UDC 547.859 : 582.284 : 545.84

52. **Toru Masuda, Toyokazu Kishi, and Mitsuko Asai :** Application of Chromatography. XXXIV.*[2] Structure of the Purple Fluorescent Substance produced by *Eremothecium ashbyii*.[1]

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

In a previous paper,[2] the authors reported the isolation, properties, and presumed structure of a purple fluorescent substance (V-compound) present in the mycelium of *Er. ashbyii* together with riboflavin, FAD, G-compound (2,4-dihydroxy-6,7-dimethyl-8-ribitylpteridine), and others. By studies conducted thereafter the structure of the photodecomposition product of V-compound was established and the presumed structure of V-compound reported previously was corrected.

Experimental

Synthesis of 2,4-Dihydroxypteridine-6-carboxylic Acid (III)—This compound was synthesized from folic acid through N-[4-{(2,4-dihydroxy-6-pteridylmethyl)nitrosamino}benzoyl]glutamic acid and N-[4-{(2,4-dihydroxy-6-pteridylmethyl)amino}benzoyl]glutamic acid according to the method of Angier, *et al.*,[3] m.p. 345°(decomp.). *Anal.* Calcd. for $C_7H_6O_4N_4$: C, 40.39; H, 1.94; N, 26.92. Found : C, 40.20; H, 2.15; N, 26.58.

Synthesis of 2,4-Dihydroxypteridine-7-carboxylic Acid (IV)[4]—Following the method of Weijlard, *et al.*,[5] hydroxyiminoacetone was boiled with H_2SO_4 and then reacted with 4,5-diaminouracil[6] to give 2,4-dihydroxy-7-methylpteridine. A portion of 1.4 g. of the product was suspended in 50 cc. of water and dissolved by warming with 4 cc. of 2*N* NaOH on a water bath. Two g. of $KMnO_4$ was added in small portions to the solution with stirring and warming on a water bath, when it was decolorized, precipitating MnO_2. After 3 hrs. the precipitate was filtered while hot and extracted repeatedly with hot water. The filtrate was combined with the extracts and, after being adjusted to pH 2.0 with dil. HCl, concentrated under reduced pressure, whereupon about 1 g. of a white precipitate was obtained. The product was recrystallized from dil. HCl and dissolved in dil. NaOH solution, and to the solution was added the same volume of 40% NaOH solution, precipitating the Na salt. This was collected, dissolved in water, and the precipitate deposited by acidifying the solution with dil. HCl was recrystallized again from dil. HCl, m.p. 340°(decomp.). *Anal.* Calcd. for $C_7H_6O_4N_4$: C, 40.39; H, 1.94;N, 26.92. Found : C, 40.15; H, 2.05; N, 26.90.

Table I. Comparison of the Photodecomposition Product of V-compound with 2,4-Dihydroxy-6- and -7-carboxylic Acid in Rf Value in Paper Chromatography and Migration Distance in Paper Ionophoresis

Solvent / Substance	Rf Value (Toyo Roshi No. 5B) AcOH·BuOH·H_2O (1:4:5)	EtOH·BuOH·H_2O (15:50:35)	Pyridine·BuOH·H_2O (3:4:7)	Distance of migration[a] (cm.)
Photodecomposition product of V-compd.	0.34	0.37	0.41	+4.5
2,4-Dihydroxypteridine-6-carboxylic acid (III)	0.19	0.29	0.36	+7.0 cm
2,4-Dihydroxypteridine-7-carboxylic acid (IV)	0.16	0.33	0.33	+7.0 cm

[a] Whatman Filter paper No. 1, pH 7.0 (Theorell buffer), $\mu=0.1$, 300 V, 3 hrs.

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* This constitutes a part of a series entitled "Application of Chromatography" by Satoru Kuwada.

** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨, 貴志豊和, 浅井満子).

1) This Bulletin, **6**, 113(1958).

