EXAMINATION OF THE STRUCTURE OF AN UNKNOWN GREEN FLUORESCENT COMPOUND, COMPOUND G₂, ACCUMULATED IN NON-GROWING CELLS OF *EREMOTHECIUM ASHBYII* BY THE ADDITION OF DIMERIC DIACETYL¹

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Summary The addition of dimeric diacetyl to *Eremothecium ashbyii* caused simultaneous accumulation of two green fluorescent compounds with the inhibition of riboflavin formation in non-growing cells. One compound, referred to as Compound G₁, was identified as 6, 7-dimethyl-8-ribityllumazine as reported previously and the other is referred to as Compound G₂. The latter compound was considered to be 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine because a violet fluorescent compound, 6-methyl-7-hydroxy-8-ribityllumazine was derived from Compound G₂ in the presence of *p*-quinone and because the known action mechanism of dimeric diacetyl as a trapping agent of possible intermediates in the biosynthetic pathway.

The results indicate that an immediate intermediate to 6, 7-dimethyl-8-ribityllumazine in the biosynthetic pathway of riboflavin is 4-ribitylamino-5-amino-2, 6-dihydropyrimidine, whose pyrimidine portion is derived by the elimination of a dimeric diacetyl fragment from the molecule of the isolated lumazine.

In a preceding paper (1), it was found that dimeric diacetyl inhibited riboflavin formation in non-growing cells of *Eremothecium ashbyii* at the higher concentrations (0.2-1.0%), accompanied by the accumulation of two green fluorescent compounds inside and outside of the cells. One of them (referred to as "Compound G₁") was accumulated in large amounts and the other (referred to as "Compound G₂") was detected in small amounts. Compound G₁ was identified as 6, 7-dimethyl-8-

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ribityllumazine by means of fluorometric, spectrophotometric and enzymic analyses with a highly purified compound from the mycelia. Furthermore, it was proved by experiments with riboflavin synthetase from *E. ashbyii* that the accumulation of the fluorescent compounds was caused by the inhibition of the riboflavin synthetase with dimeric diacetyl or its derivative. This was supported also by an inverse correlation between the formation of riboflavin and the accumulation of the fluorescent compounds in the presence of dimeric diacetyl.

In the present paper, studies were carried out to clarify the structure of Compound G₂ and its relation to the biosynthesis of riboflavin.

MATERIALS AND METHODS

Materials. Nutrients used for fermentation were of reagents grade. 6, 7-Dimethyl-8-ribityllumazine and 6-methyl-7-hydroxy-8-ribityllumazine in crystalline forms were gifts from Dr. Kuwada, Research Laboratories, Takeda Pharmaceutical Industries, Ltd. Dimeric diacetyl was prepared by self-condensation of diacetyl in an alkaline solution as follows. Diacetyl (50 ml) purchased from Nakarai Chemicals, Ltd. was mixed with distilled water (200 ml). 1 N potassium hydroxide solution (170 ml) was added to the mixture dropwise at a rate of 3 ml/min with a gentle stirring in the cold. The mixed solution was adjusted to pH 4 with H₂SO₄ and extracted with ethyl ether (450 ml). The ether fraction was dehydrated by mixing with certain amounts of anhydrous sodium sulfate, followed by evaporation to an oily substance (2). The resultant material was used as the dimeric diacetyl preparation throughout the experiments.

All other chemicals used were of best quality available.

Cultivation. Cultivation was carried out in the manner described in a previous paper (1). *Eremothecium ashbyii* IFO 0944 cells, which were obtained through the courtesy of the Institute for Fermentation, Osaka, were cultivated on agar slants and subcultured every seven days. A fragment of the mycelia was suspended in the precultural medium and cultured for one day. An aliquot of the mycelial suspension was transferred to the basal medium with the following nutrients, glucose, peptone, yeast extract, Tween 80 and salts, and cultured for one day. The obtained mycelia were starved for 8 hr and, after vacuum infiltration of dimeric diacetyl, were further incubated in phosphate buffer (pH 6.8) for 18 hr with a moderate shaking, as previously reported (3).

