Specific Enzyme Inhibitors in Vitamin Biosynthesis. Part 6.† Identification of an Affinity Chromatography Ligand for the Purification of Riboflavin Synthase

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The carbodi-imide-mediated condensation of the 7-oxolumazine (**1**)† with amino-functionalised Sepharose at pH 4—5 gave a polymeric material which was effective in the purification of riboflavin synthase by affinity chromatography. The u.v. chromophore of this polymer-supported material was different from that of the 7-oxolumazine. Investigation of an analogous model reaction in free solution has led to a tentative identification of this affinity ligand. An explanation for the ability of this compound to bind to the enzyme is presented which suggests that it may be a 'reaction co-ordinate' analogue.

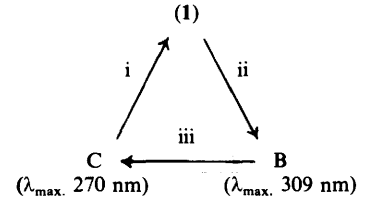
We have described the purification of riboflavin synthase (E.C. 2.5.1.9) using affinity chromatography[1,2] in which a specific competitive inhibitor (**1**) of the enzyme was developed for use as an affinity ligand. The 6-(2-carboxyethyl) side-chain of this molecule was used for coupling to an amino-functionalised Sepharose[3] by a water-soluble carbodi-imide [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC. HCl)] mediated condensation reaction. When this process was carried out at pH 6.7, the resulting polymer-supported product exhibited bright blue fluorescence, characteristic of 7-oxolumazines, and showed a u.v. spectrum [$\lambda_{max.}$(pH 1), 329, 281 nm] superimposable on that of the free ligand (**1**). This material was used successfully to purify riboflavin synthase from baker's yeast. In contrast, coupling of compound (**1**) to the amino-Sepharose at pH 4.5—4.6 gave an immobilised product which showed a very different u.v. spectrum [$\lambda_{max.}$(pH 1) 270 nm] from that of the 7-oxolumazine. Surprisingly, this material was also very effective as an affinity chromatography support for the purification of the enzyme. Our attempts to identify the product of coupling at pH 4.5—4.6 and to rationalise the inhibition characteristics of the resulting structure form the subject of this paper.

Results and Discussion

It was quickly established that the transformation of the lumazine (**1**) into a compound with the new chromophore did not require the presence of the amino-functionalised Sepharose. An analogous reaction occurred under the same conditions in the absence of Sepharose and this was conveniently monitored by u.v. spectroscopy. Clear and sharp isosbestic points were observed for the change of compound (**1**) to an intermediate B ($\lambda_{max.}$ 309 nm) (Figure *a*) and for a subsequent slow change of B to the product C ($\lambda_{max.}$ 270 nm) (Figure *b*). At pH 11 the change from B to C was rapid. Acidification of C to pH 1 slowly regenerated the 7-oxolumazine chromophore of (**1**), thus showing that the process is cyclic (See Scheme 1).

Consideration of this information led to two mechanistic hypotheses (Schemes 2 and 3) which seemed compatible with the known chemistry of the 7-oxolumazines and with the u.v. data.

In an attempt to distinguish between these possibilities, a systematic study of the structural requirements for the conversion was carried out by varying one of R^1, R^2, R^3, R^4, and X of the 7-oxolumazines (**2**) in turn, while maintaining the others constant, and checking each compound for its ability to undergo transformation to the product absorbing at 270 nm under the standard coupling conditions at pH 4.5—4.6.



Scheme 1. i, pH 1, slow; ii, EDC.HCl, pH 4.5; iii, pH 11 (fast) or pH 4.5 (slow)

Variations of R^4 (**1**), (**2a**), and (**2b**) gave products which underwent the transformation shown in Scheme 1, whereas variation of R^3 (**2c—e**) showed a critical requirement for the 2-carboxyethyl side-chain (Table 1). Blocking of N-1 and N-3 by Me [compounds (**2f—h**)] gave compounds which did not react with the carbodi-imide; however the lumazine specifically blocked at N-3 only (**2i**) did undergo the transformation where the N-1 blocked compound (**2j**) did not. This establishes that a proton is necessary on N-1 for the sequence of Scheme 1 to occur.