2) Part XXXIII. T. Masuda, T. Kishi, M. Asai : This Bulletin, **5**, 598(1957).

3) R.B. Angier, J.H. Boothe, J.H. Mowat, C.W. Waller, J. Semb : J. Am. Chem. Soc., **74**, 408(1952).

4) C.K. Cain, M.F. Mallette, E.C. Taylor : *Ibid.*, **70**, 3026(1948).

5) J. Weijlard, M. Tishler, A.E. Erickson : *Ibid.*, **67**, 804(1945).

6) M.T. Bogert, D. Davidson : *Ibid.*, **55**, 1667(1933).

Purification of the Photodecomposition Product of V-Compound—An alkaline solution of V-compound was photodecomposed in the presence of H_2O_2 as in the previous report[2] to give white crystals. The crystals were dissolved in water, the solution was adjusted to pH 3~4 with glacial AcOH, and the resulting fine white crystals were recrystallized from hot water into colorless fine needles. This product as well as those obtained thereafter by repeating the same method all gave only analytical values corresponding to $C_7H_6O_4N_4$ even after repeated recrystallizations from water as reported in the previous paper.[2] However, when the product was recrystallized several times from 2*N* HCl and the resulting slightly yellow and rather large needles were subjected to analysis after drying at 160° for 2 hrs. under reduced pressure, they gave a value almost completely agreeing with $C_7H_6O_3N_4$. *Anal.* Calcd. for $C_7H_6O_3N_4$: C, 43.30; H, 3.12; N, 28.86. Found : C, 43.28; H, 3.22; N, 28.52.

Attempted Oxidation of the Photodecomposition Product of V-Compound with $KMnO_4$—To a solution of 25 mg. of the photodecomposition product in 4 cc. of 0.05*N* NaOH, 1.5 cc. of 2% $KMnO_4$ solution was added. The mixture was heated on a water bath for 30 mins., then 2 cc. of EtOH was added, and the resulting MnO_2 was filtered while hot and thoroughly washed with hot water. The filtrate was combined with the washings, concentrated to 4~5 cc. under reduced pressure, and acidified with dil. AcOH to deposit a white precipitate. Paper partition chromatography of the product proved that it was nothing but the starting material as shown in Table II. The mother liquor gave several fluorescent spots on the paper chromatogram but their study was given up because of their small quantity.

Table II.

Substance \ Solvent	Rf Value AcOH·BuOH·H_2O (1:4:5)	 EtOH·BuOH·H_2O (15:50:35)	 Pyridine·BuOH·H_2O (3:4:7)
Photodecomposition product of V-compound	0.34	0.37	0.40
Attempted oxidation product	0.34	0.38	0.40

Chelate Compound of V-Compound—Making reference to the study of Turner, *et al.*[7] on the chelate compound between sulfosalicylic acid and copper ion, a chelate compound of V-compound with Cu ion was studied. V-Compound and $CuSO_4$ were dissolved in water in various concentrations as shown in Table III, but the concentration of [A]+[B] was 0.02 mole in all the solutions ([A] is the molar concentration of Cu^{++} ($CuSO_4 \cdot 5H_2O$ was used) and [B] is the molar concentration of the chelate reagent (V-compound)). The solutions were adjusted to pH 11.5 with dil. NaOH solution and the resulting bluish green color-tones were measured by their absorption, E, at 680 mμ. As the largest E-value was obtained at [A]/([A]+[B])=0.5, the chelate compound was considered to be composed of 1 mole of V-compound and 1 mole of Cu ion.

Table III.

