Specific Enzyme Inhibitors in Vitamin Biosynthesis. Part 3.[1] The Synthesis and Inhibitory Properties of some Substrates and Transition State Analogues of Riboflavin Synthase

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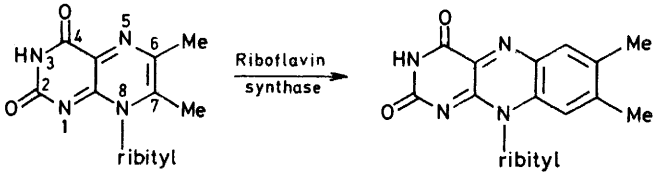
Syntheses of potential inhibitors of riboflavin synthase are described. The tolerance of the enzyme to bulky substituents was investigated by the synthesis of substrate analogues which included lumazines and pyrido[2,3-*d*]-pyrimidines prepared by condensation of α-diketones and β-keto-aldehydes respectively with appropriate amino-substituted uracils. Potential transition-state analogues, including 7-oxolumazines, 7-oxopyrido[2,3-*d*]pyrimidines, and 6,7-dioxolumazines were also prepared by similar condensations using α-keto-acid derivatives, dimethyl acetylenedicarboxylate, and oxalate derivatives. Two possible dual affinity inhibitors were also prepared. The potential inhibitors were tested using riboflavin synthase from yeast or from *E. coli*, and their effectiveness is discussed in relation to the bulk and electronic character of the substituents.

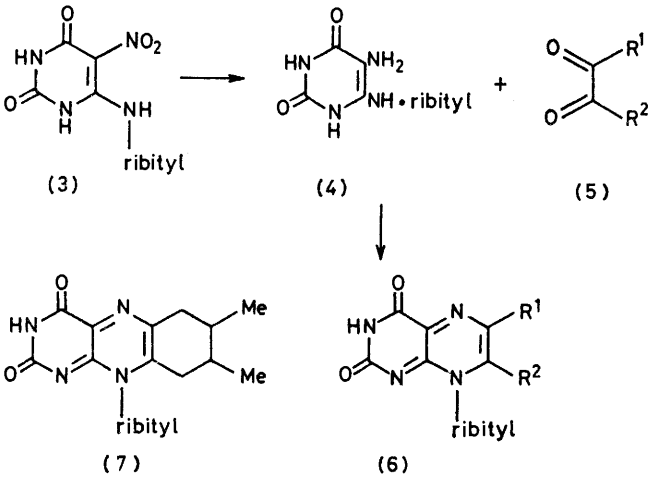
THE inhibition of an enzyme which is present in a parasite but not in a host is a rational approach to chemotherapy. Many suitable target enzymes can be found in the biosynthesis of vitamins. For example, previous studies [1,2] in these laboratories have shown that methyl substituted pyrido[2,3-*d*]pyrimidines are inhibitors of yeast riboflavin synthase (6,7-dimethyl-8-*D*-ribityl-lumazine: 6,7-dimethyl-8-D-ribityl-lumazine butane-2,3-diyltransferase, E.C. 2.5.1.9). This enzyme catalyses the conversion of two molecules of the lumazine (1) into riboflavin (2). The compounds described in the present paper are also potential inhibitors of this enzyme. They include analogues of the substrate with substituents at positions 6 and/or 7 which are larger than the substrate's methyl groups, and also analogues of a presumed transition state or intermediate [3] in the reaction catalysed by the enzyme. The effectiveness of the inhibitors sheds light upon the steric and electronic demands of the active site of riboflavin synthase. Such information is most important in the design of potent inhibitors of chemotherapeutic value.

Substrate Analogues: Lumazines.—We sought initially to investigate the effect on bonding to the enzyme of introducing bulky groups in place of one or both of the



(1) (2)

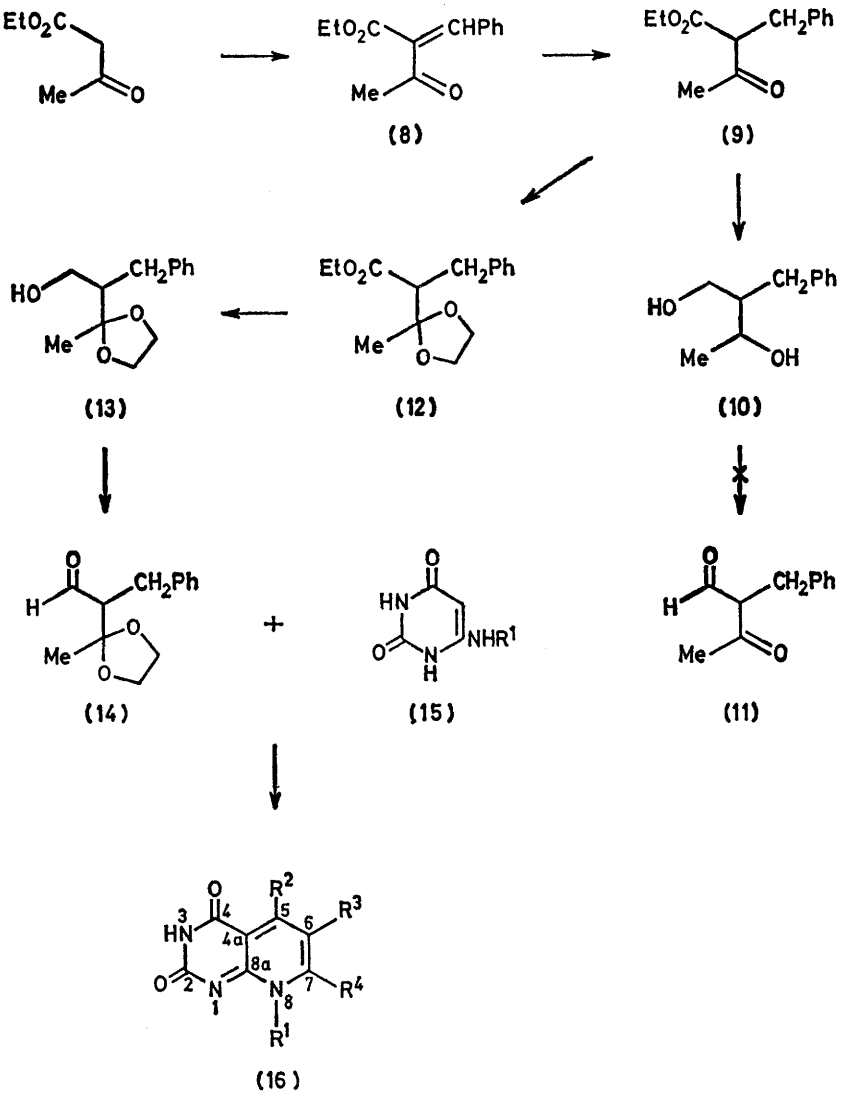
methyl groups at positions 6 and 7 of the natural substrate (1). Some work along these lines has been published by Plaut and his co-workers.[4,5] Routes to 6,7-disubstituted-8-D-ribityl-lumazines † are well established.[4,6-9] Our preferred method (Scheme 1) involved condensation of 5-amino-6-D-ribitylaminouracil (4) prepared freshly from the corresponding 5-nitrouracil (3) with the appropriate β-diketone (5a—c) thus affording the required lumazines (6a—c). With the unsymmetrical diketones (5b and c) there are two possible products from this reaction (6b and d) and (6c and e) respectively. However with each diketone only one product was isolated and the structures of the lumazines were defined unambiguously by ^{1}H n.m.r. spectroscopy as follows. Paterson and Wood [3] have shown that the 7-methyl protons of lumazine (1) undergo base-catalysed exchange in D_2O whereas the 6-methyl protons do not. In the lumazine formed from diketone (5b), a triplet at δ 0.95 (3H), a multiplet at δ 1.73 (2 H), and a triplet at δ 2.43 (2H) were observed in the ^{1}H n.m.r. spectrum in NaOD–D_2O but no singlet corresponding to the 7-methyl protons was observed. The structure of the product is therefore



a; $R^1 = R^2 = Ph$
b; $R^1 = Pr^n$, $R^2 = Me$
c; $R^1 = CH_2Ph$, $R^2 = Me$
d; $R^1 = Me$, $R^2 = Pr^n$
e; $R^1 = Me$, $R^2 = CH_2Ph$

SCHEME 1

† The nomenclature used in the discussion section is that used in the majority of biochemical papers dealing with riboflavin biosynthesis and in Chemical Abstracts prior to 1972. Compounds are named in the Experimental section using current preferred nomenclature.



a; $R^1 = CH_2CH_2OH$, $R^2 = H$, $R^3 = CH_2Ph$ $R^4 = Me$
b; R^1 = D-ribityl, $R^2 = H$, $R^3 = CH_2Ph$, $R^4 = Me$
c; R^1 = D-ribityl, $R^2 = H$, $R^3 = R^4 = Me$
d; R^1 = D-ribityl, $R^2 = R^3 = H$, $R^4 = Me$
e; R^1 = D-ribityl, $R^2 = R^3 = Me$, $R^4 = H$
f; R^1 = D-ribityl, $R^2 = R^4 = H$, $R^3 = Me$
g; R^1 = D-ribityl, $R^2 = Me$, $R^3 = R^4 = H$
h; $R^1 = CH_2CH_2OH$, $R^2 = R^4 = H$, $R^3 = NO_2$
i; R = D-ribityl, $R^2 = R^4 = H$, $R^3 = NO_2$

SCHEME 2

the 7-methyl-6-propyl isomer (6b). A similar method has been used [10] to establish the structure of a related compound. The benzyl substituted pteridine similarly showed a singlet (2H) at δ 1.56 which did not undergo exchange in NaOD–D_2O and thus the structure of the benzyl compound is 6-benzyl-7-methyl-8-D-ribityl-lumazine (6c). Tetrahydroriboflavin (7) was prepared by catalytic hydrogenation of riboflavin in trifluoroacetic acid.[11]

Substrate Analogues: Pyrido[2,3-d]*pyrimidines.*— Earlier studies [1,2] in these laboratories have shown that 6,7-dialkyl-8-D-ribitylpyrido[2,3-*d*]pyrimidines can be prepared by acid-catalysed condensation of 6-D-ribitylaminouracil with β-keto-aldehydes. We have extended this synthesis to give the 6-benzylpyrido-[2,3-*d*]pyrimidines (16a and b). These compounds not only contain a bulky group at position 6 but the benzene ring is an attractive location for placing a reactive group that would convert a competitive inhibitor into an active-site-directed irreversible inhibitor.[12]

