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6. Mitsuko Asai, Kōmei Mizuno, and Satoru Kuwada : Application of Chromatography. XLV.*1 On the Mechanism of Biosynthesis of 6-Methyl-7-hydroxyribolumazine.

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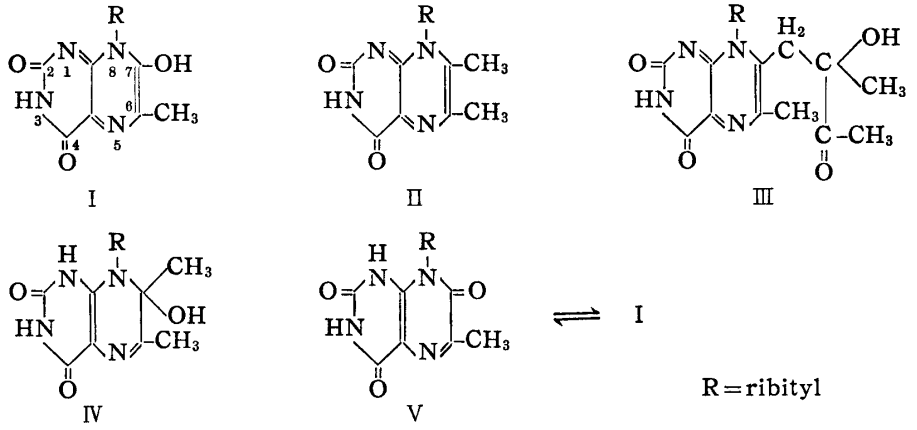
Formerly the authors[1] isolated a crystalline substance, whose solution was purple fluorescent, from the mycelium of *Eremothecium ashbyii* and called it V-compound, which was later found[2] to be 6-methyl-7-hydroxy-8-ribityllumazine (I). The authors also observed that action of the enzyme of the same microorganism on 6,7-dimethylribolumazine (G-compound, II), which is a green fluorescent substance, produced, besides riboflavin, 6-methyl-7-hydroxyribolumazine (I) as a by-product.[3] Korte, *et al.*[4] considered previously that formation of 6-methyl-7-hydroxyribolumazine might be effected by the oxidation of the 7-methyl group of 6,7-dimethylribolumazine to an OH-group during the culture of the microorganism, because the 6-methyl-7-hydroxy compound was produced by the prolonged air-oxidation of an alkaline solution of the 6,7-dimethyl compound. On the other hand, Mitsuda, *et al.*[5] assumed that enzymic degradation of 6,7-dimethylribolumazine gives 4-ribitylamino-5-aminouracil and a four carbon compound, and that the dimer of the latter (an aldol compound) reacts with the uracil compound to afford the intermediate III, which is then hydrolyzed to 6-methyl-7-hydroxyribolumazine.



Recently Rowan, Wood, and Hemmerick[6] inferred from their studies on the absorption spectrum of 6,7-dimethylribolumazine (II) that such a quinoid-type pteridine as II would receive a nucleophilic attack at the position 7 since they observed in fact that the absorption maximum of II underwent a considerable hypsochromic shift probably due to the conversion of II to the carbinolamine compound IV in the alkaline solvent. Also they reported preliminarily that for the formation of I from II a pyruvate reacts with the intermediate IV and replaces the four carbon system constituting the pyrazine

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*1 Part XLIV : This Bulletin, **11**, 18(1963).

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1) T. Masuda : This Bulletin, **4**, 72 (1956).
2) T. Masuda, T. Kishi, M. Asai : *Ibid.*, **6**, 291 (1958).
3) S. Kuwada, T. Masuda, T. Kishi, M. Asai : *Ibid.*, **6**, 618 (1958).
4) F. Korte, H.U. Aldag, G. Ludwig, W. Paulus, K. Störiko : Ann., **619**, 70 (1958).
5) H. Mitsuda, F. Kawai, J. Suzuki : Vitamins (Japan), **23**, 415 (1961).
6) T. Rowan, H.C.S. Wood, P. Hemmerick : Proc. Chem. Soc., **1961**, 260.

ring to give V as evidenced by the expriment *in vitro*, and that a repetition of the experiment of Korte, *et al.*[4] ended in failure.