[A]/([A]+[B])	[A]	[B]	E at 680 mμ
0.2	0.004	0.016	0.148
0.3	0.006	0.014	0.255
0.4	0.008	0.012	0.340
0.5	0.010	0.010	0.415
0.6	0.012	0.008	0.307
0.7	0.014	0.006	0.103
0.8	0.016	0.004	0.037

Decarboxylation of the Photodecomposition Product of V-Compound—a) By heating: A portion of 3 mg. of the photodecomposition product in a small test tube was heated in a metal bath at 200° for 4 hrs., at 250~275° for 7 hrs., or at 280~285° for 5 hrs. The product was examined by paper chromatography and paper ionophoresis, but the Rf value and distance of migration were almost the same as those of the original substance. A sample heated up to 360° also gave the same result.

b) By heating in quinoline : A solution of 20 mg. of the photodecomposition product in 3 cc. of quinoline was refluxed with ca. 50 mg. of Cu powder for 3 hrs. After cool, the mixture was shaken with ether and dil. NaOH solution, the alkaline aqueous layer was concentrated, and subjected to paper chromatography and paper ionophoresis to give the same result as in a). 2,4-Dihydroxypteridine-6-carboxylic acid (III) and -7-carboxylic acid (IV) were also treated in the same way, and the alkaline aqueous layers were examined by paper chromatography. As a result it was found that 2,4-dihydroxypteridine was produced by decarboxylation of the materials.

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[7] E. Turner, R.C. Anderson : *Ibid.*, **71**, 912(1949); cf. P. Job : Compt. rend., **180**, 928(1925); Ann. Chim., [10] **9**, 113(1928).

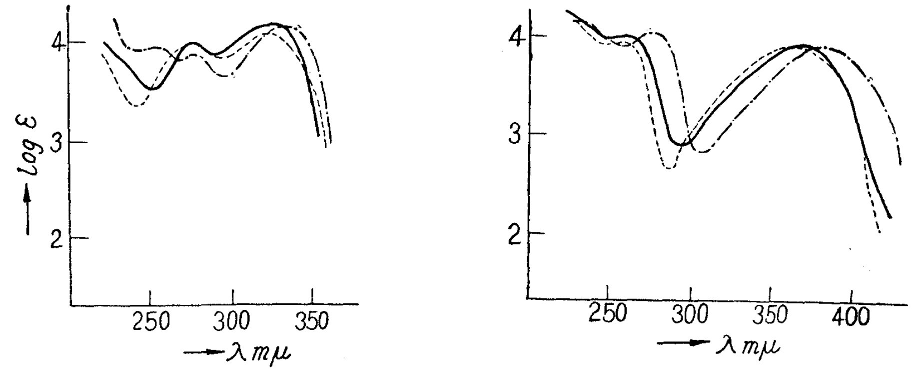
TABLE IV.

Substance \ Solvent	Rf Value		Distance of migration, Whatman filter paper No. 1, pH 7.0 (Theorell buffer), $\mu=0.1$, 300 V, 3 hrs.
	$AcOH \cdot BuOH \cdot H_2O$ (1:4:5)	$EtOH \cdot BuOH \cdot H_2O$ (15:50:35)	
2,4-Dihydroxypteridine-6-carboxylic acid (III)	0.19	0.37	+7 cm
2,4-Dihydroxypteridine-7-carboxylic acid (IV)	0.16	0.29	+7 〃
Decarboxylation product of (III)	0.31	0.56	±0 〃
Decarboxylation product of (IV)	0.32	0.55	±0 〃
2,4-Dihydroxypteridine	0.31	0.56	±0 〃

Synthesis of 2,4,7-Trihydroxy-6-methylpteridine (VIII)—A portion of 500 mg. of 4,5-diaminouracil sulfate[6] was dissolved in 50 cc. of 5% AcOH with warming. To the solution was added 2 cc. of 80% hydrazine hydrate, followed by 1.5 cc. of pyruvic acid, the mixture was heated on a water bath for 1.5 hrs., and left to stand, when pale yellow crystals separated out. The product crystallized from 2*N* HCl into slightly yellow needles, m.p. >360°. Yield, ca. 120 mg. *Anal.* Calcd. for $C_7H_6O_3N_4$: C, 43.30; H, 3.12; N, 28.86. Found: C, 43.18; H, 2.95; N, 28.64.