Condensation of benzaldehyde with ethyl acetoacetate afforded the benzylidene keto-ester (8) (Scheme 2). In this way, the substituent was placed unambiguously at C-2 avoiding the need for alkylation of an unstable β-keto-aldehyde. Catalytic hydrogenation of (8) led to the keto-ester (9) which was further reduced to the diol

TABLE 1

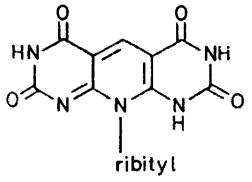
^{13}C N.m.r. spectra of pyrido[2,3-*d*]pyrimidines in D_2O plus LiOD or NaOD (δ values in p.p.m. from tetramethylsilane)

Compound	C-2 + C-4	C-4a + C-8a	C-5	C-6	C-7	CH_3	Benzyl	$CH_2{\cdot}CH_2OH$	Ribityl
(16a)	174.2 (s) 171.0 (s)	158.1 (s) 155.6 (s)	142.5 (d)	125.6 (s)	113.6 (s)	18.6 (s) *	139.6 (s) 129.5 (d) 129.2 (d) 127.3 (d) 38.6 (t)	60.0 (t) 50.2 (t)	
(16b)	174.7 (s) 171.0 (s)	158.3 (s) 156.5 (s)	142.7 (d)	126.1 (s)	113.5 (s)	19.1 (s) *	139.8 (s) 129.7 (d) 129.4 (d) 127.5 (d) 38.8 (t)		74.7 (d) 74.2 (d) 72.4 (d) 64.0 (t) 51.7 (t)
(16c)	173.7 (s) 170.2 (s)	157.6 (s) 156.3 (s)	142.1 (d)	123.9 (s)	112.6 (s)	19.7 (q) 18.9 (s) *			74.5 (d) 73.5 (d) 71.7 (d) 63.8 (t) 51.1 (t)
(16d)	174.5 (s) 170.8 (s)	159.0 (s) 157.9 (s)	141.1 (d)	115.9 (d)	113.7 (s)	22.6 (s) *			74.4 (d) 73.5 (d) 71.4 (d) 63.7 (t) 50.8 (t)

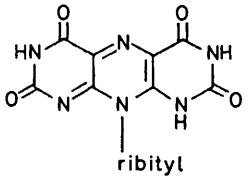
* Protons exchange with solvent.

(10) with lithium aluminium hydride in ether. Despite many attempts using a variety of oxidising agents (see Experimental section) it was not possible to convert the diol (10) into the required β-keto-aldehyde (11). However it was possible to overcome this difficulty by protecting the ketone in (9) as its ethylene acetal (12) and subsequently converting the ester into the corresponding aldehyde (14) *via* the alcohol (13) by lithium aluminium hydride reduction and oxidation in dichloromethane with the chromium trioxide dipyridine complex.[13] Condensation of the protected aldehyde with the appropriate aminouracils (15) under acidic conditions afforded the pyrido[2,3-*d*]pyrimidines (16a and b). A further pyrido-pyrimidine (17) was obtained by condensing triethyl orthoformate with ribitylaminouracil (15b): the aza-analogue (18) of this compound has been prepared previously in this laboratory.[14]

Our earlier work [1] showed that the acid-catalysed condensation of a β-oxo-aldehyde or the corresponding acetal with a 6-aminouracil was regiospecific and gave the product resulting from attack at C-5 of the pyrimidine by the *aldehyde* carbonyl group. That the same regiospecificity applied in the condensation of the aldehydoketal (14) with the pyrimidines (15) was confirmed by ^{13}C n.m.r. studies on the pyrido[2,3-*d*]pyrimidines (16a and b) (Table 1). That the methyl group was attached to C-7 (rather than C-5) was established by the



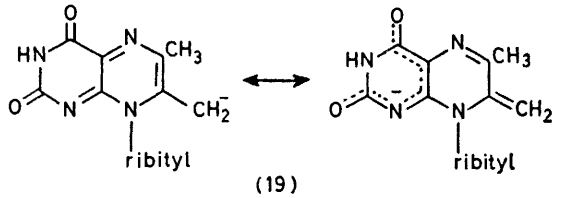
(17)



(18)

resonance of C-5 which appeared as a doublet in the single frequency off-resonance decoupled spectrum. The pyridopyrimidines (16c and d), whose structures were determined in our earlier work,[1] were used as reference compounds in interpretation of the ^{13}C n.m.r. spectra. Two additional pyrido[2,3-*d*]pyrimidines (16h and i) were prepared by condensation of the appropriate aminouracil (15) with nitromalondialdehyde in dilute hydrochloric acid.

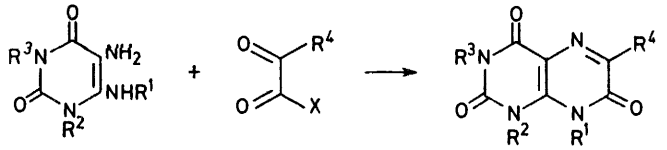
Transition State Analogues: 7-Oxolumazines.—It is thought that enzymes have maximal affinity for the transition states of the reactions that they catalyse.[15] If a chemical mechanism for an enzyme-catalysed reaction can be formulated it becomes possible to design compounds that will mimic the transition state of the reaction; such compounds should be potent inhibitors of the enzyme. On the basis of ^1H n.m.r. evidence, Paterson and Wood [3] suggested that formation of a carbanion (19), by loss of a proton from the C-7 methyl



(19)

group of the substrate, initiated transfer of the four-carbon atom unit from donor lumazine to acceptor lumazine in the reaction catalysed by riboflavin synthase. The 7-oxolumazine (20a) which is a mimic of this anion, may thus be regarded as an analogue of an intermediate or early transition state in the reaction. It is known [5,16] that the naturally occurring 7-oxolumazine (20a) is a good competitive inhibitor of riboflavin synthase and we were encouraged to explore the properties of related compounds. Accordingly, we have synthesised a series of 7-oxolumazines and have studied their potency as inhibitors of the enzyme. All the compounds, except the 6-carboxy-7-oxolumazine (20e), were prepared by condensation of the appropriate α-keto-ester or α-keto-acid with a 5-amino-6-substituted

aminouracil which was prepared *in situ* by reduction of the corresponding 5-phenylazo-, 5-nitroso-, or 5-nitrouracil (Scheme 3). The 6-carboxy-derivative (20e) was obtained by hydrolysis of the pyrimido[5,4-*g*]pteridine (18).



(20)

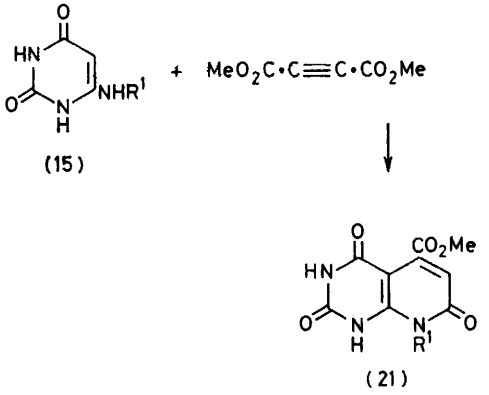
X = OH or OEt

a; R^1 = D- ribityl, R^2 = R^3 = H, R^4 = Me
b; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = Et
c; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = CH_2Ph
d; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = $CH_2CH_2CO_2H$
e; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = CO_2H
f; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = 2′-nitrobenzyl
g; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = 4′-nitrobenzyl
h; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = 2′,4′-dichlorophenyl
i; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = 4′-hydroxyphenyl
j; R^1 = D-ribityl, R^2 = R^3 = H, R^4 = Ph
k; R^1 = CH_2CH_2OH, R^2 = R^3 = H, R^4 = Me
l; R^1 = CH_2CH_2OH, R^2 = R^3 = H, R^4 = CH_2Ph
m; R^1 = CH_2CH_2OH, R^2 = R^3 = H, R^4 = 2′-nitrobenzyl
n; R^1 = $(CH_2)_5$·OH, R^2 = R^3 = H, R^4 = Me
o; R^1 = CH_2CH_2OH, R^2 = H, R^3 = R^4 = Me
p; R^1 = CH_2CH_2OH, R^2 = R^3 = Me, R^4 = $CH_2CH_2CO_2H$
q; R^1 = R^2 = R^3 = Me, R^4 = $CH_2CH_2CO_2H$
r; R^1 = R^3 = Me, R^2 = H, R^4 = $CH_2CH_2CO_2H$
s; R^1 = H, R^2 = R^3 = Me, R^4 = $CH_2CH_2CO_2H$

SCHEME 3

Transition State Analogues: 7-*Oxopyrido*[2,3-d]*pyrimidines.*—Our earlier studies,[1,2] and those reported below, show that the pyrido[2,3-*d*]pyrimidine ring system is acceptable to the enzyme as an analogue of the pteridine ring. We have therefore prepared some 8-substituted-7-oxopyrido[2,3-*d*]pyrimidines as potential transition-state analogues. These compounds (21a—c) were prepared by the method of Broom *et al.*[17] and involved condensation of the appropriate aminouracil (15) with dimethyl acetylenedicarboxylate (Scheme 4).