Thus, there seem to be three possibilities for the formation of 6-methyl-7-hydroxyribolumazine (I). The compound may be formed (1) by oxidative removal of the 7-methyl group of 6,7-dimethylribolumazine, or (2) by degradation of the condensation product of an aldol with the diaminouracil produced by the decomposition of 6,7-dimethylribolumazine, or (3) by a nucleophilic attack at the position 7 of 6,7-dimethylribolumazine followed by the reaction with a pyruvate, replacing the four carbon system constituting the pyrazine ring.

As reported in a previous paper, the authors synthesized the lumazine compound of Cresswell and Wood[7] and allowed it to react with the enzyme of *Er. ashbyii*, but as neither riboflavin nor 6-methyl-7-hydroxyribolumazine was detected in the reaction mixture the possibility (2) seems to be at first excluded.

Next, an attempt was made to see what compound other than riboflavin and 6-methyl-7-hydroxyribolumazine would be formed by the reaction of 6,7-dimethylribolumazine with the above enzyme at 37° for a definite period. To see if any carbonyl compound was produced or not, the reaction mixture was treated with 2,4-dinitrophenylhydrazine and then subjected to paper partition chromatography by the method of Gasparic, *et al.*,[8] whereby a spot corresponding to the 2,4-dinitrophenylhydrazone of formaldehyde was detected (Table II). On the other hand, when the reaction mixture was treated with an ion-exchange resin to collect acid substances and the substances adsorbed on the resin were chromatographed, formic acid was detected. As a control, the enzyme solution alone, or together with 4-ribitylamino-5-aminouracil, diacetyl, or acetoin was processed in the same way. In this case, the formation of formic acid was observed only in the presence of acetoin, and neither formaldehyde nor formic acid was detected in the other cases.

Table I. Influence of pH on the Enzymic Reaction
(concentration of substrate, $25.0 \times 10^{-4} M$)

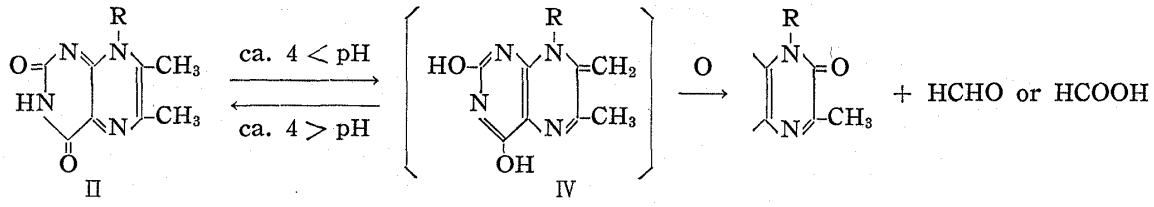
Substrate	pH 6.4			pH 7.0			pH 7.8		
	detected γ	10^{-7} mole	%	detected γ	10^{-7} mole	%	detected γ	10^{-7} mole	%
6,7-Dimethylribolumazine	266.25	8.17	32.6	120.0	3.99	15.9	71.5	2.2	8.38
Riboflavin	219.65	5.85	23.4	235.9	6.26	25.0	228.4	6.08	24.3
6-Methyl-7-hydroxyribolumazine	160.0	4.85	19.5	262.5	8.0	32.0	307.5	9.37	37.4

Table II. Rf-values of 2,4-Dinitrophenylhydrazone Derivatives

Substrate	1) Method of Hawary, *et al.* EtOH-BuOH-0.5*N* NH_3OH (10:70:20) Rf		2) Method of Gasparic, *et al.* Rf		
6,7-Dimethylribolumazine	0.00	0.85	0.00	0.22	0.42
4-Ribitylamino-5-aminouracil	0.00	0.85	0.00		0.42
Enzyme soln.	0.00	0.85	0.00		0.42
Acetoin	0.00	0.85	0.00		0.42
Diacetyl	0.00	0.85	0.00		0.42
	0.42 (Pyruvic acid)			0.20 (Formaldehyde)	

From the results mentioned above it seems reasonable to assume that the 7-methyl group of 6,7-dimethylribolumazine (II) is liable to be converted into a methylene group and, therefore, action of an enzyme on VI produces formaldehyde or formic acid as shown below.