The product dissolves in $NaHCO_3$ solution with foaming. It is sparingly soluble in water and its aqueous solution exhibits purple fluorescence. When applied on filter paper and sprayed with 5% $AgNO_3$ solution, the spot takes yellow color as observed with V-compound, its photodecomposition product, and other pteridine compounds. It is evident that the product is the expected 6-methyl compound from the fact that its ultraviolet spectrum is in accord with that of the corresponding compound given in the literature[8,9] and also from the theory of Forrest, *et al.*[10] that an α-ketol reacts with a 4,5-diaminopyrimidine compound in the presence of hydrazine to give the corresponding 6-substituted pteridine compound.

Synthesis of 2,4,6-Trihydroxy-7-methylpteridine (XIII)—A solution of 2 g. of 4,5-diaminouracil sulfate in 200 cc. of 2*N* H_2SO_4 was heated with 5 cc. of pyruvic acid on a water bath for 1 hr. The pale yellow precipitate was purified by recrystallization from 250 times its weight of 2*N* HCl to slightly yellow scales, m.p. >360°. Yield, ca. 0.5 g. *Anal.* Calcd. for $C_7H_6O_3N_4$: C, 43.30; H, 3.12; N, 28.86. Found: C, 43.58; H, 2.95; N, 28.58.



2,4,7-Trihydroxy-6-methylpteridine 2,4,6-Trihydroxy-7-methylpteridine

Fig. 1. Ultraviolet Absorption Spectra of 2,4,7-Trihydroxy-6-methylpteridine and 2,4,6-Trihydroxy-7-methylpteridine at various pH's.

——— in aq. soln. – – – – in 0.05*N* HCl —·—·— in 0.05*N* NaOH

Discussion

In the preceding paper[2] the authors reported the extraction, purification, and properties of the purple fluorescent substance (V-compound) produced by *Er. ashbyii* and assigned the structures (I) and (II), respectively, to the substance and its photodecomposition product, based on various experimental results.

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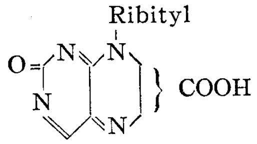
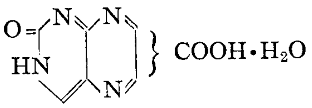
[8] R. Tschesche, F. Korte: Chem. Ber., **84**, 801(1951).

[9] Y. Hirata, *et al.*: Experientia, **8**, 339(1952); J. Am. Chem. Soc., **75**, 4446(1953).

[10] H. S. Forrest, J. Walker: J. Chem. Soc., **1949**. 2077.

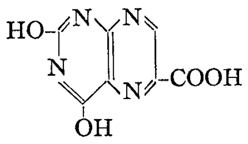


(I)
V-Compound

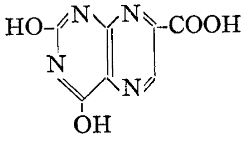


(II)
Photodecomposition product

V-Compound occurs as colorless needles, m.p. 263°(decomp.), $[\alpha]_D^{20}$ +4.5°(H_2O). The molecular weight of the compound is about 300 when measured by the Barger method, titration, and polarography, and its analytical values correspond to $C_{12}H_{16}O_7N_4$. From its ultraviolet spectrum and color reaction with $AgNO_3$ the compound is assumed to be a pteridine derivative. Also, judging from its $[\alpha]_D$, formation of a tetraacetate, formation of formaldehyde by treatment with lead tetraacetate, and photodecomposition, it seems to have a sugar group, ribityl. As the compound produces urea by its alkali decomposition, it appears to have a hydroxyl group at C-2 of the pteridine ring and a side chain at one of the nitrogens in the ring. The photodecomposition product of the compound seems to have been produced by splitting off of the sugar group. From its paper ionophoresis, infrared spectrum, and property to dissolve in $NaHCO_3$ solution with foaming, the product is assumed to be an acid substance probably due to the presence of a carboxyl group. However, as there was a doubt that the product might be 2,4-dihydroxypteridine-6-carboxylic acid (lumazine-6-carboxylic acid)(III) or 2,4-dihydroxypteridine-7-carboxyl acid (IV), the two compounds were synthesized and compared with the product, but neither of them coincided with the latter in infrared spectrum, decomposition point, behaviors, and paper ionophoresis and paper chromatography by various solvent systems.