The final structural variation imposed on the lumazine was to make the corresponding pyrido[2,3-*d*]pyrimidine (**2k**). This compound was prepared by condensation of 6-(2-hydroxyethyl-amino)uracil with diethyl 2-formylglutarate and subsequent hydrolysis as outlined in Scheme 4. This pyridopyrimidine failed to undergo the carbodi-imide-mediated transformation under the standard conditions.

The major difference between Schemes 2 and 3 is that, in the former, the first step requires proton loss from N-1; this is not a requirement in the latter. The above evidence thus strongly supports the mechanism of Scheme 2 with the qualification that the pyrido[2,3-*d*]pyrimidine (**2k**) might have been expected to undergo this transformation (but not that of Scheme 3) whereas in fact it did not. Although we have no clear explanation for this apparent anomaly in relation to Scheme 2 it is likely that replacement of N-5 by CH [to give (**2k**)] causes a major change in the electron availability in the molecule. Circumstantial evidence to support this argument comes from two sources: firstly we have found a difference in pK_a between the lumazine series (*ca.* 3.7) and the pyridopyrimidine (**2k**) (*ca.* 4.5), and secondly there is a large difference in the u.v. spectra [the 7-oxolumazine (**2b**), $\lambda_{max.}$(pH 13) 351 nm and the pyridopyrimidine (**2k**), $\lambda_{max.}$(pH 13) 329 nm].

† In this paper, the name 'lumazine' is used to refer to the pteridine-2,4(1*H*,3*H*)-dione ring system.

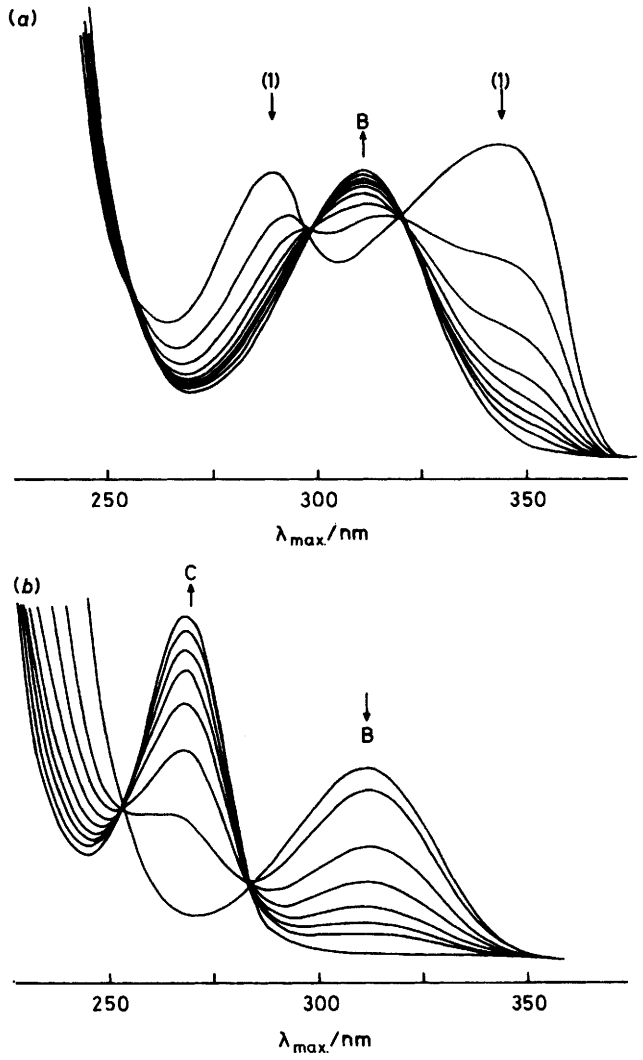
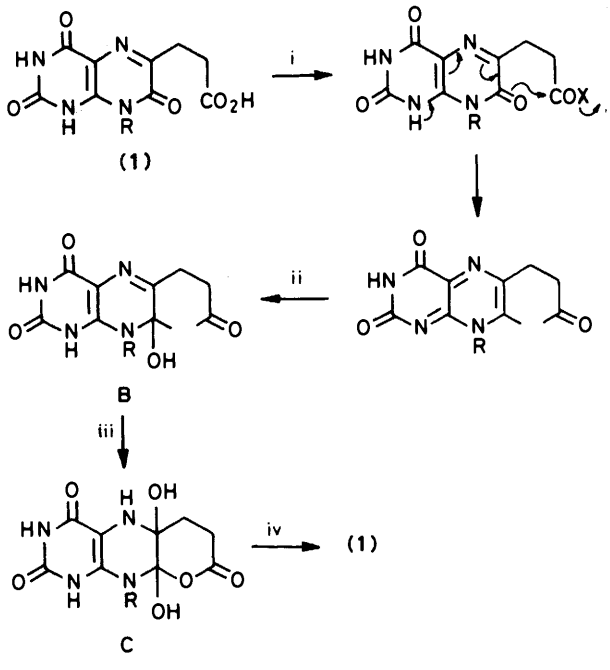
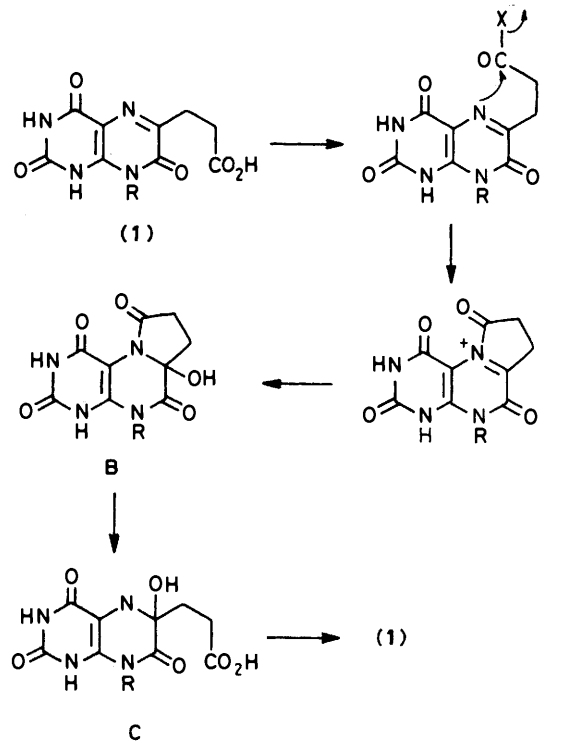


