of the supernatant with cold acetone (140 ml) precipitated more protein. After centrifugation this was dissolved in 0·04M-phosphate buffer (pH 7; 50 ml) containing 2-mercaptoethanol (0·2 mol l^{-1}) and was freeze-dried to an active powder (401 mg).

The substrate, 6,7-dimethyl-8-D-ribityl-lumazine (1) was synthesised by the method of Rowan and Wood.[12]

Assay method. For measurement of the K_i values of the inhibitors (9), (13), (10), (11), and (14) a modification of the assay method [11] of Plaut was used. The initial rate of production of riboflavin (assayed by increase in absorbance at 470 nm) was recorded spectrophotometrically for various concentrations of substrate and inhibitor at constant enzyme concentration. From these measurements the K_i values were determined by the Lineweaver–Burk method.[13]

Thus a mixture consisting of 0·2M-phosphate buffer (pH 7; 0·5 ml), 0·3M-sodium hydrogen sulphite (0·3 ml) $4{\cdot}5 \times 10^{-4}$M 6,7-dimethyl-8-*D*-ribityl-lumazine (1), the inhibitor, and water to a final volume of 2·9 ml was incubated at 37° in a 1 cm quartz u.v. cuvette, placed in an SP 8000 grating spectrophotometer set to measure increase in absorbance at 470 nm (A_{470}). The reaction was initiated by the addition of riboflavin synthetase (0·1 ml) made up as an aqueous solution (10 mg ml^{-1}) from the freeze-dried extract. The rate of increase in A_{470} was then recorded on an SP21 'flat-bed' recorder. The inhibition constants obtained are recorded in Table 1.

Nuclear Overhauser Effect Studies.—The solutions used for these studies were 4—5% w/v in [$^2H_6$]dimethyl sulphoxide, 10% of deuterium oxide being added for the exchange experiments. The solutions were not degassed.

It was first necessary to assign shift frequencies for the four compounds (9), (13), (16), and (17). These are given in Table 3.

Assignments for the 8-hydroxyethyl compounds (16) and (17) were straightforward, but deuterium exchange, de-

[11] R. A. Harvey and G. W. E. Plaut, *J. Biol. Chem.*, 1966, **241**, 2120.

[12] T. Rowan and H. C. S. Wood, *J. Chem. Soc.* (*C*), 1968, 452.

[13] M. Dixon and E. C. Webb, 'Enzymes,' 2nd edn., Longmans, London, 1967, p. 69 *et seq.*

TABLE 3

N.m.r. data (τ values; J in Hz)

Compound:	(16)	(17)	(9)	(13)
Solvent:	$(CD_3)_2SO$	$(CD_3)_2SO$	10% D_2O–$(CD_3)_2SO$	10% D_2O–$(CD_3)_2SO$
Group				
3-H	−0·57 (s)	−0·68 (s)	−0·66 (s) [a]	−0·80 (s) [a]
5-H		1·79 (s)		1·75 (s)
5-CH_3	7·23 (s)		7·22 (s) [b]	
6-CH_3	7·82 (s)	7·72 (s)	7·78 (s)	7·67 (s)
7-H	1·86 (s)		1·90 (s)	
7-CH_3		7·34 (s)		7·29 (s) [b]
C(1′)H_2	5·65 (t, J 5·4)	5·43 (t, J 5·6)	6·10— 5·80 (m) [c]	5·90— 5·30 (m) [c]
C(5′)H_2			6·60— 6·25 (m)	6·60— 6·25 (m)
C(2′)H			5·30— 5·05 (m) [d]	5·30— 5·05 (q) [d]
C(3′)H				
C(4′)H			6·60— 6·25 (m) [d]	6·60— 6·25 (m) [d]
C(2′)H_2·OH	6·35 (q, J 5·4) [a]	6·21 (q, J 5·6) [a]		
C(2′)H_2·OH	5·06 (t, J 5·4)	4·98 (t, J 5·5)		

[a] Before D_2O exchange. [b] Slowly exchanges with D_2O of solvent. Assigned from N.O.E./frequency plot. [d] Assignment not certain. [e] Irradiation at quartet frequencies decouples both triplets.

coupling, and the N.O.E. experiments themselves were necessary to assign the frequencies of the 8-D-ribityl compounds (9) and (13).

N.O.E. is reported here as:

$$f_{\text{shift of observed peak}}\ (\text{shift of } h_2 \text{ field}) = x\%$$

where h_2 represents the second, decoupling r.f. field. The frequency of the second field (h_2) was located by noting its interference beat while sweeping the observed field h_1. The symmetrical equilibrium (or non-effect) integral value was determined with the h_2 field at the same power as used for the N.O.E. integral value and placed equidistant from, and on the other side of, the observed signal.