Determination of the amounts of riboflavin, Compound G and Compound G₂. Non-growing cells of *Eremothecium ashbyii* were incubated at 27°C for 18 hr at indicated concentrations of dimeric diacetyl in Fig. 1. The mycelial mixtures were separated to the mycelia and the incubation fluid by filtration. The mycelia were heated at 80°C for 10 min in distilled water at a rate of 2.5 ml/g wet mycelia. The mixture was centrifuged and a clear mycelial extract was obtained. The riboflavin content of the mycelial extract and the incubation fluid were spectrophotometri-

cally determined using a millimolar absorptivity 11.2 at 450 nm, and the values were expressed as total flavins ($\mu\text{g/g}$ wet mycelia).

For determination of Compound G and Compound G_2 content, the mycelial extract (15 ml) was applied to a Dowex 50W $\times$ 4(H^+) column(0.9 $\times$ 14 cm), which was developed with distilled water at a flow rate of 3 ml/5 min. The contents of both were calculated from the volume of the eluate and the mean absorbance using the millimolar absorptivity of Compound G, 10.2, based on the supposition that Compound G_2 had the same millimolar absorptivity as that of Compound G.

Isolation and purification of Compound G_2 . Isolation and purification of accumulated Compound G_2 were carried out by the same methods as those for Compound G_1 (I). However, the yields of purified Compound G_2 were very low because of the losses during purification due to its instability. The preparation obtained was used for paper chromatography. For the conversion reaction and the preparation of a violet fluorescent compound, a preparation obtained by means of Dowex 50W $\times$ 4 column chromatography was used.

Preparation of a violet fluorescent compound from isolated Compound G_2 . Isolated Compound G_2 was adsorbed on acid-washed charcoal in acid solution and filtered through filter paper. The charcoal was thoroughly washed with distilled water and suspended in a solution of 10% pyridine in 50% ethanol. The suspension was then stirred for 30 min. After filtration, the filtrate was evaporated to dryness. The dried matter was dissolved in a minimum volume of distilled water and placed in a line on paper. For chromatography, the paper was developed overnight by the ascending method at room temperature with a solvent consisting of butanol: pyridine: H_2O (6: 4: 3), and completely dried by means of an air flow. Using an ultraviolet light the violet fluorescent area was located and cut out. The violet fluorescent compound was extracted by heating in distilled water (10 ml) at 80°C for 10 min. The extract (around 10 ml) was applied to a Dowex 50W $\times$ 4(H^+ , 200–400 mesh) column (0.9 $\times$ 14 cm) and eluted with distilled water at a flow rate of 3 ml/5 min. The eluate was submitted to identification of a violet fluorescent compound by means of spectrophotometric and fluorometric analyses.

Determination of ultraviolet and visible light absorption spectra and excitation and emission spectra of the violet fluorescent compound. Ultraviolet and visible light absorption spectra of the violet fluorescent compound derived from Compound G_2 were measured in acid, neutral and alkaline solutions together with an authentic compound by means of a Shimadzu multipurpose recording spectrophotometer MPS-50L.

The excitation spectra of the violet fluorescent compound were followed by the fluorescence intensity at 500 nm emitted with the indicated wavelengths for excitation in Fig. 2. Emission spectra were obtained by following the fluorescence intensity at wavelengths shown in Figs. 3 and 4 emitted with an excitation wavelength of 290 nm. The fluorescence spectra were measured respectively in 2.5 $\times$

10^{-2} M phosphate buffer (pH 7.0) using a Hitachi fluorescence spectrophotometer MPF-2A.

Reaction of Compound G₂ with p-quinone. Isolated Compound G₂ in a certain amount was added to 10^{-3} M *p*-quinone solution in 2.5×10^{-2} M phosphate buffer (pH 7.0) (4). The emission spectra were followed by measurement of fluorescence intensity in a 360–580 nm wavelength emitted by exciting the above solution at a wavelength of 320 nm immediately after the addition of *p*-quinone.