Transition State Analogues: 6,7-*Dioxolumazines.*—The detailed mechanism put forward by Beach and Plaut [18] to explain the regiospecificity of the reaction catalysed by

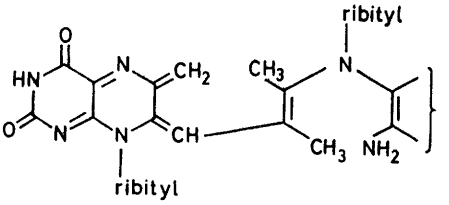


(15) (21)

a; R^1 = H
b; R^1 = CH_2CH_2OH
c; R^1 = D-ribityl

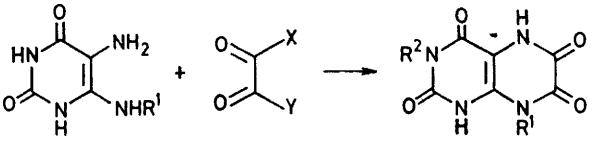
SCHEME 4

riboflavin synthase is similar to that proposed by us [3] but differs in detail in the final steps. In particular, Beach and Plaut suggest formation of an intermediate (22) which subsequently cyclises to form the dimethylbenzene ring of riboflavin. Just as 7-oxolumazines can be regarded as mimics of the carbanion (19) formed in the early stages of the enzymic reaction, 6,7-dioxolumazines can be considered to be mimics, or transition-state analogues, of the intermediate (22). Indeed the 6,7-dioxo-8-D-ribityl-lumazine (23a) is already known [5] to



(22)

be a potent inhibitor of riboflavin synthase and its efficacy can be rationalised in this way. We have, therefore, prepared a series of 6,7-dioxolumazines with a view to exploring the tolerance of the enzyme to variation of the N-8 substituent. The compounds were prepared by condensation of the appropriate 5-amino-4-substituted aminouracil with either ethoxalyl chloride or diethyl oxalate (Scheme 5) with the exception



(23)

X = Y = OEt
X = OEt, Y = Cl

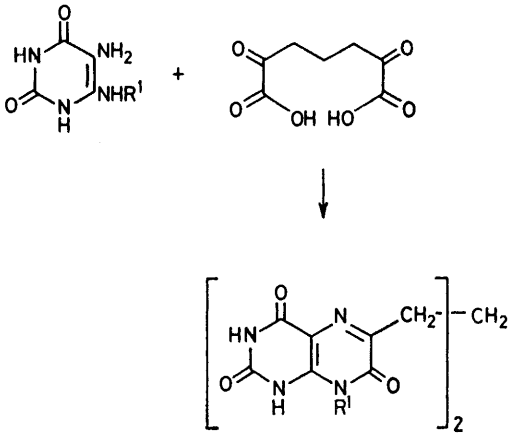
a; R^1 = D-ribityl, R^2 = H
b; R^1 = CH_2CH_2OH, R^2 = H
c; R^1 = CH_2CH_2O·$COCH_2Cl$, R^2 = H
d; R^1 = CH_2·CH_3, R^2 = H
e; R^1 = CH_2CO_2H, R^2 = H
f; R^1 = CH_2·$CH_2NHCOCH_3$, R^2 = H
g; R^1 = CH_2·CH_2·CH_2OH, R^2 = H
h; R^1 = $CH_2CH_2CH_2$·O·CO·CH_2Cl; R^2 = H
i; R^1 = CH_2·CH(OH)·CH_3, R^2 = H
j; R^1 = CH_2·CH(OH)·CH_2NHCHO, R^2 = H
k; R^1 = CH_2·CH(OH)·CH_2NHCHO, R^2 = H
l; R^1 = CH_2·CH(OH)·CH_2NH_2, R^2 = H
m; R^1 = $[CH_2]_5OH$; R^2 = H
n; R^1 = $[CH_2]_5CO_2H$; R^2 = H
o; R^1 = CH_2CH_2OH; R^2 = Me
p; R^1 = D-ribityl, R^2 = Me

SCHEME 5

of the chloro-acetoxy-derivatives (23c) and (23h) which were prepared from the corresponding hydroxy-compounds (23b) and (23g) by treatment with chloroacetyl chloride, and the amine (23l) which was prepared by acid hydrolysis of the corresponding formamido-derivative (23k).

Transition-state Analogues: Dual Affinity Inhibitors.—Lienhard and Secemski [19] have suggested that inhibition

of a two-substrate enzymic reaction should be particularly effective when the inhibitor possesses, within a single molecule, the binding determinants for both substrates. We have made a preliminary study of this concept in relation to riboflavin synthase by the synthesis of two dual-affinity analogues based on the 7-oxolumazine derivatives discussed above. These compounds (24a) and (24b) were prepared by condensation of the appropriate 5-amino-4-substituted aminouracil with 2,6-dioxoheptane-1,7-dicarboxylic acid (Scheme 6).



(24)

a; R^1 = D-ribityl
b; R^1 = CH_2CH_2OH

SCHEME 6

Enzymic Studies.—The potential inhibitors were tested using either a partially purified preparation of riboflavin synthase from baker's yeast [1] or a similar preparation from *E. coli*. Most of the compounds tested were found to be competitive inhibitors and the results are recorded in Table 2. Inhibition constants (K_i) were measured for the yeast enzyme using one of the two assays described in the Experimental section. These assays differed mainly in that sodium hydrogen sulphite was omitted from the second assay which resulted in control values dropping to zero. The percentage inhibition results were obtained using the enzyme from *E. coli*. From these results a number of generalisations can be made.

The most effective inhibitors are those with a D-ribityl group attached to N-8, and this observation is in accord with the work of Plaut and his group.[5,20] Given this proviso, the substrate analogues (lumazines and pyrido[2,3-*d*]pyrimidines) are less effective inhibitors than are the transition-state analogues and this lends further support to this general approach [15] to specific enzyme inhibitors. Variation of the groups attached to positions 6 and 7 of the substrate analogues confirms the tolerance of the enzyme to bulky substituents at position 6, and the activity of the 6-benzylpyrido[2,3-*d*]pyrimidine (16b) against the yeast enzyme is particularly significant. The activity of such compounds is understandable in view of the fact that the active site of the enzyme must accommodate two molecules of substrate, and indeed some tricyclic compounds, (7), (18), and (17) of similar size to riboflavin are inhibitors.

The best transition-state analogues were the 7-oxolum-

TABLE 2

Enzyme inhibition data

Compound type	Comp. no.	Inhibition constant[a] K_i (μmol l^{-1}) Assay 1	Assay 2	% age Inhibition[b] (conc. μmol l^{-1})
Lumazine	(1) = Substrate	30 = K_m[c]	9 = K_m	
	(6a)	100		
	(6b)	60		
	(6c)	20		
	(7)	24		
	(18)	30		50 (62)
Pyrido[2,3-*d*]-pyrimidine	(16a)		17	20 (100)
	(16b)		2.2	50 (167)
	(16c)	20[d]		50 (40)
	(16d)	17[d]		50 (10)
	(16e)	>300[d]		0 (1 660)
	(16f)	120[d]		50 (420)
	(16g)	350[d]		0 (300)
	(16h)			
	(16i)	>3 000		
	(17)	30		
7-Oxolumazine	(20a)	0.53[e]		50 (6)
	(20b)	2.2		
	(20c)	2.2		
	(20d)	10		22 (350)
	(20e)	1		50 (50)
	(20f)			50(90)
	(20g)			50 (13)
	(20h)			22 (100)
	(20i)			33 (100)
	(20j)			0 (100)
	(20k)	140		
	(20l)	75		0 (246)
	(20m)			6 (104)
	(20n)			11 (112)
	(20o)			0 (400)
	(20p)		28	0 (100)
	(20q)		*	0 (100)
	(20r)		*	0 (100)
	(20s)		2.0	0 (100)
7-Oxopyrido[2,3-*d*]-pyrimidine	(21a)			0 (200)
	(21b)			0 (200)
	(21c)			0 (200)
6,7-Dioxolumazine	(23a)	0.025 *[f]		50 (0.038)
	(23b)	60		50 (100)
	(23c)	250		
	(23d)	*ca.* 1 500		
	(23e)	200		
	(23f)	300		
	(23g)		1.8	50 (200)
	(23h)		3.7	
	(23i)		0.8	
	(23j)	20 *		
	(23k)	200		
	(23l)	450		
	(23m)			25 (125)
	(23n)	*ca.* 1 500		
	(23o)			0 (400)
	(23p)			50 (300)
Dual affinity inhibitor	(24a)			57 (200)
	(24b)			14 (200)

* Mixed inhibition.

[a] Determined using riboflavin synthase isolated from baker's yeast. [b] Determined using riboflavin synthase isolated from *E. coli*. [c] Harvey and Plaut (R. A. Harvey and G. W. E. Plaut, *J. Biol. Chem.*, 1966, **241**, 2120) report K_m = 10 μmol l^{-1} for yeast enzyme. [d] Data from ref. 1. [e] Winestock, Aogaichi, and Plaut [5] give 2.0 for *A. gossypii* enzyme. [f] Winestock, Aogaichi, and Plaut [5] give 0.009* for *A. gossypii* enzyme.

azine (20a) and the 6,7-dioxolumazine (23a). Variation of the substituent at position 6 of the 7-oxolumazines confirms the general tolerance of the enzyme to bulk in this position and one of these compounds (20d) has been used by us [21] as the specific ligand in the purification of riboflavin synthase by affinity chromatography. Inhibition of the yeast enzyme by the N-1, N-3 dimethyl derivatives (20p) and (20s) was completely unexpected, the more so since the latter compound had *no* hydroxyalkyl substituent at position 8. The very effective inhibition displayed by the 6,7-dioxolumazine (23a) provided a good basis for a study of N-8 substituent effects. The results show that a full ribityl side-chain is not required for significant inhibition. Compounds (23g) and (23i) which have a hydroxy-group at position 2 or 3 of a three-carbon side-chain are effective inhibitors of the yeast enzyme. This fact supports the suggestion made by Plaut and Beach [20] that the corresponding hydroxy-groups in the ribityl side-chain play an important role in the catalytic process.

In summary, there appears to be considerable flexibility available in the groups attached at positions 6 and 8 of potential lumazine and lumazine analogue inhibitors of riboflavin synthase. This opens the way for the synthesis of inhibitors bearing reactive functional groups thereby transforming competitive inhibitors, such as those described in this paper, into active-site directed irreversible inhibitors. There are also major differences between the enzyme isolated from yeast and that from *E. coli* as revealed by strong inhibition of one and weak inhibition of the other by a given compound, *e.g.* (23g). The possibility thus exists for the synthesis of other selective inhibitors of this enzyme which could have an important role in chemotherapy.

EXPERIMENTAL

U.v. spectra were determined with Unicam SP 8000 A or Perkin-Elmer 402 spectrophotometers for aqueous solutions of standard pH. ^{1}H N.m.r. spectra were recorded using Perkin-Elmer R12 B (60 MHz), or Perkin-Elmer R14 (100 MHz) spectrometers operating in the continuous wave mode and Bruker HFX 90 or JEOL PS100: PFT100 instruments in the Fourier transform mode (tetramethylsilane as standard). ^{13}C N.m.r. spectra were obtained on the JEOL instrument at 25.15 MHz using wide band decoupling of protons. Interpretation was aided by use of single frequency off-resonance decoupling. Resonances are reported as p.p.m. from tetramethylsilane; position numbers indicating downfield shifts (*i.e.* δ scale). For aqueous solutions the internal reference was dioxan whose resonance was assumed to be at $\delta = 67.4$.