Observed N.O.E.s for the 8-hydroxyethyl compounds are as follows:

For (16):

Irradiation with h_2 at 6-CH_3: $f_{1\cdot86}(7\cdot82) = 12 \pm 3\%$
Irradiation with h_2 at C(1′)H_2: $f_{1\cdot86}(5\cdot65) = 33 \pm 4\%$

thus confirming the 5,6-dimethyl structure.

For (17):

Irradiation with h_2 at 6-CH_3: $f_{1\cdot79}(7\cdot72) = 35 \pm 2\%$
Irradiation with h_2 at C(1′)H_2: $f_{1\cdot79}(5\cdot43) = 0\%$

thus confirming the 6,7-dimethyl structure.

As assignment of the C(1′)H_2 signal for the 8-D-ribityl compounds (9) and (13) was more difficult, N.O.E.s were determined at high power for constant h_2 fields at regular frequency intervals across the aliphatic absorption area of the spectra. The plot of N.O.E. *versus* h_2 frequency showed maxima where true N.O.E.s occurred.

For (9), the N.O.E./h_2 frequency plot showed maxima at τ 7·78 (6-CH_3) and 5·91 [C(1′)H_2] identifying (9) as the 5,6-dimethyl isomer. This was confirmed by repeating the experiment at low power and irradiating at the above frequencies.

Irradiation with h_2 at 6-CH_3: $f_{1\cdot90}(7\cdot78) = 13 \pm 2\%$
Irradiation with h_2 at C(1′)H_2: $f_{1\cdot90}(5\cdot91) = 21 \pm 3\%$

thus confirming (9) as the 5,6-dimethyl derivative.

For (13), the N.O.E./h_2 frequency plot showed only one maximum at τ 7·67. The experiment was repeated at low power.

Irradiation with h_2 at 6-CH_3: $f_{1\cdot75}(7\cdot67) = 36 \pm 2\%$
Irradiation with h_2 at C(1′)H_2: $f_{1\cdot75}(5\cdot90—5\cdot30) = 0\%$

thus confirming (13) as the 6,7-dimethyl derivative.

Photolytic Degradation Studies.—Photolyses were performed in a Pyrex tube with a tap for sample removal and a condenser attachment for cooling purposes, by using a Hanovia photochemical Reading Reactor having two straight arc medium-pressure mercury vapour lamps of 500 W each. The reactions were followed by observing the change in u.v. spectrum at pH 3.

Photolysis of 6,7-*Dimethyl*-8-D-*ribitylpyrido*[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (13).—The pyridopyrimidine [1] (13) (20 mg) in dilute hydrochloric acid (4 ml; pH 3) was irradiated for 1 h. The reaction was followed by observing the decrease in absorbance at 366 nm at pH 3. The solid which precipitated on cooling was collected by centrifugation, washed, and recrystallised thrice from water to give a white powder (5·2 mg), m.p. 317—327° (decomp.), $\lambda_{max.}$ (pH 1) 316, 245sh, and 220 nm, (pH 13) 346sh, 324, and 265 nm, identical with 6,7-dimethylpyrido[2,3-*d*]pyrimidine-2(1*H*),-4(3*H*)-dione (20) [provided by Dr. G. H. Hitchings, Burroughs Wellcome Co., North Carolina], m.p. 320—327° (decomp.) as judged by u.v. and i.r. spectra (KBr) and by paper chromatography in three different solvent systems.

Photolysis of 8-(2-*Hydroxyethyl*)-6,7-*dimethylpyrido*[2,3-d]-*pyrimidine*-2(8H),4(3H)-*dione* (17).—The pyridopyrimidine (17) (50 mg) was dissolved in dilute hydrochloric acid (5 ml; pH 2) and irradiated for 6 h. The white product (15 mg) which precipitated on cooling was identical with the photolysis product of (13) and the authentic 6,7-dimethylpyridopyrimidine (20).

Photolysis of 5,6-*Dimethyl*-8-D-*ribitylpyrido*[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (9).—The pyridopyrimidine [1] (9) (32 mg) was dissolved in dilute hydrochloric acid (7 ml; pH 3) and irradiated for 2·5 h. On cooling, a yellow-brown solid was obtained by centrifugation. This was purified by repeatedly dissolving in 0·1M-sodium hydroxide and reprecipitating by acidification. The purified material was identical with the pyridopyrimidine (21) as judged by u.v. and i.r. spectra (KBr) and by paper chromatography in three different solvent systems.

Photolysis of 8-(2-*Hydroxyethyl*)-5,6-*dimethylpyrido*[2,3-d]-*pyrimidine*-2(8H),4(3H)-*dione* (16).—The pyridopyrimidine [1] (16) (30 mg) was dissolved in dilute hydrochloric acid (13 ml; pH 3) and irradiated for 5 h. The product obtained on cooling was purified by repeated precipitation from aqueous acid and was identical with the product of photolysis of (9) and the synthetic 5,6-dimethylpyridopyrimidine (21).