Figure. (*a*) Conversion of (**1**) into intermediate **B** with EDC.HCl. (*b*) Conversion of B into C with aq. NH_4OH



Scheme 2. X = $EtNHC(O)=N(CH_2)_2NMe_2$, R = D-ribityl. *Reagents:* i, EDC.HCl; ii, H_2O; iii, HO^- (fast) or H_2O (slow); iv, pH 1



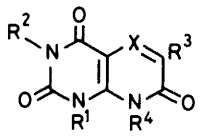
Scheme 3. X = $EtNHC(O)=N(CH_2)_2NMe_2$, R = D-ribityl

In view of the doubts raised by this observation, attempts to identify B and C by isolation and characterisation were carried out. The high water solubility of the 8-D-ribityl-7-oxolumazine (**1**) precluded the isolation of products in this series, but the corresponding 8-(2-hydroxyethyl)lumazine (**2b**), on suspension in water and treatment with EDC.HCl, proceeded smoothly to give intermediate **B** without complete solution ever being achieved. Microanalysis established that the empirical formula of **B** was isomeric with the starting material (**2b**). Both representations of **B** (Schemes 2 and 3) are consistent with this fact. The ^1H n.m.r. spectrum was not very informative, but showed that the 8-(2-hydroxyethyl) substituent protons were unchanged from those of (**2b**) while the protons on the C-6 side-chain appeared as an unresolved multiplet in **B** (δ 2.0—2.8). In (**2b**), the spectrum showed two second-order multiplets at δ 2.96 and 2.55 suggesting that some change (perhaps ring formation) had occurred in this region. The ^{13}C n.m.r. spectrum was more useful. Provisional assignments of some of the carbons of compound (**2b**) together with the corresponding assignments for compound **B** are shown in Table 2.