All products were homogeneous when examined by paper chromatography (Whatman No. 1 paper) using the ascending technique with (A) butan-1-ol, glacial acetic acid, water (5 : 2 : 3), (B) propan-1-ol, ammonia, water

TABLE 3

Preparation of 6-substituted amino-5-nitropyrimidine-2,4(1*H*,3*H*)-diones

	Reaction conditions	Yield (%)	M.p. (°C)	Formula	Found (%) C	Found (%) H	Found (%) N	Requires (%) C	Requires (%) H	Requires (%) N
6-D-Ribitylamino-	0°, 24 h, 50% aq. MeOH	72	235—238 [a]	$C_9H_{14}N_4O_8$	35.4	4.4	18.0	35.3	4.6	18.3
6-(5-Hydroxypentylamino)-	Reflux, 30 min., EtOH	17	223—227 (decomp.)	$C_9H_{14}N_4O_5$	41.5	5.55	22.1	41.9	5.45	21.7
6-Ethylamino-	20°, 24 h, 50% aq. EtOH	57	310 [b] (decomp.)	$C_6H_8N_4O_4$	36.0	4.0	27.6	36.0	4.0	28.0
6-Carboxymethylamino-	20°, 30 min., 50% aq. EtOH	68	>320	$C_6H_6N_4O_6 \cdot H_2O$	28.6	3.2	22.8	29.0	3.25	22.6
6-(2-Aminoethylamino)-	20 °C, 30 min., H_2O	95	>320	$C_6H_9N_5O_4$	33.6	4.2	32.8	33.5	4.2	32.6
6-(2-Acetamidoethylamino)- [c]		70	289 (decomp.)	$C_8H_{11}N_5O_5$	37.5	4.4	26.7	37.4	4.3	27.2
6-(3-Hydroxypropylamino)-	20 °C, 24 h, 50% aq. EtOH	62	269—270	$C_7H_{10}N_4O_5$	36.6	4.3	24.3	36.5	4.3	24.3
6-(2-Hydroxypropylamino)-	20 °C, 24h, 50% aq. EtOH	80	240	$C_7H_{10}N_4O_5 \cdot H_2O$	34.2	5.1	22.7	33.9	4.8	22.6
6-(3-Amino-2-hydroxypropylamino)-	20 °C, 1 h, H_2O	70	300 (decomp.)	$C_7H_{11}N_5O_5$	34.4	4.7	28.5	34.3	4.5	28.6
6-(2-Hydroxy-3-*p*-toluenesulphonamidopropylamino)- [d]		82	236—238	$C_{14}H_{17}N_5O_7S$	42.1	4.2	17.8	42.1	4.3	17.6
6-(3-Formamido-2-hydroxypropylamino)- [e]		47	230—232 (decomp.)	$C_8H_{11}N_5O_6 \cdot 0.75 H_2O$	33.4	4.2	24.7	33.8	4.4	24.5
6-(5-Carboxypentylamino)-	20 °C, 4 h, 50% aq. EtOH	73	250—253	$C_{10}H_{14}N_4O_6$	42.1	5.0	19.7	42.0	4.9	19.6
3-Methyl-6-(2-hydroxyethylamino)-	Reflux, 30 min., EtOH	59	217	$C_7H_{10}N_4O_5$	36.3	4.4	24.1	36.6	4.4	24.4
3-Methyl-6-methylamino-	O °C, 1 h	75	312—314	$C_6H_8N_4O_4$	36.1	4.1	28.3	36.0	4.05	28.0
1-Methyl-6-methylamino-	0 °C, 1 h EtOH	79	263—265	$C_6H_8N_4O_4$	36.1	3.95	28.2	36.0	4.05	28.0
3-Methyl-6-D-ribitylamino-	Reflux, 30 min., EtOH	32	172—173	$C_{10}H_{16}N_4O_8$	37.3	5.0	17.2	37.5	5.0	17.5

[a] Lit.[24] 203—204 °C. [b] Lit. (J. C. Davis, H. H. Ballard, and J. W. Jones, *J. Heterocyclic Chem.*, 1970, **7**, 405), 299—301 °C. [c] Prepared from the corresponding amine by treatment (30 min) with acetic anhydride (3 mol) in aq. alkali at room temperature. [d] Prepared from the corresponding amine by treatment (1 h) with toluene-*p*-sulphonyl chloride (2 mol) in aq. alkali at room temperature. [e] Prepared from the corresponding amine by heating (100 °C, 5 min) with acetic anhydride and formic acid.

(6 : 3 : 1), and (C) 3% ammonium chloride as solvents, or by t.l.c. on silica-gel plates using (D) chloroform, methanol, 32% acetic acid (120 : 90 : 40), or (E) chloroform, methanol, ammonia (120 : 90 : 40) as solvents. Spots were detected with filtered u.v. light (λ 254 and 365 nm).

6-Substituted Amino-5-nitropyrimidine-2,4(1H,3H)-diones.—These compounds were prepared from the 6-chloro-5-nitropyrimidine-2,4(1*H*,3*H*)-dione by reaction with the appropriate primary amine as exemplified by the preparation of 6-(2-hydroxyethylamino)-5-nitropyrimidine-2,4(1*H*,3*H*)-dione given below. The intermediate amine salts were converted into the free pyrimidines by recrystallisation at pH 4. Details of reaction conditions, yields, analytical data *etc.* are given in Table 3. 6-Chloro-1,3-dimethyl-5-nitropyrimidine-2,4(1*H*,3*H*)-dione, m.p. 72—80 °C (lit.,[22] 75—81 °C), and 6-chloro-3-methyl-5-nitropyrimidine-2,4(1*H*,3*H*)-dione, m.p. 190 °C (lit.,[23] 195—197 °C) were prepared by published methods, and 6-chloro-1-methyl-5-nitropyrimidine-2,4(1*H*,3*H*)-dione, m.p. 121—123 °C, as described below.

6-Chloro-5-nitropyrimidine-2,4(1H,3H)-dione.—This compound was prepared by a modification of the literature method [24] which resulted in more reproducible results. 6-Chloropyrimidine-2,4(1*H*,3*H*)-dione (2.5 g) which had been twice recrystallised, was *dissolved* in concentrated sulphuric acid (3 ml) at 0 °C with constant stirring. Fuming nitric acid (2.7 ml) was added dropwise, the temperature being kept at 5—10 °C. The mixture was stirred for 30 min at room temperature when a yellow precipitate formed. Crushed ice (10 g) was added and the product collected immediately, washed with ice-cold water (5 ml) and ethanol–ether (1 : 2; 15 ml) and the solid (3.7 g, 60%) dried immediately over phosphorus pentaoxide *in vacuo*, m.p. 222—223 °C (lit.,[24] 220—222 °C).

6-(2-Hydroxyethylamino)-5-nitropyrimidine-2,4(1H,3H)-dione.—2-Aminoethanol (ethanolamine) (0.635 g, 0.01 mol) and 6-chloro-5-nitropyrimidine-2,4(1*H*,3*H*)-dione (1 g, 0.005 mol) in ethanol (50 ml) were refluxed for 30 min. Water was added to give a clear solution which was then allowed to cool. The *pyrimidine ethanolamine* salt (0.93 g, 66%) separated as yellow needles, m.p. 215—217 °C (Found: C, 34.8; H, 5.1; N, 25.0. $C_8H_{15}N_5O_6$ requires C, 34.7; H, 5.45; N, 25.3%). Recrystallisation from ethanol–water acidified to pH 4 with 2M-HCl gave the *pyrimidinedione* as colourless needles, m.p. 264—266 °C (decomp.). The literature value [24] of 217—219 °C is, in fact, the m.p. of the corresponding ethanolamine salt (Found: C, 33.7; H, 3.9; N, 25.9. $C_6H_8N_4O_5$ requires C, 33.3; H, 3.7; N, 25.9%), $\lambda_{max.}$ (ε) (pH 1) 322 nm (12 100). (pH 13) 337 nm (16 000).

6-Substituted Aminopyrimidine-2,4(1H,3H)-diones.—These compounds were prepared by published methods as follows: 6-(2-hydroxyethylamino)pyrimidine-2,4(1*H*,3*H*)-dione, m.p. 249—251 °C (lit.,[25] 249 °C), 6-(2-hydroxyethylamino)-1,3-dimethylpyrimidine-2,4-dione, m.p 182 °C (lit.,[26] 182 °C), 1,3-dimethyl-6-methylaminopyrimidine-2,4-dione, m.p. 240—244 °C (lit.,[26] 245 °C).

6-D-Ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione was prepared by a modification of the method of Maley and Plaut [27] as follows. Aqueous D-ribitylamine [28] (25 ml, 0.04 mol) and 6-chloropyrimidine-2,4(1*H*,3*H*)-dione [24] (2.95 g, 0.02 mol) were refluxed for 12 h, cooled, and poured into methanol (150 ml) with stirring. After being allowed to stand overnight at −20 °C, the precipitate was collected, dried, and recrystallised from acetic acid–ethanol (4 : 1) to give the ribityl-aminopyrimidine as colourless crystals (3.4 g, 60%), m.p. 196—198 °C (lit.,[27] 197 °C) (Found: C, 41.5; H, 5.8; N, 16.4. Calc. for $C_9H_{15}N_3O_6$ C, 41.4; H, 5.8; N, 16.1%), $\lambda_{max.}$ (ε) (pH 1) 267 nm (21 300); (pH 13) 267 nm (16 900).

6-(2-Hydroxyethylamino)-1,3-dimethyl-5-nitrosopyrimidine-2,4-dione.— 6-(2-Hydroxyethylamino)-1,3-dimethylpyrimidine-2,4-dione (3 g) was suspended in ethanol (21 ml) and isopentyl nitrite (9 ml) was added. The mixture was stirred at 50—60 °C; conc. hydrochloric acid (2 drops) was added, and the mixture stirred for a further 15 min. After 1 h at 0 °C, the solid was collected, washed with ethanol, and dried to give the *nitrosopyrimidine* (2.21 g, 65%) as purple prisms, m.p. 153—154 °C (decomp.), $\nu_{max.}$ 1 520 (NO), $\lambda_{max.}$ (pH 1) 322 and 256 nm (Found: C, 42.1; H, 5.4; N, 24.5. $C_8H_{12}N_4O_4$ requires C, 42.1; H, 5.3; N, 24.6%).