7) R.M. Cresswell, H.C.S. Wood : Proc. Chem. Soc., **1959**, 387; J. Chem. Soc., **1960**, 4768.
8) J. Gasparic, M. Vecera : J. Chromatography, **1**, xviii (1958).



II IV

It was observed that when 6,7-dimethylribolumazine (II) was subjected to the enzymic reaction at a pH causing dissociation of the OH group (above ca. pH 4), 6-methyl-7-hydroxyribolumazine (I) was formed for a short time (30~60 min.), and its quantity was increased with the rise of the pH values (pH 6.4, 7, 7.8). On the contrary, I was not produced in the control experiment using no enzyme.

The authors also oxidized 6,7-dimethylribolumazine with air in 0.1*N* alkali solution for four days, subjected the reaction mixture to paper partition chromatography, and detected a purple fluorescent spot corresponding to the Rf-value of 6-methyl-7-hydroxyribolumazine. This fact led the authors to justify the experiment of Korte, *et al.*, and to agree with the possibility 1.

Finally, in order to investigate the assumption of Rowan, *et al.* 4-ribitylamino-5-aminouracil was allowed to react with pyruvic acid in the presence of the enzyme solution. At the same time, similar reactions were performed after addition of formic acid, formaldehyde, or acetoin as a carbon donor. As seen in Table IV, the formation of 6-methyl-7-hydroxyribolumazine as well as riboflavin and 6,7-dimethylribolumazine was observed only in the presence of acetoin as reported frequently by the present authors.[9] Incidentally, when pyruvic acid was added as a carbon donor, a purple fluorescent spot was detected in paper partition chromatography of the reaction mixture, but the ultraviolet spectrum of an extract of the spot indicated that the substance was quite different from 6-methyl-7-hydroxyribolumazine as shown in Fig. 1. The amount of the lumazine compound was found to be nearly zero when calculated from the absorption at 345 mμ (Table V). From these results the possibility 3 proposed by Rowan, *et al.* is denied and, for the explanation of their experiment *in vitro* another mechanism seems to be required.

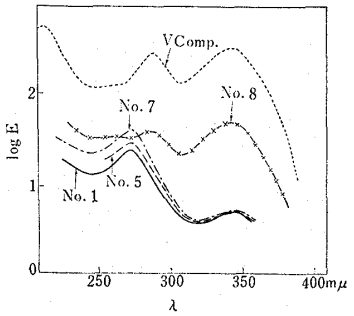


Fig. 1.

---------- log $E^{1\%}_{1cm}$ of 6-Methyl-7-hydroxyribolumazine
——— log $E^{1\%}_{1cm}$ of No. 1
— — — — 〃 of No. 5
— — — — — 〃 of No. 7
—×—×— 〃 of No. 8

Table III. Rf-values of Acid Substances

Substrate	3) Method of Kennedy, *et al.* (95% EtOH 100 cc. + 28% NH_4OH 1 cc.) Rf	
6,7-Dimethylribolumazine	0.00	0.21
4-Ribitylamino-5-aminouracil	0.00	
Enzyme soln.	0.00	
Acetoin	0.00	0.21
Diacetyl	0.00	0.21 (Formic acid)

9) M. Asai, T. Masuda, S. Kuwada: This Bulletin, **9**, 496 (1961).