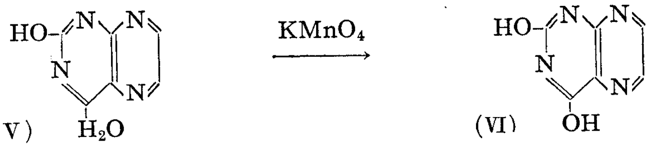


(III)
Lumazine-6-carboxylic acid



(IV)
Lumazine-7-carboxylic acid

Brown and Mason[11] formerly reported that when 2-hydroxypteridine (V) has fast-combining water of crystallization, its mild oxidation gives lumazine by the entrance of a hydroxyl group into the 4-position. If the photodecomposition product has the structure of (II), its oxidation ought to give 2,4-dihydroxypteridine-6- or -7-carboxylic acid according to the above theory, but only the starting material was recovered in the experiment.



(V) 2-Hydroxypteridine·H_2O

(VI) Lumazine

According to Tschesche and Korte,[8] 2,4,7-trihydroxypteridine-6-acetic acid (VII) and 2,4,7-trihydroxypteridine-6-carboxylic acid (IX) are decarboxylated to 2,4,7-trihydroxy-6-methylpteridine (VIII) and 2,4,7-trihydroxypteridine (X), respectively, only by heating at 280°, and 2,4-dihydroxypteridine-6-carboxylic acid (III) and -7-carboxylic acid (IV) are readily decarboxylated to lumazine (VI) by heating with copper powder in quinoline, whereas the photodecomposition product of V-compound is never affected under such conditions.

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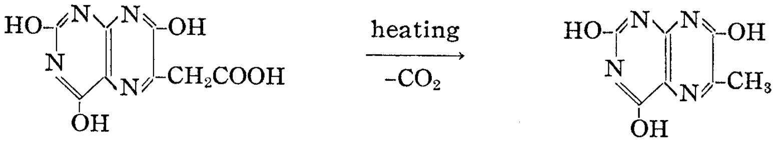
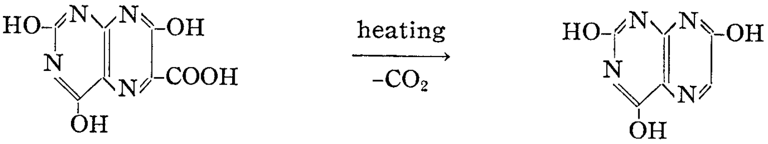
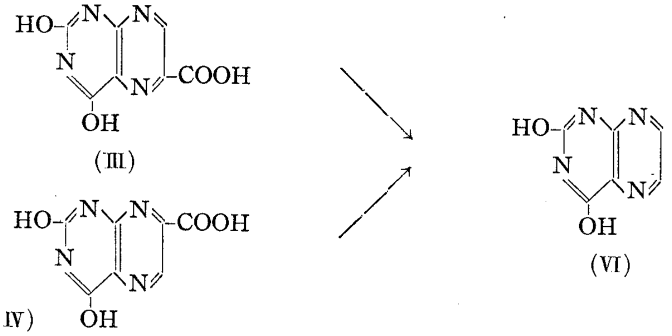
[11] D. J. Brown, S. F. Mason: J. Chem. Soc., **1956**, 3443.