Prepared similarly were 1,3-dimethyl-6-methylamino-5-nitrosopyrimidine-2,4-dione, m.p. 150 °C (decomp.) (lit.,[26] 148—150 °C), and 6-amino-1,3-dimethyl-5-nitrosopyrimidine-2,4-dione, m.p. 266—267 °C (lit.,[29] 233 °C for monohydrate) (Found: C, 39.4; H, 4.5; N, 31.4. $C_6H_8N_4O_3$ requires C, 39.2; H, 4.4; N, 30.5%).

5-Phenylazo-6-D-ribitylaminopyrimidine-2,4(1H,3H)-dione.—This compound was prepared by a modification of the literature method [30] as follows. Benzenediazonium chloride,[31] from aniline (4 g) was added dropwise during 30 min to an ice-cold solution of 6-D-ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione (5.75 g) in water (30 ml). The temperature was kept below 10 °C and the pH >7 by the addition of solid sodium hydrogen carbonate. The mixture was stirred for a further 30 min and the red solid was collected. Recrystallisation from water gave the 5-phenylazopyrimidine (6—7.6 g, variable yield), m.p. 237—238 °C (lit.,[30] 232 °C), $\lambda_{max.}$ (pH 1) 404 and 233 nm; (pH 13) 403sh, 370, 260, and 245 nm.

7-Methyl-6-propyl-8-D-ribitylpteridine-2,4(3H,8H)-dione (6b).— 5-Nitro-6-D-ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione (1 g) in water (60 ml) was reduced over pre-reduced palladium on charcoal (10%; 300 mg) at room temperature and pressure. The catalyst was filtered off, the filtrate was run directly into a solution of sodium dithionite (50 mg) in water (1 ml), and the colourless solution was adjusted to pH 3 with hydrochloric acid and placed under nitrogen. A solution of hexane-2,3-dione (3.5 ml) in ethanol (60 ml) was added and the mixture was refluxed under nitrogen for 30 min. After cooling, charcoal was added and the solution was filtered. The filtrate was concentrated to *ca.* 5 ml *in vacuo* and hot ethanol (70 ml) was added. After refrigeration the *pteridine* was obtained as yellow needles (200 mg, 17%), m.p. 283 °C (decomp.) (lit.,[4] 267—268 °C) (Found: C, 50.8; H, 6.3; N, 15.7. $C_{15}H_{22}N_4O_6$ requires C, 50.8; H, 6,3; N, 15.8%), $\lambda_{max.}$ (ε) (pH 1) 257 (13 600), 275sh (9 500), and 405 nm (8 500); (pH 13) 282 (10 600) and 315 nm (7 500); δ(D_2O–NaOD) 0.95 (3 H, t), 1.73 (2 H, m), 2.43 (2 H, t), and 3.74 (m, ribityl).

The following compounds were prepared similarly.

6-Benzyl-7-methyl-8-D-ribitylpteridine-2,4(3H,8H)-dione (6c).—Nitropyrimidine and 4-phenylbutane-2,3-dione [32] gave yellow needles (12%), m.p. 236—238 °C (decomp.) (Found: C, 56.5; H, 5.6; N, 13.9. $C_{19}H_{22}N_4O_6$ requires C, 56.7; H, 5.5; N, 13.9%), $\lambda_{max.}$ (ε) (pH 1) 258 (18 800), 278sh (13 200), and 405 nm (12 100); (pH 13) 281 (16 500) and 316 nm (12 800); δ(D_2O–NaOD) 1.56 (2 H, s), 3.67 (m, ribityl), and 7.65 (m, ArH).

6,7-*Diphenyl*-8-D-*ribitylpteridine*-2,4(3H,8H)-*dione* (6*a*).—Nitropyrimidine and benzil gave yellow needles (44%), m.p. 220—223 °C (lit.,[4] 212—218 °C) (Found: C, 61.1; H, 5.1; N, 12.6. $C_{23}H_{22}N_4O_6$ requires C, 61.1; H, 4.9; N, 12.5%).

6,7,8,9-*Tetrahydroriboflavin* (7).—The following method is based on the report [11] of Hemmerich *et al.*

Riboflavin (1 g) in trifluoroacetic acid (80 ml) was hydrogenated over platinum oxide (250 mg) at room temperature and pressure for 7 days when the uptake of hydrogen was *ca.* 4 mol equiv. The colourless solution turned red instantly on admission of air. Filtration and evaporation to dryness (with addition of methanol) gave a brown gum. The crude hexahydroriboflavin was dissolved in acetic acid (50 ml) and oxygen was bubbled through the solution for 3 days at room temperature. Evaporation to dryness and recrystallisation from methanol gave 6,7,8,9-tetrahydroriboflavin (220 mg), m.p. 254—256 °C (decomp.) (lit.,[33] 264—266 °C), $\lambda_{max.}$ (pH 1) 370, 310, and 247 nm; (pH 13) 376, 318, 270sh, and 245 nm.

10-D-*Ribitylpyrimido*[5,4-g]*pteridine*-2,4,6,8(3H,7H,9H,-10H)-*tetraone* (18).—Prepared from 5-phenylazo-6-D-ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione by the method of Cresswell *et al.*[14]

Aliphatic Precursors: Ethyl 2-*Acetyl*-3-*phenylacrylate* (8).[34,35]—A solution of ethyl acetoacetate (195 g) and benzaldehyde (174.9 g) in dry benzene (300 ml) was treated with piperidine (6 ml) and glacial acetic acid (18 ml). The mixture was heated under reflux (Dean and Stark) for 1 h during which time the theoretical quantity of water (27 ml) was collected. After being heated for a further 1 h, the solution was cooled, the solvent and excess benzaldehyde were removed *in vacuo*, and the residue was distilled to give the acrylate (305 g, 93%) as a pale yellow liquid, b.p. 129 °C/0.7 Torr (lit.,[34] 125—126 °C/0.9 Torr) which crystallised with time to give a yellow solid, m.p. 60 °C (lit.,[35] 61 °C), δ($CDCl_3$), 7.2—7.7 (6 H, m), 4—4.2 (2 H, m), 2.41 (3 H, s), 2.34 (3 H, s), 1.46 (3 H, t), and 1.1 (3 H, t). The ^1H n.m.r. spectrum is consistent with a mixture of geometrical isomers and this was confirmed by t.l.c. ($CHCl_3$).

Ethyl 2-*Benzyl*-3-*oxobutanoate* (9).—The above acrylate (58.3 g) in ethyl acetate (300 ml) was hydrogenated over palladium on charcoal (10%, 3.5 g) for 30 min at 3 atm. Filtration (Kieselguhr) and evaporation of the filtrate gave an oil which was distilled to afford the *keto-ester* as a pale yellow oil (57.3 g, 97%) (Found: C, 70.9; H, 7.4. $C_{13}H_{16}O_3$ requires C, 70.9; H, 7.3%), δ($CDCl_3$) 7—7.4 (5 H, m), 4.22 (2 H, q, *J* 7 Hz), 3.76 (1 H, t, *J* 8 Hz), 3.15 (2 H, d, *J* 8 Hz), 2.17 (3 H, s), and 1.18 (3 H, t, *J* 7 Hz).

2-*Benzylbutane*-1,3-*diol* (10).—A suspension of lithium aluminium hydride (9.5 g) in dry ether (1l) was cooled to −5 °C and the above keto-ester (55 g) was added dropwise with stirring during 1 h, the temperature being maintained in the range 0—5 °C. After the mixture had been stirred at room temperature for 18 h, water (9.5 ml), sodium hydroxide solution (15%, 9.5 ml), and water (28.5 ml) were added successively. The mixture was filtered (Kieselguhr), dried (Na_2SO_4), and evaporated to yield a yellow oil which was distilled *in vacuo*. The *diol* was obtained as an oil (36.1 g, 80%), b.p. 128—130 °C/0.7 Torr (Found: C, 73.1; H, 9.05. $C_{11}H_{16}O_2$ requires C, 73.3; H, 8.95%), δ($CDCl_3$) 7—7.4 (5 H, m), 3.5—4.15 (3 H, m), 3.04 (2 H, s), 2.5—2.8 (2 H, m), 1.8—2.2br (1 H), and 1.15 (3 H, dd, *J* 7 Hz and 2 Hz).

Attempted oxidation of the diol (10) to give the keto-aldehyde (11) did not yield the required product. Reagents used included dicyclohexylcarbodi-imide in dimethyl sulphoxide,[36] *N*-chlorosuccinimide and dimethyl sulphide,[37] chromium trioxide on graphite (Seloxcette),[38] and chromium trioxide–dipyridine complex in glacial acetic acid [39] and in methylene chloride.[13]

Ethyl 2-*Benzyl*-3,3-*ethylenedioxybutanoate* (12).—A solution of the keto-ester (9) (50 g) in dry benzene (500 ml) containing ethane-1,2-diol (28.1 g) and toluene-*p*-sulphonic acid (1 g) was heated under reflux (Dean and Stark) for 22 h. The reaction mixture was evaporated to dryness and the residue dissolved in chloroform (500 ml). The chloroform solution was washed with water (50 ml), saturated aqueous sodium hydrogen carbonate (50 ml), and water (3 × 50 ml), and then dried (Na_2SO_4). Evaporation gave the required *acetal* (54.2 g, 90%) as a colourless liquid, b.p. 128 °C/0.5 Torr which was pure by g.l.c. on Apiezon at 170 °C (Found: C, 67.8; H, 7.75. $C_{15}H_{20}O_4$ requires C, 68.2; H, 7.6%), δ(CCl_4) 6.8—7 (5 H, m), 3.69—3.98 (6 H, m), 2.58—2.9 (3 H, m), 1.38 (3 H, s), and 1.0 (3 H, t, *J* 7 Hz).