It is clear from these data that the only large change is at C-7. Although there is no model which accounts for the chemical shift data for C-7 of compound **B** as shown in Scheme 2, hemiacetals appear typically at 93—97 p.p.m. and C-1 (the anomeric carbon) of nucleosides appears at 85—87 p.p.m.[4] This gives some support to the transformation of the sp^2 carbon at C-7 in (**2b**) to an sp^3 species with electronegative substituents.

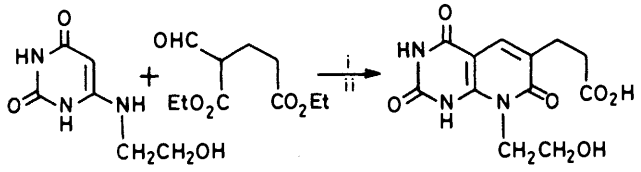
There is a similar lack of data for the assignment of the u.v. spectrum of compound **B** [$\lambda_{max.}$ (pH 1) 309 nm] although there is good correlation with the spectrum of a hydrated pteridine (**3**) reported by Pfleiderer.[5]

Table 1. Effect of varying the substituents on the production of compounds C ($\lambda_{max.}$ 270 nm)

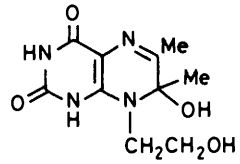


	R^1	R^2	X	R^3	R^4	Transformation[a]
(1)	H	H	N	$CH_2CH_2CO_2H$	D-Ribityl	+
(2a)	H	H	N	$CH_2CH_2CO_2H$	Me	+
(2b)	H	H	N	$CH_2CH_2CO_2H$	CH_2CH_2OH	+
(2c)	H	H	N	CO_2H	D-Ribityl	–
(2d)	H	H	N	$CH_2CH_2CO_2Me$	CH_2CH_2OH	–
(2e)	H	H	N	$CH_2CH_2CONHNH_2$	CH_2CH_2OH	–
(2f)	Me	Me	N	$CH_2CH_2CO_2H$	H	–
(2g)	Me	Me	N	$CH_2CH_2CO_2H$	Me	–
(2h)	Me	Me	N	$CH_2CH_2CO_2H$	CH_2CH_2OH	–
(2i)	H	Me	N	$CH_2CH_2CO_2H$	Me	+
(2j)	Me	H	N	$CH_2CH_2CO_2H$	Me	–
(2k)	H	H	CH	$CH_2CH_2CO_2H$	CH_2CH_2OH	–

[a] + Indicates transformation to the product C took place, – that C was not obtained.



(2k)



(3) $\lambda_{max.}$(pH 1) 306, 275 nm

Scheme 4. *Reagents:* i, pH 3, aq. soln.; ii, 1M-HCl

Table 2. Provisional ^{13}C n.m.r. assignments for compounds (2b) and B

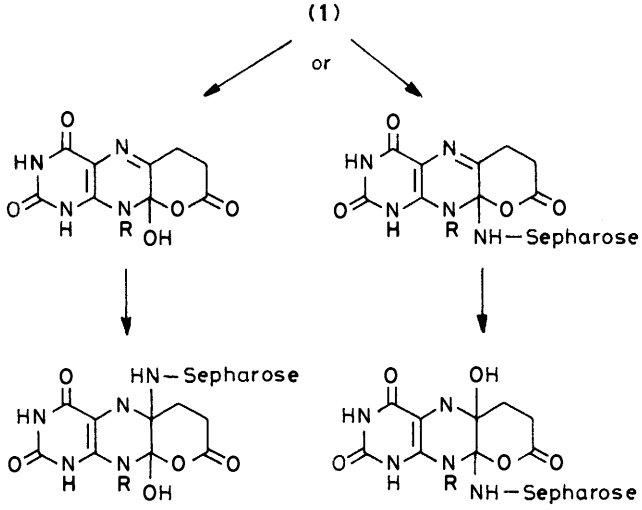
	δ_C (p.p.m.)						
Compd.	C-2	C-4	C-4a	C-6	C-7	C-8a	CO_2R
(2b)	159.6	148.6	107.7	149.1	154.3	143.0	173.0 (R = H)
B	166.0	158.6	94.5	149.6	84.5*	141.5	171.2 (R = ?)

* See text.