(VII) (VIII)



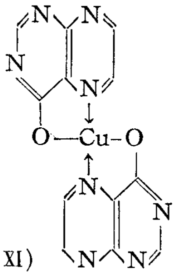
(IX) (X)



(III) (IV) (VI)

As already described in a previous report the authors attempted the above experiment, but the negative result made the authors hesitate to draw a definite conclusion about the structure of the photodecomposition product. Since, however, the same negative result was obtained by subsequent repeated experiments, it has become doubtful that the product has a carboxyl group.

According to Albert,[12] pteridine having a hydroxyl group at the 4-position forms such a chelate compound as shown by (XI) with various metal ions, for example, Cu^{2+}, Fe^{2+}, and Co^{2+}. As mentioned in the experimental part, the authors conducted the following experiment according to the method of Turner, *et al.*[7] Copper ion was added at pH 11.5 to an aqueous solution of V-compound, which exhibits absorption maxima at 289 and 346 mμ in an alkaline pH, when the latter came to have a new absorption at 680 mμ. The photodecomposition product of V-compound also gave the same result. In this case, if [A]/([A]+[B]) was changed, E-value of V-compound at the wave length became maximum when [A]/([A]+[B]) was 0.5. This fact shows that V-compound formed a chelate compound with copper ion in a form other than that reported by Albert. The structure of the chelate compound is now under study, but at least the formation of this compound makes it appropriate to think that V-compound has a hydroxyl group at C-4 of the pteridine ring.



(XI)

Albert[13] listed melting points and solubilities in water of pteridine and its various

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[12] A. Albert: Biochem. J., **47**, ix, xxvii (1950); **54**, 646(1953).

[13] A. Albert: Quart. Revs. (London), **6**, 197(1952).

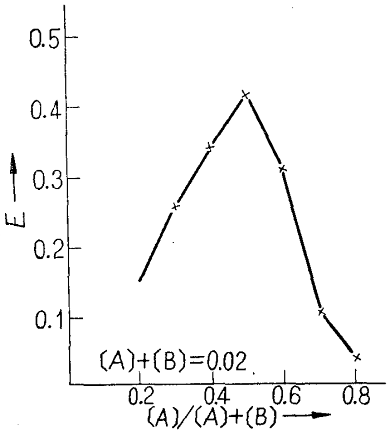


Fig. 2.
Formation of a Chelate Compound of V-Compound with Cu^{2+}

[A] = mol. concn. of Cu^{2+}
[B] = mol. concn. of V-compound

derivatives and showed that when these compounds have no substituent involving hydrogen bonding, they are readily soluble in water and have comparatively low melting points, whereas when they have an amino or hydroxyl group, they melt at high temperatures with decomposition, and especially when they have more than two of such substituents, they come to decompose at temperatures over 300° and their solubilities in water are markedly decreased. The fact that while the photodecomposition product of V-compond has a m.p. above 360°, V-compound itself melts at 263°, may be explained by thinking that the latter is an N-substituted compound of the former. The same relation was observed between 7-hydroxypteridine and its N-methyl derivative. Also, the high melting point and sparing solubility in water of the photodecomposition product seem to show that it is a di- or tri-substituted compound. In general, hydroxypteridines are acidic compounds and can be titrated with alkali. For example, 2,4,7-trihydroxy-6-methylpteridine has a pK of 3.95 and behaves like an acid (Fig. 3). V-Compound is also an acid substance and its 3% aqueous solution exhibits pH 2.42, and its pK calculated from the curve obtained by titration with 0.1*N* NaOH is 3.95.