2-*Benzyl*-3,3-*ethylenedioxybutan*-1-*ol* (13).—The above acetal (12) (64 g) was added dropwise with stirring over 1 h to a suspension of lithium aluminium hydride (9.25 g) in dry ether (1 l) at 0 °C). After being stirred for 60 h at room temperature, water (9.3 ml) followed by 15% aqueous sodium hydroxide (9.3 ml) and water (27.9 ml) were added slowly and the mixture was stirred for a further 1 h. The mixture was filtered (Kieselguhr), dried (Na_2SO_4), and evaporated *in vacuo* to give the *alcohol* (49 g, 90%), b.p. 140 °C/1.4 Torr, m.p. 35 °C, which was pure by g.l.c. on Apiezon at 170 °C (Found: C, 70.3; H, 8.05. $C_{13}H_{18}O_3$ requires C, 70.3; H, 8.15%), δ($CDCl_3$) 7.02 (5 H, s), 3.87 (4 H, s), 3.5br (2 H, s), 2.84 (2 H, dd, *J* 10 Hz and 3 Hz), 2.35 (1 H, t, *J* 10 Hz), 1.8—2.15 (1 H, m), and 1.35 (3 H, s).

2-*Benzyl*-3,3-*ethylenedioxybutanal* (14).—Dry chromium trioxide (40 g) was added in portions with mechanical stirring to a solution of dry pyridine (62.7 g) in dichloromethane (750 ml) at 0 °C so that the solution temperature remained less than 20 °C. After being stirred for 1 h at 20 °C the deep red colour of the mixture indicated formation of the dipyridine–chromium trioxide complex. A solution of the alcohol (13) (11 g) in dry dichloromethane (10 ml) was added dropwise during 10 min with continued stirring, again with the temperature being kept below 20 °C. After a further 1.5 h, the reaction mixture was poured into ether (500 ml) and the solution washed successively with 5% aqueous sodium hydroxide (4 × 250 ml), 5% aqueous sodium hydrogen carbonate (250 ml), and water (2 × 250 ml). The solution was then dried (Na_2SO_4) and evaporated *in vacuo* to give a pale yellow oil which was distilled to give the *aldehyde* (9 g, 82%), b.p. 134 °C/1.5 Torr (Found: C, 70.7; H, 7.35. $C_{13}H_{16}O_3$ requires C, 70.9; H, 7.3%), δ($CDCl_3$), 9.45 (1 H, d, *J* 2 Hz), 7 (5 H, m), 3.86 (4 H, s), 2.6—3.1 (3 H, m), and 1.29 (3 H, s).

Pyrido[2,3-d]*pyrimidines*: 6-*Benzyl*-7-*methyl*-8-D-*ribityl*-*pyrido*[2,3-d]*pyrimidine*-2,4(3H,8H)-*dione* (16b).—A solution of 6-D-ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione (2.6 g) in water (10 ml), concentrated hydrochloric acid (0.5 ml), and ethanol (5 ml) was heated to 100 °C and a solution of the above aldehyde (14) (2.4 g) in ethanol (10 ml) was added dropwise with stirring during 3 h. After being heated for a further 1 h, the solution was chilled and the solid which formed was collected. Recrystallisation from aqueous ethanol (50%) afforded the *pyrido*[2,3-d]*pyrimidine* (1.7 g, 42%) as cream coloured prisms, m.p. 144 °C (resolidi-

fies and remelts at 241 °C) (Found: C, 57.7; H, 6.0; N, 10.6. $C_{20}H_{25}N_3O_7 \cdot H_2O$ requires C, 57.3; H, 6.0; N, 10.0%), $\lambda_{max.}$ (pH 1) 365sh, 332, 278sh, and 255sh nm; (pH 13) 373 and 259 nm; δ[$(CD_3)_2SO$] 8.04 (1 H, s), 6.9—7.20 (5 H, m), 4.05—5 (13 H, m), 3.97 (2 H, s), and 2.60 (3 H, s); δ(CF_3CO_2H), 8.63 (1 H, s), 6.75—7.4 (5 H, m), 3.8—5.2 (15 H, m), and 2.96 (3 H, s). The ^{13}C n.m.r. spectrum is recorded in Table 1.

6-*Benzyl*-8-(2-*hydroxyethyl*)-7-*methylpyrido*[2,3-d]*pyrimidine*-2,4(3H,8H)-*dione* (16a).—This compound was prepared as above from 8-(2-hydroxyethylamino)pyrimidine-2,4(1*H*,3*H*)-dione in 30% yield, m.p. 289 °C (Found: C, 65.4; H, 5.6; N, 13.5. $C_{17}H_{17}N_3O_3$ requires C, 65.6; H, 5.5; N, 13.5%), $\lambda_{max.}$ (pH 1) 365sh, 331, 278, and 259sh nm; (pH 13) 377, 277sh, and 270 nm, δ[$(CD_3)_2SO$] 8.0 (1 H, s), 6.9—7.3 (5 H, m), 4.3—4.6 (2 H, m), 3.98 (2 H, s), 3.55—3.85 (2 H, m), and 2.6 (3 H, s); δ(CF_3CO_2H) 8.64 (1 H, s), 6.8—7.4 (5 H, m), 4.8—5 (2 H, m), 4.3—4.5 (2 H, m), 4.2 (2 H, s), and 2.9 (3 H, s). The ^{13}C n.m.r. spectrum is recorded in Table 1.

6-*Nitro*-8-D-*ribitylpyrido*[2,3-d]*pyrimidine*-2,4(3H,8H)-*dione* (16i).—6-D-Ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione (522 mg) was suspended in water (10 ml) and sodium nitromalondialdehyde monohydrate [40] (450 mg) was added with stirring. Concentrated hydrochloric acid was added to pH 2 and the mixture was heated on a steam-bath for 5 min. The mixture was cooled and the precipitate collected and recrystallised from water to give the *pyridopyrimidine hemihydrate* (175 mg, 25%) as light yellow crystals, m.p. 240 °C (decomp.) (Found: C, 41.1; H, 4.1; N, 15.8. $C_{12}H_{14}N_4O_8 \cdot 0.5\ H_2O$ requires C, 41.0; H, 4.25; N, 15.9%), $\lambda_{max.}$ (ε) (pH 1) 350 nm (14 400); (pH 13) 246sh (8 100), 265sh (5 900), and 463 nm (25 200).

8-(2-*Hydroxyethyl*)-6-*nitropyrido*[2,3-d]*pyrimidine*-2,4-4(3H,8H)-*dione* (16 h).—This compound was prepared as for the ribityl analogue above with 15 min heating to give yellow needles (32%), m.p. 295 °C (decomp.) (Found: C, 42.8; H, 3.4; N, 22.1. $C_9H_8N_4O_5$ requires C, 42.8; H, 3.2; N, 22.2%), $\lambda_{max.}$ (ε) (pH 1) 354 nm (14 200); (pH 13) 265sh (7 300) and 463 nm (24 600); δ[$(CD_3)_2SO$] 9.45 (1 H, d, *J* 3 Hz), 8.71 (1 H, d, *J* 3 Hz), 5.0 (1 H, t, *J* 6 Hz), 4.5 (2 H, t, *J* 6 Hz), and 3.77 (2 H, m).

10-D-*Ribitylpyrido*[2,3-d:6,5-d′]*dipyrimidine*-2,4,6,8-(3H,7H,9H,10H)-*tetraone* (17).—6-D-Ribitylaminopyrimidine-2,4(1*H*,3*H*)-dione (1.05 g) and triethyl orthoformate (redistilled; 6 ml) were refluxed in ethanol (30 ml) for 4 h. After the mixture had been chilled for several hours, the yellow powder was collected and recrystallised from dilute hydrochloric acid (pH ~ 2) and then from water to give the *pyridodipyrimidine* (160 mg, 21%) as needles, m.p. >340 °C (Found: C, 44.4; H, 4.3; N, 18.8. $C_{14}H_{15}N_5O_8$ requires C, 44.1; H, 4.0; N, 18.4%), $\lambda_{max.}$ (ε) (pH 1) 262 (6 000), 298 (7 600), and 372 nm (15 700); (pH 13) 278 (10 100) and 393 nm (27 300).

6-*Substituted Pteridine*-2,4,7(1H,3H,8H)-*triones* (20).—These compounds were prepared by condensation of the appropriate α-keto-acid or α-keto-ester with the required 5-amino-6-substituted aminopyrimidine-2,4(1H,3H)-diones. The latter were generated *in situ* by reduction of the corresponding 5-phenylazo-, 5-nitroso-, or 5-nitro-pyrimidines

TABLE 4

Preparation of 6-Substituted pteridine-2,4,7(1*H*,3*H*,8*H*)-triones (20)