Table 3. U.v. spectroscopic study of the effect of aqueous solvents on the reactions of the intermediate B with bases and nucleophiles

Reagent	Solvent	Product $\lambda_{max.}$/nm	Time (h)	Comment
	H_2O	309	>1	B stable
KOH (pH 11)	H_2O	270	1	B rapidly ⟶ C
NH_4OH (0.1M)	H_2O	268[a]	1	B rapidly ⟶ C
NH_4OH (2M)	H_2O	268	1	B rapidly ⟶ C
HCl (0.1M)	H_2O	303	No change	B stable

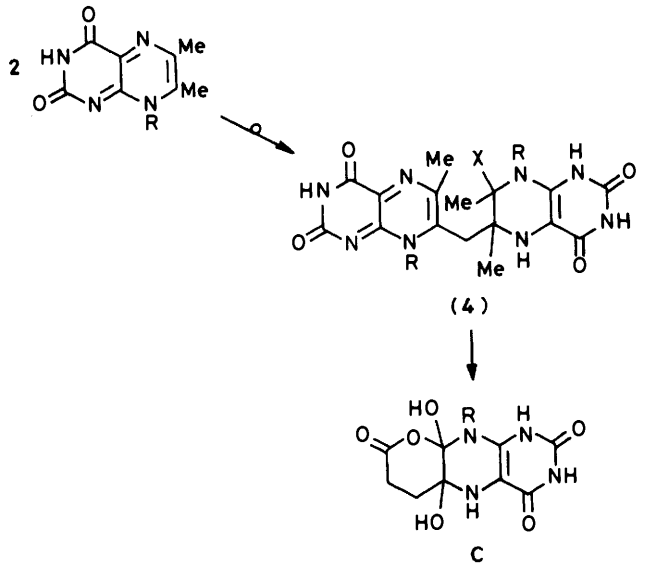
[a] Peak at 315 nm also observed.



Scheme 5. R = D-Ribityl

The conversion of B into a compound having the chromophore of C was achieved under a variety of aqueous basic conditions (see Experimental section). For example, treatment of B [R = CH_2CH_2OH in Scheme 2, *i.e.* derived from (2b)] with aqueous ammonium hydroxide produced the characterstic u.v. spectral shift to 270 nm (Table 3). Evaporation of the solution to dryness gave a yellow powder which had an empirical formula of $C_{11}H_{17}N_5O_7$ from microanalysis. This could be formulated as an ammonium salt of C. Compound B was stable in distilled water, 0.1M-HCl, and in non-aqueous systems (acetonitrile, dimethyl sulphoxide, dimethylformamide) even in the presence of non-aqueous bases. This information is cited as supporting evidence for the conversion of compound B into C involving a further hydration at C-6 (see Scheme 2) which generates a pyrimidine-like u.v. chromophore ($\lambda_{max.}$ 270 nm).

Our interpretation of all the evidence presented above is that Scheme 2 adequately describes the transformation shown in Scheme 1, and that the corresponding reaction with the amino-functionalised Sepharose can now be represented as in Scheme 5.



Scheme 6. R = D-Ribityl

Product C (R = D-ribityl; Scheme 2) was tested as an inhibitor of riboflavin synthase and showed competitive inhibition of the enzyme with an approximate K_i of 2×10^{-4}M. The fact that this molecule has an affinity for the enzyme explains why the analogous immobilised material was effective as an affinity chromatography ligand. Furthermore, consideration of the probable structure of C in relation to one of the proposed intermediates[6] (4) for the enzymic conversion of 6,7-dimethyl-8-D-ribityl-lumazine into riboflavin catalysed by riboflavin synthase, shows some elements of similarity between the two. This suggests that C may have some features of a 'reaction co-ordinate' or transition state analogue (Scheme 6). The design of compounds to test this hypothesis is in progress.

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 a Perkin-Elmer R12 B (60 MHz) or Perkin-Elmer R14 (100 MHz) spectrometer operating in the continuous wave mode and a Bruker HFX 90 or JEOL PS100: PFT100 instrument 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. The interpretation was aided by the use of single frequency off-resonance decoupling. Resonances are reported as p.p.m. from tetramethylsilane (δ scale). Ether refers to diethyl ether.