Table IV. Rf-values of Products in Various Enzymic Reactions

Sample No.	4-Ribitylamino-5-aminouracil $9.5\times10^{-3}M$ (cc.)	Pyruvic acid $1.36\times10^{-1}M$ (cc.)	Formic acid $3.6\times10^{-1}M$ (cc.)	Formaldehyde $4\times10^{-1}M$ (cc.)	Acetoin $1.36\times10^{-1}M$ (cc.)	Enzyme soln. Protein N 159.6 γ/cc. (cc.)	Heat treated enzyme soln. (cc.)	Reaction products: EtOH-BuOH-H_2O (15:50:35) Rf			Reaction products: AcOH-BuOH-H_2O (1:4:5) Rf		
1	0.5	0.5	—	—	—	0.5				0.30 V+			0.18 V+
2	0.5	—	0.5	—	—	0.5					0.05 W±		
3	0.5	—	—	0.5	—	0.5					0.03 W±		
4	0.5	—	—	—	—	0.5							
5	0.5	0.5	0.5	—	—	0.5				0.31 V+			0.16 V+
6	0.5	—	—	—	0.5	0.5		0.26 G+	0.28 V+	0.40 Y+	0.13 G+	0.15 V+	0.25 Y+
7	0.5	0.5	—	—	—	—	0.5		0.29 G+			0.18 V+	
8	6,7-Dimethylribolumazine ($4.7\times10^{-3}M$) 1					0.5		0.26 G+ (A)	0.28 V## (B)	0.40 Y## (C)	0.13 G+ (A)	0.15 V## (B)	0.25 Y## (C)

A: 6,7-Dimethylribolumazine B: 6-Methyl-7-hydroxyribolumazine C: Riboflavin
G: Green fluorescence V: Violet fluorescence W: White fluorescence
Y: Yellow fluorescence ±, +, ##, ##: Intensity of fluorescence

Table V. Quantitative Estimation on Some Samples in Table IV

	Substrate		Quantity of 6-methyl-7-hydroxyribolumazine detected	Product (%)
No. 1	4-Ribitylamino-5-aminouracil	4.7×10^{-6} mole	1.46×10^{-7} mole	0
〃 5	〃	〃	1.43×10^{-7}	0
〃 7	〃	〃	1.49×10^{-7}	—
〃 8	6,7-Dimethylribolumazine	〃	1.51×10^{-6}	32

The authors[10] previously found that one mole each of riboflavin and 6-methyl-7-hydroxyribolumazine was produced from 3 moles of 6,7-dimethylribolumazine and the view of Plaut[11] for the formation of riboflavin, but did not examine the formation mechanism of 6-methyl-7-hydroxyribolumazine. As evident from Table I, while the formation of riboflavin from 6,7-dimethylribolumazine by the enzyme reaction is hardly influenced by pH, the formation of 6-methyl-7-hydroxyribolumazine is strongly affected by pH. This fact seems to suggest that the formation of both compounds takes quite different mechanisms. For the formation of riboflavin the authors agree with the view of Plaut, but assume that 6-methyl-7-hydroxyribolumazine is probably produced by the oxidative rupture of the 7-methyl group of 6,7-dimethylribolumazine. From this point of view the present paper furnishes a powerful experimental ground to the possibility 1.

Experimental

I. Preparation of an Enzyme Solution from the Mycelium of *Er. ashbyii*——Twenty grams of the mycelium obtained by a 67 hr. culture of *Er. ashbyii* was fractionated with $(NH_4)_2SO_4$ by the method of Part XXXVI,[3] and the fractions of 0.3~0.7 saturation were dissolved in 100 cc. of $M/15$

10) M. Asai, T. Masuda, S. Kuwada: This Bulletin, **9**, 503 (1961).
11) G. W. E. Plaut: J. Biol. Chem., **235**, PC 41 (1960).

phosphate buffer (pH 7.0) and dialyzed against the same buffer for 24 hr. Each lot of the solution thus obtained contained about 160 γ/cc. of protein nitrogen.

II. Influence of pH on the Enzymic Conversion of 6,7-Dimethylribolumazine to 6-Methyl-7-hydroxyribolumazine——To 1 cc. each of a solution ($25.0 \times 10^{-4}M$) of 6,7-dimethylribolumazine were added 0.5 cc. of the above enzyme solution and 0.5 cc. each of phosphate buffers of pH 5.0, 7.0 and 9.0, and the resulting mixtures showing pH 6.4, and 7.8, respectively, were incubated at 37° for 1 hr. A 0.2 cc. portion each of the reaction mixtures was applied on two pieces of filter paper, 4×45 cm., and developed with BuOH-EtOH-H_2O (50:15:35). The spots of 6,7-dimethylribolumazine and 6-methyl-7-hydroxyribolumazine were extracted and determined by the method of Part XLIII,[10] giving the results shown in Table I.