Compound	Method	α-Keto-acid or α-keto-ester	Yield (%)	M.p. (°C)	Formula	Found (%) C	Found (%) H	Found (%) N	Requires (%) C	Requires (%) H	Requires (%) N
6-Ethyl-8-D-ribityl (20b)	(a)	2-Oxobutanoic	13	158—160	$C_{13}H_{18}N_4O_7$	45.6	5.3	16.2	45.6	5.3	16.4
6-Benzyl-8-D-ribityl (20c)	(a)	Phenylpyruvic acid	73	258—259	$C_{18}H_{20}N_4O_7$	53.9	5.2	13.8	53.6	5.0	13.9
6-Carboxyethyl-8-D-ribityl (20d)	(a)	2-Oxoglutaric acid	50	155—158	$C_{14}H_{18}N_4O_9$	43.8	4.65	14.3	43.5	4.6	14.5
6-(2-Nitrobenzyl)-8-D-ribityl (20f)	(b)	Ethyl(2-nitrophenyl) pyruvate [d]	24	207—208 (decomp.)	$C_{18}H_{19}N_5O_9$	47.8	4.4	15.3	48.1	4.2	15.6
6-(4-Nitrobenzyl)-8-D-ribityl (20g)	(b)	Ethyl(4-nitrophenyl) pyruvate [e]	17	173—175	$C_{18}H_{19}N_5O_9 \cdot 1H_2O$	46.1	4.1	15.0	46.2	4.5	15.1
6-(2,4-Dichlorophenyl)-8-D-ribityl (20h)	(b)	2,4-Dichlorophenyl glyoxylic acid [a]	50	218—219 (decomp.)	$C_{17}H_{16}Cl_2N_4O_7 \cdot 1.5H_2O$	41.7	3.6	11.1 [b]	42.0	3.9	11.5
6-(4-Hydroxyphenyl)-8-D-ribityl (20i)	(b)	4-Hydroxyphenyl-glyoxylic acid	20	250—252 (decomp.)	$C_{17}H_{18}N_4O_8 \cdot 2H_2O$	46.4	4.45	12.9	46.6	4.1	12.8
6-Phenyl-8-D-ribityl (20j)	(b)	Phenylglyoxylic acid	46	265—267	$C_{17}H_{18}N_4O_7 \cdot 0.66H_2O$	50.7	4.7	13.6 [c]	50.7	4.8	13.9
8-(2-Hydroxyethyl)-6-methyl (20h) [f]	(c)	Pyruvic acid	85	>350	$C_9H_{10}N_4O_4$	45.2	4.5	23.8	45.4	4.2	23.5
8-(2-Hydroxyethyl)-6-(2-nitrobenzyl) (20m)	(c)	Ethyl(2-nitrophenyl) pyruvate [d]	10	256 (decomp.)	$C_{15}H_{13}N_5O_6 \cdot 1.5H_2O$	46.9	4.4	18.1	46.6	4.2	18.1
8-(5-Hydroxypentyl)-6-methyl (20n)	(c)	Ethyl pyruvate	75	250—280 (slow decomp.)	$C_{12}H_{16}N_4O_4 \cdot 0.5H_2O$	50 0	5 6	19 3	49.9	5.9	19.4
8-(2-Hydroxyethyl)-3,6-dimethyl (20o)	(c)	Ethyl pyruvate	54	251—252 (decomp.)	$C_{10}H_{12}N_4O_4$	47.4	4.8	22.0	47.6	4.8	22.2
6-Carboxyethyl-8-hydroxyethyl-1,3-dimethyl (20p)	(b)	2-Oxoglutaric acid	24	195—196	$C_{13}H_{16}N_4O_6$	48.1	5.1	17.1	48.2	5.0	17.3
6-Carboxyethyl-3,8-dimethyl (20r)	(b)	2-Oxoglutaric acid	71	299—304 (decomp.)	$C_{11}H_{12}N_4O_5$	47.4	4.45	20.4	47.2	4.3	20.0
6-Carboxyethyl-1,3-dimethyl (20s)	(b)	2-Oxoglutaric acid	71	247—248	$C_{11}H_{12}N_4O_5$	47.0	4.4	20.1	47.2	4.3	20.0

[a] This compound was a gift from Mr. D. Sawyer of the Wellcome Research Laboratories. [b] Loss at 100 °C = 5.99%. Required for 1.5 H_2O 5.55%. [c] Loss at 100 °C = 3.02%. Required for 0.66 H_2O 2.98%. [d] W. B. Wright and K. H. Collins, *J. Amer. Chem. Soc.*, 1956, **78**, 221. [e] P. Cogniant, *Ann. Chim. (France)*, 1952, **7**, 442. [f] R. D. Sprenger, P. M. Ruoff, and A. H. Frazer, *J. Amer. Chem. Soc.*, 1950, **72**, 2874.

using zinc in dilute sulphuric acid [30] for the 5-phenylazo-derivatives, hydrogenation over palladium on charcoal for the 5-nitroso-[26] and 5-nitro-6-D-ribitylamino-compounds, and aqueous sodium dithionite for the remaining 5-nitro-derivatives. Typical experimental details are given below for each method and the precise reaction conditions, yields, and analytical data for individual pteridinetriones are given in Table 4.

(a) 6-*Methyl*-8-D-*ribitylpteridine*-2,4,7(1H,3H,8H)-*trione* (20a). 5-Phenylazo-6-D-ribitylaminopyrimidine-2,4(1H,-3H)-dione (1 g) and zinc powder (0.5 g) in water (15 ml) were heated to boiling when 5N-sulphuric acid (*ca.* 3 ml) was added dropwise until the solution became colourless. The hot solution was filtered and the filtrate acidified to pH 1 with 5N-sulphuric acid. Ethyl pyruvate (1 ml) was added and the mixture heated to 100 °C for 1 h in the dark under nitrogen. After 1 h the hot solution was taken to pH 7 with 10N-potassium hydroxide and allowed to cool. The solid which separated was collected, redissolved in the minimum of hot 0.1N-hydrochloric acid, filtered hot, and ethanol added to produce a slight turbidity. On cooling, the pteridinetrione (0.53 g, 59%) separated and was recrystallised from water to give crystals, m.p. 252—253 °C (lit.,[30] 250 °C) (Found: C, 44.0; H, 4.5; N, 17.0. $C_{12}H_{16}N_4O_7$ requires C, 43.9; H, 4.85; N, 17.0%), $\lambda_{max.}$ (pH 1) 328 and 282 nm; (pH 13) 349 and 285 nm.

(b) 6-*Carboxyethyl*-1,3,8-*trimethylpteridine*-2,4,7(1H,3H,-8H)-*trione* (20q). 1,3-Dimethyl-6-methylamino-5-nitrosopyrimidine-2,4(1*H*,3*H*)-dione (2 g) in methanol (100 ml) was hydrogenated over palladium on charcoal (10%, 0.2 g) for 45 min at 3 atm. The solution was filtered (Kieselguhr) and evaporated under reduced pressure to give the 5-aminopyrimidine (1.8 g, 97%) as cream prisms, m.p. 141 °C (lit.,[26] 143 °C). This was dissolved in water (15 ml), 2-oxoglutaric acid (1.53 g) was added, and the solution was acidified to pH 2 with concentrated hydrochloric acid. The mixture was heated to 100 °C for 4 h, filtered hot, and cooled. The solid which separated was collected and recrystallised from glacial acetic acid to give the *pteridinetrione* (1.35 g, 46%), m.p. 249—253 °C (Found: C, 49.0; H, 4.8; N, 19.3. $C_{12}H_{14}N_4O_5$ requires C, 49.0; H, 4.8; N, 19.1%), $\lambda_{max.}$ (pH 1) 333 and 291 nm; (pH 13) 294 nm; δ(CF_3CO_2H) 3.77 (3 H, s), 3.7 (3 H, s), 3.48 (3 H, s), 3.18—3.38 (2 H, m), and 2.98—3.18 (2 H, m).

(c) 6-*Benzyl*-8-(2-*hydroxyethyl*)*pteridine*-2,4,7(1H,3H,-8H)-*trione* (20l).—6-(2-Hydroxyethylamino)-5-nitropyrimidine-2,4(1H,3H)-dione (0.5 g) in water (10 ml) was heated to 100 °C and solid sodium dithionite was added until the colour changed from pale yellow to colourless. Ethyl phenylpyruvate [41] (0.5 g) in methanol (5 ml) was added and the mixture was heated at 100 °C for a further 10 min. On cooling a colourless solid separated and this was collected and recrystallised from water to give the 6-*benzylpteridinetrione* (0.48 g, 66%), m.p. 236—260 °C (decomp.) (Found: C, 57.4; H, 4.7; N, 18.2. $C_{15}H_{14}N_4O_4$ requires C, 57.3; H, 4.5; N, 17.9%), $\lambda_{max.}$ (ε) (pH 1) 332 (14 300) and 283 nm (10 800); (pH 13) 353 (15 700) and 290 nm (9 800); δ[$(CD_3)_2SO$] 7.22 (5 H, s), 4.16 (2 H, t, *J* 7 Hz), 3.88 (2 H, s), and 3.5 (2 H, t, *J* 7 Hz).

6-*Carboxy*-8-D-*ribitylpteridine*-2,4,7(1H,3H,8H)-*trione* (20e).— 10-D-Ribitylpyrimido[5,4-*g*]pteridine-2,4,6,8(3*H*,-7*H*,9*H*,10*H*)-tetraone (18) (0.5 g) and 0.1M-sodium hydroxide (5 ml) in water (10 ml) were heated at 100 °C for 10 h. 0.1M-Sodium hydroxide (3 ml) was added to pH 10 and heating was continued for a further 7 h. Acidification to pH 3—4 with acetic acid and concentration of the solution to *ca.* 10 ml gave a yellow solid on cooling. Filtration and fractional recrystallisation to remove traces of the less-soluble starting material gave the 6-*carboxypteridine* as its sodium salt (0.3 g, 60%), m.p. 197 °C (decomp.) (Found: C, 37.4; H, 3.6; N, 15.0: $C_{12}H_{13}N_4NaO_9$ requires C, 37.8; H, 3.45; N, 14.7%), $\lambda_{max.}$ (pH 1) 362 and 277 nm; (pH 13) 376 and 263 nm.

5-*Methoxycarbonyl*-8-(2-*hydroxyethyl*)*pyrido*[2,3-d]*pyrimidine*-2,4,7(1H,3H,8H)-*trione* (21b).—6-(2-Hydroxyethylamino)pyrimidine-2,4(1*H*,3*H*)-dione (900 mg) and dimethyl acetylenedicarboxylate (900 mg) were heated to reflux in water (30 ml) for 4 h. The resulting suspension was filtered hot to give the *pyridopyrimidine* (0.24 g, 16%), m.p. 243—245 °C) (Found: C, 46.7; H, 3.75; N, 14.9. $C_{11}H_{11}N_3O_6$ requires C, 47.0; H, 3.9; N, 14.9%), $\lambda_{max.}$ (pH 1) 316 and 282 nm; (pH 13) 342, 286, and 255 nm; δ[$(CD_3)_2SO$] 6.15 (1 H, s), 4.32 (2 H, t), 3.80 (3 H, s), and 3.66 (2 H, t).

The following were prepared similarly.

5-*Methoxycarbonyl*-8-D-*ribitylpyrido*[2,3-d]*pyrimidine*-2,4,7(1H,3H,8H)-*trione* (21c) in 70% yield, m.p. 199—200 °C (Found: C, 44.4; H, 4.75; N, 11.0. $C_{14}H_{17}N_3O_9 \cdot 0.5H_2O$ requires C, 44.2; H, 4.7; N, 11.1%), $\lambda_{max.}$ (pH 1) 317 and 281 nm; (pH 13) 344, 285, and 256 nm; δ[$(CD_3)_2SO$] 6.13 (1 H, s), 3.78 (3 H, s), and 3.55—4.38 (m); and 5-*methoxycarbonylpyrido*[2,3-d]*pyrimidine*-2,4,7(1H,3H,8H)-*trione* (21a) in 38% yield, m.p. >300 °C (lit.,[17] 52%, m.p. 320 °C).