Photolysis of 8-(2-*Hydroxyethyl*)-5-*methylpyrido*[2,3-d]*pyrimidine*-2(8H),4(3H)-*dione* (12).—The pyridopyrimidine [1] (12) (40 mg) in dilute hydrochloric acid (5 ml; pH 2) was irradiated for 1·5 h. On cooling a solid was produced. This was collected by centrifugation and recrystallised twice from aqueous methanol to give a product which was identical [paper chromatography in three solvent systems and i.r. (KBr) and u.v. spectra] with the pyridopyrimidine (22).

Photolysis of 8-(2-*Hydroxyethyl*)-7-*methylpyrido*[2,3-d]-*pyrimidine*-2(8H),4(3H)-*dione* (15).—The pyridopyrimidine [1] (15) (50 mg) in dilute hydrochloric acid (10 ml; pH 2) was irradiated for 2 h. The solid produced on cooling was collected and recrystallised as before to give material identical with the pyridopyrimidine (23).

5,6-*Dimethylpyrido*[2,3-d]*pyrimidine*-2(1H),4(3H)-*dione* (21).—6-Aminouracil (4) (1·27 g, 0·01 mol) and 2-methylbut-2-enal (5) (1·5 ml) in 20% hydrochloric acid (40 ml) were stirred at room temperature in air for 36 h. The resulting solid was filtered off, washed with water, and recrystallised from aqueous ethanol giving the *pyridopyrimidine* (21) (1·08 g, 57%) as white plates, m.p. >310° (Found: C, 56·5; H, 4·7; N, 21·5. $C_9H_9N_3O_2$ requires C, 56·5; H, 4·7; N, 21·9%), $\lambda_{max.}$ (pH 1) 312 and 243 nm, (pH 13) 343sh, 324, and 264 nm, τ (CF_3·CO_2H) 7·43 (3H, s, 6-CH_3), 6·87 (3H, s, 5-CH_3), and 1·47 (1H, s, 7-H).

5-*Methylpyrido*[2,3-d]*pyrimidine*-2(1H),4(3H)-*dione* (22).—6-Aminouracil (4) (800 mg) and crotonaldehyde (6) (1 ml) in 20% hydrochloric acid (20 ml) were stirred in air at room temperature for 0·5 h; a white solid was produced. The mixture was then heated on a water-bath for 12 h, cooled, and neutralised to pH 7—8 with concentrated ammonium hydroxide, and the cream solid was filtered off and dried. This material was adsorbed on alumina (5 g) and chromatographed on an alumina column (29 × 3·7 cm) (elution with 50% ethanol–water). The progress of the chromatography was monitored by u.v. and fluorescence spectroscopy. The first product obtained was an unidentified aromatic pyrido-[2,3-*d*]pyrimidine (20 mg), m.p. 225° (decomp.), $\lambda_{max.}$ (pH 1) 318 nm, (pH 13) 350sh, 326, and 269 nm. This was followed by a mixture of the above compound and the product (22). The 5-*methylpyridopyrimidine* (22) (65 mg) was obtained from the later fractions and was recrystallised from water; m.p. >300° (Found: C, 54·25; H, 4·05; N, 23·45%; M^+, 177·0540. $C_8H_7N_3O_2$ requires C, 54·25; H, 3·95; N, 23·6%; *M*, 177·0538), $\lambda_{max.}$ (pH 1) 303 nm, (pH 13) 333sh, 317, and 264 nm, τ (CF_3·CO_2H) 6·78 (3H, s, 5-CH_3), 2·33 (1H, d, *J* 7 Hz, 6-H), and 1·29 (1H, d, *J* 7 Hz, 7-H).

7-*Methylpyrido*[2,3-d]*pyrimidine*-2(1H),4(3H)-*dione* [1] (23).—6-Aminouracil (4) (600 mg) and methyl vinyl ketone (8) (1 ml) in 20% hydrochloric acid (20 ml) were stirred in air at room temperature for 12 h. On evaporation a brown solid was produced. This was collected by filtration and redissolved in hot 2M-sodium hydroxide. The solution was treated with charcoal, filtered, and acidified; a pale brown solid precipitated. This was collected (249 mg) and recrystallised twice from water to give the pyridopyrimidine (23), m.p. 312—315° (lit.,[1] 315°), $\lambda_{max.}$ (pH 1) 306 and 240sh nm, (pH 13) 338sh, 317, and 265 nm, τ (CF_3,CO_2H) 7·03 (3H, s, 7-CH_3), 2·34 (1H, d, *J* 9 Hz, 6-H), and 0·95 (1H, d, *J* 9 Hz, 5-H).

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