III. Action of the Enzyme Solution on 6,7-Dimethylribolumazine——A mixture of 370 mg. of 6,7-dimethylribolumazine, 40 cc. of the enzyme solution and 560 cc. of distilled H_2O was incubated at 37° for 5 hr., the reaction mixture was poured on a column (18×180 mm.) of carbon powder, and the effluent (600 cc.) was divided into two parts, which were subjected to tests for carbonyl compounds and acids.

a) Carbonyl Compounds: To 300 cc. of the effluent was added a solution of 10 mg. of 2,4-dinitrophenylhydrazine in 3 cc. of 35% HCl, and the mixture was heated on the water bath for 30 min., left standing overnight, and shaken with AcOEt. The AcOEt layer was separated, washed with H_2O, dried over anhyd. Na_2SO_4, and concentrated to 10 cc. to give a sample for paper partition chromatography.

1) Method by Hawary, *et al.*[12]: Paper, Tōyō filter paper No. 7; solvent, BuOH-EtOH-0.5*N* NH_4OH (70:10:20).
2) Method by Gasparic, *et al.*[8]: Tōyō filter paper No. 7 was soaked in a 25% solution of dimethylformamide. The excess solvent on the paper was removed by absorption with filter paper, and 15 min. later the sample was applied and developed with cyclohexane.

Under ultraviolet rays the 2,4-dinitrophenylhydrazone compound appeared as a black spot, which, however, turned orange in 1% EtOH solution of KOH.

The method 1) detected no spot corresponding to the 2,4-dinitrophenylhydrazone of pyruvate (Rf 0.42), but the method 2) spotted the 2,4-dinitrophenylhydrazone of HCHO (Rf 0.22) (Table II).

The mother liquor obtained after removal of acid components by means of an ion-exchange resin was allowed to react with 2,4-dinitrophenylhydrazine and submitted to the same procedure as above. Also in this case, the method 2) detected the 2,4-dinitrophenylhydrazone of HCHO.

b) Acids: The remaining half of the effluent was passed first through a column (14×150 mm.) of Amberlite IR-120 (H-form) and then through a column (14×130 mm.) of Amberlite IR-4B(OH-form). The latter column was washed with H_2O and eluted with 300 cc. of *N* NH_4OH, and the eluate was concentrated in a reduced pressure. The residue was dissolved in 0.5 cc. of distilled H_2O and chromatographed by the method of Kennedy and Barker[13] to detect the spot of HCOOH as shown in Table III. The spot was blue when treated with a solution of Bromphenol Blue acidified with citric acid, and yellowish brown with Brown's NH_4OH-$AgNO_3$ solution.[14]

IV. Action of the Enzyme solution on 4-Ribitylamino-5-aminouracil——A mixture of 110 mg. of 4-ribitylamino-5-aminouracil·$\frac{1}{2}H_2SO_3$,[15] 20 cc. of the enzyme solution, and 280 cc. of distilled H_2O was incubated at 37° for 5 hr., and the reaction mixture was passed first through a column (14×150 mm.) of Amberlite IR-120 (H-form) and then through a column (14×130 mm.) of Amberlite IR-4B (OH-form). The effluent was tested for carbonyl compounds a), and the fractions adsorbed on the second column for acids b).

a) The effluent was allowed to react with 2,4-dinitrophenylhydrazine and processed as before, but HCHO was not detected as shown in Table II.

b) The fractions were tested for acids as in the case of III, but no spot of HCOOH was detected as shown in Table III.

V. Blank (Control) Test with the Enzyme Solution——To 20 cc. of the enzyme solution was added 280 cc. of distilled H_2O and the mixture was incubated at 37° for 5 hr., but neither HCHO nor HCOOH was detected in the reaction mixture as shown in Tables II and III.

VI. Action of the Enzyme Solution on Acetoin——A mixture of 100 mg. of acetoin, 5 cc. of the enzyme solution and 50 cc. of phosphate buffer was incubated at 37° for 5 hr., and 10 cc. and 40 cc. of the reaction mixture were tested for carbonyl compounds and acids, respectively.

a) To 10 cc. of the sample was added a solution of about 50 mg. of 2,4-dinitrophenylhydrazine in 10 cc. of 35% HCl, and the mixture was diluted with H_2O to 100 cc. and left standing overnight.