RESULTS

1. Relation between the formation of riboflavin and the accumulation of 6, 7-dimethyl-8-ribityllumazine (Compound G) and Compound G₂ in the presence of dimeric diacetyl

Effect of dimeric diacetyl on the formation of riboflavin and the accumulation of Compound G and Compound G₂ was examined with non-growing cells of *E. ashbyii* although the former compound was shown to be 6, 7-dimethyl-8-ribityllumazine in a previous paper (1). The results are given in Fig. 1. The addition of dimeric diacetyl up to a concentration of 0.3% rather stimulated riboflavin formation, but the formation gradually decreased in a higher concentration range. The accumulation of Compound G and Compound G₂ was started with the decrease of riboflavin formation. Under the experimental conditions, the compounds showed simultaneous accumulation behaviour with maximum accumulation at 1.0% of dimeric diacetyl.

2. Properties of the violet fluorescent compound derived from Compound G₂

Paper chromatographic analysis of the prepared violet fluorescent compound was done with various solvent systems indicated in Table 1. The results show the good coincidence of *R_f* values between the isolated violet fluorescent compound and 6-methyl-7-hydroxy-8-ribityllumazine (Compound V) on the paper chromatograms.

Table 1. Paper chromatography of the violet fluorescent compound derived from Compound G₂ and Compound V. Paper chromatography was done overnight at room temperature by the ascending method in various solvent systems. The fluorescent compounds were detected as violet spots under an ultraviolet light.

Solvents ^a	A	B	C	D	E	F
	<i>R_f</i> Values					
Sample V ^b	0.19	0.41	0.04	0.36	0.44	0.32
Authentic V ^c	0.20	0.40	0.04	0.36	0.44	0.32

^a A, BuOH: pyridine: H₂O (6: 4: 3); B, *t*-BuOH: NH₄OH: H₂O (60: 5: 35); C, BuOH: EtOH: H₂O (50: 15: 35); D, BuOH: AcOH: H₂O (3: 2: 2); E, *t*-BuOH: H₂O (60: 40); F, *t*-BuOH: pyridine: H₂O (60: 15: 35).

^b Sample V: a violet fluorescent compound derived from Compound G₂.

^c Authentic V: 6-methyl-7-hydroxy-8-ribityllumazine (Compound V).

Ultraviolet and visible light absorption spectra were measured over a wavelength of 210–450 nm in the solutions at pH 1.0, 7.0 and 13.0, respectively. The results are given in Table 2. Absorption maxima of the isolated compound and Compound V in the solutions were quite similar, respectively.

The excitation spectra are shown in Fig. 2. Maximum absorptions of the isolated compound and authentic compound were the same, indicating main peaks at 290 nm and 350 nm in the buffer.

Table 2. Ultraviolet absorption maxima of the violet fluorescent compound derived from Compound G₂ and Compound V. Ultraviolet and visible light absorption spectra were automatically recorded over a wavelength of 210–450 nm in indicated solutions.

		Sample V			Authentic V		
		$\lambda_{\max}$ (nm)					
pH	1.0 ^a	280,	325		282,	327	
pH	7.0 ^b	253 ^s ,	286,	346	253 ^s ,	288,	346
pH	23.0 ^c	253 ^s ,	284,	348	253 ^s ,	288,	346

^a in 0.1 N HCl, ^b in 5×10^{-2} M phosphate buffer, ^c in 0.1 N NaOH, ^s shoulder.

Fig. 1

Fig. 1. Effect of dimeric diacetyl on the formation of riboflavin and the accumulation of Compound G (6, 7-dimethyl-8-ribityllumazine) and Compound G₂ in non-growing cell experiments with *E. ashbyii*.

Fig. 2

Fig. 2. Excitation spectra of the violet fluorescent compound derived from Compound G₂ and Compound V. Excitation spectra of the compounds were recorded in 5×10^{-2} M phosphate buffer (pH 7.0) at the emission wavelength at 520 nm.

Furthermore, the emission spectra of the isolated and authentic compounds were measured and the results are exhibited in Fig. 3. The emission spectra of these compounds completely coincided, indicating maximum intensity of fluorescence at 420 nm, which clearly differed from Compound G having the fluorescence maximum at 480 nm.