8-*Substituted Pteridine*-2,4,6,7(1H,3H,5H,8H)-*tetraones* (23).—These compounds were prepared by condensation of the required 5-amino-6-substituted aminopyrimidine-2,4(1*H*,3*H*)-dione with either diethyl oxalate or ethoxalyl chloride.[42] The 5-aminopyrimidines were prepared by reduction, *in situ*, of the corresponding 5-nitro-derivatives (see above). Typical experimental details are given below for each method and detailed reaction conditions, yields, and analytical data for individual pteridine tetraones are given in Table 5.

(a) 8-(2-*Hydroxyethyl*)*pteridine*-2,4,6,7(1H,3H,5H,8H)-*tetraone* (23b). 6-(2-Hydroxyethylamino)-5-nitropyrimidine-2,4(1H,3H)-dione (875 mg) in 0.1 M-sodium hydroxide (40 ml) was heated to 100 °C and solid sodium dithionite was added until the colour changed from yellow to colourless. After cooling, triethylamine (1.62 g) was added followed by ethoxalyl chloride (1.65 g) added dropwise with vigorous stirring. After the chloride had dissolved, ethanol (40 ml) was added and the mixture was refluxed for 2 h and chilled overnight. The precipitate was collected, dissolved in boiling water and the solution acidified with dilute hydrochloric acid. Refrigeration gave the *pteridine* as colourless needles (600 mg, 62%) which were recrystallised from water, m.p. >320 °C (Found: C, 39.6; H, 3.5; N, 23.2. $C_8H_8N_4O_5$ requires C, 40.0; H, 3.3; N, 23.3%), $\lambda_{max.}$ (ε) (pH 1) 294 (8 800) and 325sh nm (6 900); (pH 13) 309 (10 900) and 349 nm (8 800).

(b) 8-*Ethylpteridine*-2,4,6,7(1H,3H,5H,8H)-*tetraone* (23d). 6-Ethylamino-5-nitropyrimidine-2,4(1*H*,3*H*)-dione (0.5 g) was reduced to the 5-amino-analogue as above. Ethanol (5 ml) and diethyl oxalate (1.8 ml) were added to the cooled solution which was then refluxed for 1 h. After refrigeration overnight the pale yellow precipitate was collected and recrystallised twice from water to give the *pteridinetetraone* (0.1 g, 22%) as light yellow needles, m.p. >335 °C (Found: C, 42.0; H, 3.6; N, 24.3. $C_8H_8N_4O_4 \cdot 0.25H_2O$ requires C, 42.0; H, 3.7; N, 24.5%), $\lambda_{max.}$ (ε)

TABLE 5

Preparation of 8-substituted pteridine-2,4,6,7(1*H*,3*H*,5*H*,8*H*)-tetraones (23)

Compound	Method	Yield (%)	M.p. (°C)	Formula	Found (%) C	Found (%) H	Found (%) N	Requires (%) C	Requires (%) H	Requires (%) N
8-D-Ribityl (23a)	(b)	7	287—288 [a]	$C_{11}H_{14}N_4O_8 \cdot 0.25H_2O$	40.0	4.3	16.6	39.5	4.3	16.8
8-(2-Chloroacetoxyethyl) (23c) [b]		90	>325	$C_{10}H_9ClN_4O_6$	38.3	3.1	17.4	37.9	2.8	17.7
8-Carboxymethyl (23e)	(a)	32	>300	$C_6H_6N_4O_6$	38.1	2.4	22.3	37.8	2.4	22.1
8-(2-Acetamidoethyl) (23f)	(a)	40	>340	$C_{10}H_{11}N_5O_5$	42.2	4.3	24.8	42.7	3.9	24.9
8-(3-Hydroxypropyl) (23g)	(a)	72	>300	$C_9H_{10}N_4O_5$	42.2	4.1	22.2	42.5	4.0	22.0
8-(3-Chloroacetoxypropyl) (23h) [b]		85	>300	$C_{11}H_{11}ClN_4O_6$	39.7	3.5	17.0	39.9	3.3	16.9
8-(2-Hydroxypropyl) (23i)	(a)	60	>300	$C_9H_{10}N_4O_5 \cdot H_2O$	39.7	4.4	20.6	40.2	4.3	20.6
8-(2-Hydroxy-3-*p*-tolyl-sulphonamidopropyl) (23j)	(a)	58	>360	$C_{16}H_{17}N_5O_7S$	45.5	4.2	16.8	45.4	4.0	16.6
8-(3-Formamido-2-hydroxy-propyl) (23k)	(a)	51	>325	$C_{10}H_{11}N_5O_6 \cdot H_2O$	38.1	4.5	22.9	38.1	4.2	22.2
8-(3-Amino-2-hydroxypropyl). Hydrochloride (23l) [c]		75	304 (decomp.)	$C_9H_{12}ClN_5O_5 \cdot 0.5H_2O$	34.7	4.85	22.8	34.4	4.1	22.3
8-(5-Hydroxypentyl) (23m)	(b)	42	320 (decomp.)	$C_{11}H_{14}N_4O_5 \cdot 0.5H_2O$	45.7	4.85	19.5	45.4	5.15	19.3
8-(5-Carboxypentyl) (23n)	(a)	57	>330	$C_{12}H_{14}N_4O_6$	46.8	4.6	17.8	46.5	4.55	18.1
8-(2-Hydroxyethyl)-3-methyl (23o)	(b)	24	>320	$C_9H_{10}N_4O_5$	42.4	4.0	21.5	42.5	4.0	22.0
3-Methyl-8-D-ribityl (23p)	(b)	53	259—260 (decomp.)	$C_{12}H_{16}N_4O_8$	40.6	5.0	15.5	40.8	4.8	15.8

[a] Lit.,[5] m.p. 284—285 °C. [b] Prepared from the corresponding hydroxyalkyl pteridines (23b) and (23g) by heating (2 h) with chloroacetic acid at 160 °C. [c] Prepared by hydrolysis (0.5M-HCl; 5 min at 100 °C) of the corresponding formamidopteridine (23k).

(pH 1) 293 (8 900) and 327 nm (7 500); (pH 13) 308 (12 700) and 345 nm (11 000).

1,3-*Bis*(1,2,3,4,7,8-*hexahydro*-2,4,7-*trioxo*-8-D-*ribityl*-*pteridin*-6-*yl*)*propane* (24a).—5-Nitro-6-D-ribitylamino-pyrimidine-2,4(1*H*,3*H*)-dione (2 g) in water (50 ml) was hydrogenated over palladium on charcoal (10%, 100 mg) for 18 h. The reaction mixture was filtered, 2,6-dioxo-heptane-1,7-dicarboxylic acid [43] (0.62 g) in water (3 ml) was added, and the mixture was heated at 100 °C for 9 h. After cooling overnight, the *bis-pteridine-trione* (0.84 g, 38%) was collected as a pale yellow, slightly hygroscopic solid, m.p. 197 °C (decomp.) (Found: C, 43.7; H, 5.0; N, 16.4. $C_{25}H_{32}N_8O_4 \cdot H_2O$ requires C, 43.7; H, 5.0; N, 16.3%), $\lambda_{max.}$ (pH 1) 333 and 282 nm; (pH 13) 357, 291, and 262 nm; δ[$(CD_3)_2SO$] 3.5—4.8 (m), 2.75 (4 H, t), and 2.2 (2 H, m).

The following was prepared similarly.

1,3-*Bis*[1,2,3,4,7,8-*hexahydro*-8-(2-*hydroxyethyl*)-2,4,7-*trioxo*]*propane* (24b) in 25% yield, m.p. 235 °C (slow decomp.) (Found: C, 44.1; H, 4.6; N, 21.8. $C_{19}H_{20}N_8O_8 \cdot 1.5H_2O$ requires C, 44.3; H, 4.5; N, 21.7%), $\lambda_{max.}$ (pH 1) 333 and 280 nm; (pH 13) 356 and 289 nm.

Enzyme Inhibition Studies.—(a) *Yeast enzyme.* Riboflavin synthase was isolated from dried baker's yeast (Distillers Co. Ltd.) by the method described in our earlier work.[1] Kinetic data were obtained using the dialysate obtained after the second ammonium sulphate precipitation. Inhibition constants (K_i) were determined using this enzyme solution (0.5 ml) in the assay method described previously [1] (Assay 1) or using a modified method with omission of the sodium hydrogen sulphite solution and its replacement by an equal volume of phosphate buffer (Assay 2). K_i Values were determined by the graphical method of Dixon.[44]

(b) *Bacterial enzyme.* Frozen paste of *E. coli* B (Grain Processing Corp., Muscatine, Iowa, USA) was thawed in 2—2.5 volumes of 0.05M-phosphate buffer, pH 7.0, plus 2.5—5 mM-dithiothreitol (DTT). The cells were broken by two passages through a French Pressure Cell at 18 000 lb in^{-2} and the cellular debris removed by centrifugation at 49 000 × g for 1 h. Protamine sulphate was added dropwise (0.2 volume of a 2% solution) while the supernatant liquid was stirred at 0—5 °C, and the precipitate removed by centrifugation (49 000 × g, 30 min). Solid $(NH_4)_2SO_4$ was added gradually to this supernatant liquid to 50% saturation and the pellets obtained after centrifugation were dissolved in minimal volumes of the phosphate–DTT buffer and dialysed overnight against several hundred volumes of 10 mM phosphate buffer, pH 7.0. In one preparation, the enzyme had to be treated further, by heating at 37 °C for 15 min and removal of precipitated proteins by centrifugation, in order to prevent protein precipitation in the assay cuvettes.

The assay mixtures contained 50 mM Sorensens phosphate buffer, pH 7.0, 90 μM 6,7-dimethyl-8-D-ribityl-lumazine, ± compound, sufficient enzyme to give a $\Delta OD_{470} \sim 0.01$/min and H_2O to 1 ml. All components less enzyme were incubated for 5 min at 37 °C before the reaction was started by the addition of enzyme.

We thank the S.R.C. and Burroughs Wellcome Co. U.S.A. for Research Grants, and the Distillers Company Limited for generous gifts of dried baker's yeast. Saieba S. Al-Hassan was on leave of absence from the Science College, University of Baghdad.

[0/035 *Received*, 9*th January*, 1980]

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