12) M. F. S. El Hawary, R. H. S. Thompson: Biochem. J., **53**, 340 (1953).
13) E. P. Kennedy, H. A. Barker: Ann., **23**, 1033 (1951)
14) F. Brown: Nature, **167**, 441 (1951).
15) T. Masuda, T. Kishi, M. Asai, S. Kuwada: This Bulletin, **7**, 366 (1959).

The separated 2,4-dinitrophenylhydrazone of acetoin was filtered, the filtrate was shaken with AcOEt, and the AcOEt solution, after washing with H_2O and drying, was concentrated to 10 cc. and subjected to paper partition chromatography, but no spots corresponding to the 2,4-dinitrophenylhydrazone of HCHO and pyruvate were detected as shown in Table II.

b) The sample was tested for acids according to the method of b) in III, and HCOOH was detected as shwon in Table III.

VII. Action of the Enzyme Solution on Diacetyl——A mixture of 0.1 g. of diacetyl and 50 cc. of phosphate buffer was incubated at 37° for 5 hr., and the reaction mixture was investigated after treating as in VI, but neither carbonyl compound nor formic acid was detected as shown in Tables II and III.

VIII. Conversion of 6,7-Dimethylribolumazine in an Alkaline Solution——A solution of 10 mg. of 6,7-dimethylribolumazine in 10 cc. of *N*/10 NaOH was left standing in a dark place for 4 days and oxidized with air. The reaction mixture was passed through a column (5×50 mm.) of Amberlite IR-120 (H-form), and the effluent was concentrated and chromatographed with BuOH-EtOH-H_2O (50:15:35). The chromatogram was investigated under UV rays, giving the results shown below.

Rf	0.09	0.15	0.18	0.23	0.35	0.52
	G±	W±	G+	V‡	V+	B+

G=green fluorescence; W=white fluorescence;
V=violet fluorescence; B=blue fluorescence.

Of the above Rf values, Rf 0.23 corresponded to that of 6-methyl-7-hydroxyribolumazine. The UV spectrum of an aqueous extract of the spot was also in accord with that of the lumazine compound.

IX. Reaction of 4-Ribitylamino-5-aminouracil with Carbon Donors in the Presence of Enzyme——Various mixtures shown in Table IV were incubated at 37° for 5 hr. (in No. 7 the enzyme solution was heated beforehand), each of the reaction mixtures was developed on paper with two solvents, and the chromatograms were investigated under UV rays giving the results shown in the same Table.

Next, 0.1 cc. each of the samples Nos. 1, 5, 7 and 8 was applied on filter paper (4×45 cm.) and developed with EtOH-BuOH-H_2O (15:50:35). The purple fluorescent spots with Rf 0.29 were cut out and extracted with 5 cc. of distilled H_2O at 80° for 15 min., separately. The UV spectra of the extracts exhibited the absorption curves as shown in Fig. 1. The three curves of Nos. 1, 5 and 7 were nearly the same in form but distinctly different from that of 6-methyl-7-hydroxyribolumazine represented by the dotted line. When the amount of 6-methyl-7-hydroxy-ribolumazine in each sample was calculated from the absorption at 345 mμ, the value was almost the same as that of the control (No. 7), but far smaller than that of No. 8, to which 6,7-dimethylribolumazine was added as a substrate[3] (Table V). On the contrary, No. 6, to which acetoin was added, gave the same result as reported previously.[9]

Summary

It was evidenced that action of the enzyme of *Eremothecium ashbyii* on 6,7-dimethylribolumazine produced formaldehyde and formic acid, besides riboflavin, and 6-methyl-7-hydroxyriboumazine. The experimental data obtained support the mechanism suggested by Plaut for the formation of riboflavin. As for the formation of 6-methyl-7-hydroxyribolumazine, however, it has been proved that the compound was produced by oxidative rupture of the 7-methyl group of 6,7-dimethylribolumazine, and this result gave an experimental ground to the assumption of Korte, *et al.*

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