All products were homogeneous when examined by paper chromatography (Whatman No. 1 paper) or by t.l.c. on silica-gel plates, using the solvents described previously.[1] Spots were detected with filtered u.v. light (λ 254 and 365 nm).

Coupling of 6-*Carboxyethyl*-7-*oxo*-8-D-*ribityl-lumazine* (**1**) *to Amino-functionalised Sepharose.*—The preparation of polymer supported material was carried out as described previously.[1]

Transformation of 6-*Carboxyethyl*-7-*oxo*-8-D-*ribityl-lumazine* (**1**) *to Compounds B and C.*—A solution of compound (**1**) in water was made up such that the absorbance at 340 nm was approximately 0.6 and was placed in a u.v. cuvette. EDC.HCl (approx. 2.3 mg) was added and the u.v. spectrum from 450—250 nm (cut off point) was monitored every 5 min. A transformation of (**1**) (initially $\lambda_{max.}$ 342, 287 nm at pH *ca.* 4.5) occurred with isosbestic points at 320 and 298 nm to give material B ($\lambda_{max.}$ 309 nm). This reaction was complete at ambient temperature in approx. 30 min. The addition of 1 drop of 10M-KOH to the cuvette (final pH 11) resulted in the transformation of B, with an isosbestic point at 284 nm into material C ($\lambda_{max.}$ 269 nm) during 30 min. If the base was not added then B changed more slowly to C (3 days to completion). 6-Carboxyethyl-7-oxo-8-D-ribityl-lumazine (**1**), 6-carboxy-7-oxo-8-D-ribityl-lumazine (**2c**), 6-carboxyethyl-1,3-dimethyl-7-oxolumazine (**2f**), 6-carboxyethyl-7-oxo-1,3,8-trimethyl-lumazine (**2g**), 6-carboxyethyl-1,3-dimethyl-8-(2-hydroxyethyl)-7-oxolumazine (**2h**), 6-carboxyethyl-3,8-dimethyl-7-oxolumazine (**2i**), and 6-carboxyethyl-1,8-dimethyl-7-oxolumazine (**2j**), have all been described previously.[7] 6-Carboxyethyl-8-(2-hydroxyethyl)pteridine-2,4,7(1*H*,3*H*,8*H*)-trione (**2b**), 6-(2-methoxycarbonylethyl)-8-(2-hydroxyethyl)pteridine-2,4,7(1*H*,3*H*,8*H*)-trione (**2d**), and 6-(2-hydrazinocarbonylethyl)-8-(2-hydroxyethyl)pteridine-2,4,7(1*H*,3*H*,8*H*)-trione (**2e**) were prepared as described in Part 5.[1]

Diethyl 2-*Formylglutarate.*—Sodium wire (2 g) was placed in a round-bottom flask and covered with dry ether (60 ml). Diethyl glutarate (7.52 g, 0.04 mol) was added to freshly distilled ethyl formate (2.96 g, 0.04 mol) and this mixture was added dropwise to the sodium–ether mixture at −5 °C. The mixture was stirred and allowed to come to room temperature. Stirring was continued for 3 days at room temperature and the light brown solid was transferred to a conical flask. Water (50 ml) was added and the solution was adjusted to pH 1 by the addition of dilute hydrochloric acid. This acidic solution was extracted with chloroform (5 × 30 ml), the extracts were combined and dried (Na_2SO_4) and the chloroform was evaporated off to give the product as a pale yellow oil (1.3 g, 15%). No further purification was attempted. δ($CDCl_3$) 9.8 (1 H, d, *J* 4 Hz, CHO), 7.67 (1 H, s, *H*OCH=C), 3.48 (1 H, s, OHCC*H*), 4.25 (4 H, m, $CO_2CH_2CH_3$, superimposed), 2.45 (4 H, m, CH_2CH_2), and 1.25 (6 H, m, $CO_2CH_2CH_3$, superimposed).