The conversion of Compound G_2 to Compound V occurred in the presence of *p*-quinone. This fact was confirmed by following the changes of emission spectra of Compound G_2 with the incubation time. The results are shown in Fig. 4. The maximum intensity of the fluorescence initially observed at 520 nm, attributable to Compound G_2 , moved toward that at 420 nm, being a characteristic of Compound V with the increasing incubation time, and perfectly coincided with that of Compound V after incubation for 30 min. The changes clearly demonstrate that the dehydrogenic demethylation reaction (4) of Compound G_2 to Compound V took place under the conditions used. Moreover, it is to be noted that the maximum intensity of Compound G_2 was detected at 520 nm, being different from those of Compound G and Compound V but almost similar to that at 530 nm of riboflavin. Riboflavin itself exhibited no change at the maximum intensity of the fluorescence under the same conditions described above.

Fig. 3

Fig. 4

Fig. 3. Emission spectra of the violet fluorescent compound derived from Compound G_2 and Compound V. Emission spectra of the compounds were measured in 5×10^{-2} M phosphate buffer (pH 7.0) at the excitation wavelength at 300 nm.

Fig. 4. Change of fluorescence spectra of Compound G_2 in the presence of *p*-quinone. The reaction was followed under the experimental conditions described in MATERIALS AND METHODS with indicated incubation times using the wavelength at 300 nm for excitation.

DISCUSSION

In a previous paper it was found that dimeric diacetyl caused the concomitant accumulation of Compound G₁ and Compound G₂ in a close relation to riboflavin formation. Compound G₁ was identified as 6, 7-dimethyl-8-ribityllumazine as reported previously (1). The paper presented conclusive evidence showing that Compound G₂ has a lumazine ring, a ribityl side chain and a methyl group at carbon 6 on the molecule based on the occurrence of 6-methyl-7-hydroxy-8-ribityllumazine from Compound G₂ and the results of fluorometric and spectrophotometric analyses of the reaction products obtained with or without *p*-quinone. This indicates that Compound G₂ is the ribityllumazine derivative having an unknown carbon chain at carbon 7 on the molecule. However, Compound G₂ is clearly different from 6, 7-dimethyl-8-ribityllumazine (Compound G) with respect to the maximum intensity of fluorescence at 520 nm and the elution position with Dowex 50W × 4 column chromatography. Moreover, Compound G₂ does not appear to be derived from a condensation reaction of Compound G with added dimeric diacetyl because Compound G₂ always appeared at a constant accumulation ratio to Compound G in mycelial extracts of *E. ashbyii* obtained by different extraction methods with cold distilled water together with or without alumina, hot water, as employed in the present paper, and perchloric acid (5). Furthermore, heating a mixture of authentic Compound G and dimeric diacetyl in varied concentrations (80°C, 10 min) did not produce Compound G₂.

On the other hand, it is well known that dimeric diacetyl conjugates with 4-ribitylamino-5-amino-2, 6-dihydroxypyrimidine to form 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine *in vitro* (6).

Accordingly, it is supposed that Compound G₂ is 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine which originated from intracellular condensation of dimeric diacetyl with an intermediate, 4-ribitylamino-5-amino-2, 6-dihydroxypyrimidine, in non-growing cells of *E. ashbyii*.

Furthermore, it has been shown that the long carbon chain substituent at 7 position of the lumazine molecule makes ring closure easier so as to yield riboflavin *in vitro* in 0.1 N NaOH solution or in an acid solution of pH 1–6 (6). The cyclization of Compound G₂ also occurred through lyophilization of Compound G₂ fraction obtained by column chromatography on Dowex 50W × 4 and through the purification step on a Sephadex G-10 column chromatography in the course of the present experiments. The results of paper chromatography of the riboflavin preparation obtained through a Dowex 50W × 4 column chromatography and successive lyophilization are given in Table 3. *R_f* values of the preparation are quite identical to those of authentic riboflavin. The same evidence is provided by a similarity in maximum intensity of fluorescence at 520 nm and 530 nm between Compound G₂ and riboflavin, which may indicate the structural similarity of both compounds in the buffer used. Thus, the results indicate the strong possibility

Table 3. Paper chromatography of authentic riboflavin and the riboflavin preparation. The Compound G₂ fraction obtained through column chromatography on Dowex 50W × 4(H⁺) was lyophilized. The dried matter was dissolved in a minimum volume of distilled water and spotted on paper. Chromatography was carried out overnight at room temperature by the ascending method with the indicated solvents. The compounds with indicated R_f values were located as yellow fluorescent spots on the paper under an ultraviolet light.

Solvents ^a	A	B	C	D	E	F	G	H
Riboflavin	0.23	0.18	0.48	0.48	0.52	0.39	0.45	0.51
Preparation	0.22	0.18	0.49	0.48	0.51	0.38	0.44	0.49

^a A, BuOH: EtOH: H₂O (50: 15: 35); B, BuOH: EtOH: 2N NH₃ (20: 2: 5); C, BuOH: AcOH: H₂O (3: 2: 2); D, BuOH: pyridine: H₂O (6: 4: 3); E, *t*-BuOH: pyridine: H₂O (60: 15: 35); F, *t*-BuOH: NH₃: H₂O (60: 5: 35); G, *t*-BuOH: H₂O (60: 40); H, PrOH: NH₃: H₂O (60: 3: 1).

that Compound G₂ is 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine.

Accordingly, it appears likely that the native compound, which is trapped with dimeric diacetyl in cells, is 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine because elimination of dimeric diacetyl from the lumazine moiety of Compound G₂ gives the pyrimidine portion.

However, it remains unclear whether the pyrimidine accumulated is an immediate intermediate to Compound G or a by-product formed in the riboflavin synthetase reaction. The same behaviours in the accumulation of Compound G and Compound G₂ were found in the present work. While, dimeric diacetyl or its derivatives, possibly Compound G₂, was verified to inhibit the riboflavin synthetase reaction in the previous paper (1). If dimeric diacetyl itself inhibits the synthetase reaction, the parallel accumulation of Compound G and Compound G₂ possibly occurs in the case in which Compound G₂ is an intermediate derivative prior to Compound G being a substrate in the riboflavin synthetase reaction. Otherwise, even if a dimeric diacetyl derivative such as Compound G₂ inhibits the reaction, the same trends in the accumulation of both compounds may be observed in cells when Compound G₂ is also considered to be an immediate intermediate derivative to Compound G but not a by-product derivative. If the consideration is accepted, it is possible that the pyrimidine portion on Compound G₂ molecule, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine, is an direct precursor of Compound G in the biosynthetic pathway of riboflavin.

On the other hand, the stimulation effect in lower concentrations and the inhibition effect in higher concentrations of dimeric diacetyl on flavinogenesis were similarly observed even when glucose at 0.5% was further added to the non-growing cell medium under the same experimental conditions. That is, the parallel elevation of riboflavin formation appeared with increasing concentrations of dimeric diacetyl in the presence of 0.5% glucose. Accordingly, the action mechanism of

the dimeric diacetyl in flavinogenesis is considered to be specific, because the effects were not affected even by the addition of glucose which is most effective compound in flavinogenesis of *E. ashbyii* as carbon source and energy source necessary for flavinogenesis. For the enhancement of riboflavin formation in lower concentrations of dimeric diacetyl, the following consideration may be possible: the reutilization reaction of the by-product in the riboflavin synthetase reaction was operative as previously reported (7) so as to further increase riboflavin formation, or the 4-carbon compound required for flavinogenesis was supplied from the added dimeric diacetyl so as to stimulate riboflavin formation in which dimeric diacetyl acts as a 4-carbon compound like the diacetyl effective for the ring closure of the intermediate or the by-product to form 6,7-dimethyl-8-ribityllumazine after its degradation in cells.

In this sense, the effect of dimeric diacetyl is very important. The origin of 4-carbon compound for flavinogenesis is still unclear. Further study on effects of dimeric diacetyl on riboflavin formation may solve the problem.

Fig. 5. Proposed trapping action of dimeric diacetyl on the riboflavin biosynthetic pathway.

In conclusion, the mode of action of dimeric diacetyl on flavinogenesis is schematically depicted in Fig. 5, showing that an immediate precursor to 6,7-dimethyl-8-ribityllumazine is 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine.

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