6-*Carboxyethyl*-8-(2-*hydroxyethyl*)*pyrido*[2,3-d]*pyrimidine*-2,4,7(1H,3H,8H)-*trione* (**2k**). Diethyl 2-formylglutarate (1.5 g, 0.007 mol) and 6-(2-hydroxyethylamino)uracil[8] (1.2 g, 0.007 mol) were suspended in 0.01M-hydrochloric acid (20 ml). The mixture was heated on the steam-bath for 4 h. The resulting brown solution was refrigerated overnight, and the pink crystals which formed were collected (1.25 g). These were re-dissolved in 1M-hydrochloric acid (20 ml) and heated on the steam-bath for 4 h to hydrolyse any remaining ester. On cooling, white crystals were collected, and recrystallised from water to yield the 6-*carboxyethyl*-8-(2-*hydroxyethyl*)*pyrido*[2,3-d]*pyrimidine* as white needles (0.7 g, 34%), m.p. 254—256 °C (Found: C, 49.1; H, 4.4; N, 14.3. $C_{12}H_{13}N_3O_6$ requires C, 48.8; H, 4.4; N, 14.2%); $\lambda_{max.}$(ε) (pH 1) 289 (13 050) and 312 nm (14 750); $\lambda_{max.}$ (pH 13) 250 (7 130), 286 (8 190), and 333 nm (13 150); δ[$(CD_3)_2SO$] 7.46 (1 H, s, 5-H), 4.18 (2 H, m, CH_2CH_2OH), 3.56 (2 H, m, CH_2CH_2OH), and 2.46 (4 H, m, $CH_2CH_2CO_2H$).

Reaction of 6-*Carboxyethyl*-8-(2-*hydroxyethyl*)*pteridine*-2,4,7(1H,3H,8H)-*trione* (**2b**) *with* 3-(3-*Dimethylaminopropyl*)-1-*ethylcarbodi-imide Hydrochloride.*—The lumazine (**2b**) (0.5 g, 1.7×10^{-3} mol) was suspended in distilled water (10 ml) and EDC.HCl (0.35 g, 1.8×10^{-3} mol) was added. The mixture was stirred at room temperature for 2—3 h, the course of the reaction being followed by u.v. spectroscopy. When the peaks at 328 and 281 nm had completely disappeared, the *product* showed a single peak at 309 nm. The suspension at no time completely dissolved. The product B (0.42 g, 84%) was collected

by filtration and dried, but no successful recrystallisation solvent was found, m.p. 251—253 °C (Found: C, 44.4; H, 4.1; N, 19.1. $C_{11}H_{12}N_4O_6$ requires C, 44.6; H, 4.1; N, 18.9%); $\lambda_{max.}$(ε) (pH 7) 310 nm (9 210); δ[$(CD_3)_2SO$] 7.05 (1 H, s, $CH_2CH_2O\mathit{H}$), 3.88 (1 H, s), 3.8—3.2 (4 H, m, $C\mathit{H}_2C\mathit{H}_2OH$), and 2.7—2.0 (4 H, m, CH_2CH_2CO); *m/z* 278 (10%), 260 (100), and 232 (29).

Reaction of Intermediate B with Ammonium Hydroxide.—The intermediate B (0.2 g) was dissolved in 2M-ammonium hydroxide (20 ml) and stirred at room temperature for 2 h. The reaction was followed by u.v. spectroscopy, and was considered complete when the absorbance at 309 nm had completely disappeared. The solution was evaporated to dryness, leaving a pale yellow powder C, m.p. 275—276 °C (Found: C, 39.6; H, 5.5; N, 21.3. $C_{11}H_{17}N_5O_7$ requires C, 39.7; H, 5.4; N, 21.2%); $\lambda_{max.}$(ε) (pH 1) 268 nm (20 080); $\lambda_{max.}$ (pH 13) 270 nm (20 060); *m/z* 296 (6.3%), 252 (85), and 234 (100).

Inhibition of Riboflavin Synthase by the Ammonium Salt of Compound C derived from 6-Carboxyethyl-7-oxo-8-D-ribityllumazine (**1**).—Riboflavin synthase was isolated from dried baker's yeast and the inhibition assay was carried out as described in ref. 7. The inhibitor C concentration was calculated using the known extinction coefficient of C derived from (**2b**). A value of $K_i = 2.1 \times 10^{-4}$M was calculated and the inhibition was shown to be competitive with the substrate.

Acknowledgements

We thank the S.E.R.C. and Burroughs Wellcome for financial support.

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Received 21*st September* 1983; *Paper* 3/1661