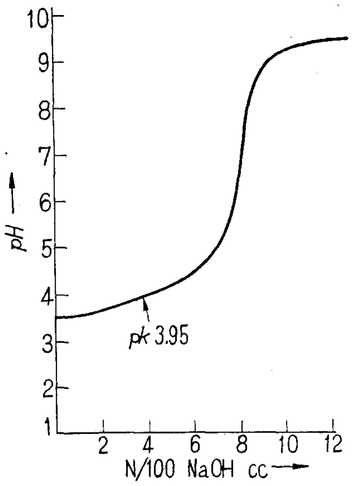


Fig. 3.
Alkali Titration Curve of 2,4,7-Trihydroxy-6-methylpteridine

In the previous paper the photodecomposition product was presumed to have a carboxyl group from the explanation of its infrared spectrum, but the carbonyl absorption at 5.9 μ may be due to an α-amide group, whose C=O absorption shifts toward a lower frequency like that of the carboxyl group on forming a salt as observed in the infrared spectrum of the Na salt of 2,4,7-trihydroxy-6-methylpteridine (cf. Figs. 5 and 6).

From the consideration there arises a strong possibility that the photodecomposition product may be a derivative of trihydroxypteridine.

Table V. Comparison by Paper Chromatography

Substance \ Solvent	Rf value			Distance of migration (cm.) $\mu=0.1$, 300 V, 3.5 hrs. (Theorell buffer)		
	$AcOH \cdot BuOH \cdot H_2O$ (1:4:5)	$EtOH \cdot BuOH \cdot H_2O$ (15:50:35)	$pyridine \cdot BuOH \cdot H_2O$ (3:4:7)	pH9.0	pH7.0	pH4.0
Photodecomposition product of V-compd.	0.34(v)	0.37(v)	0.41(v)	+4.5	+5.0	+3.2
Synthetic 6-methyl compd. (VIII)	0.33(v)	0.36(v)	0.40(v)	+4.5	+4.7	+3.2
Synthetic 7-methyl compd. (XIII)	0.38(lv)	0.42(lv)	0.42(lv)	+4.5	+4.7	+3.2

(v) purple fluorescence (lv) light purple fluorescence

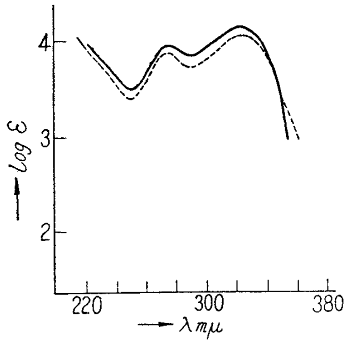


Fig. 4.
Comparison of Ultraviolet Absorption Spectra

—— Synthetic 6-methyl compound in aq. soln.
------ Photodecomposition product of V-compound

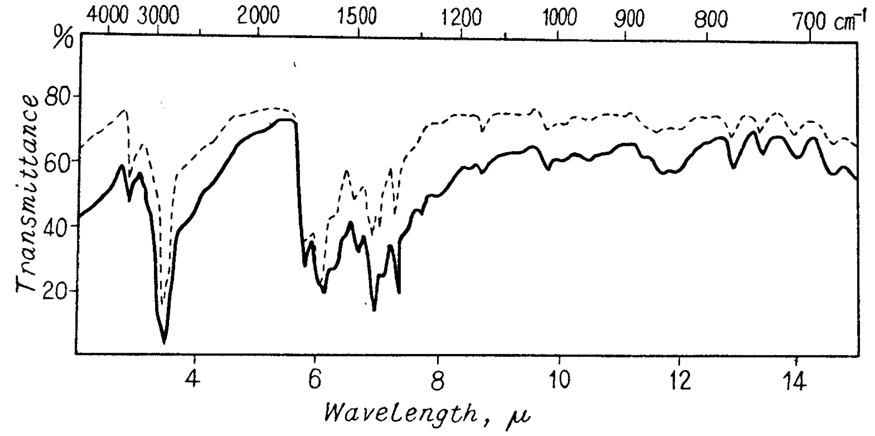


Fig. 5. Comparison of Infrared Absorption Spectra
—— Synthetic 2,4,7-trihydroxy-6-methylpteridine
------ Photodecomposition product of V-compound

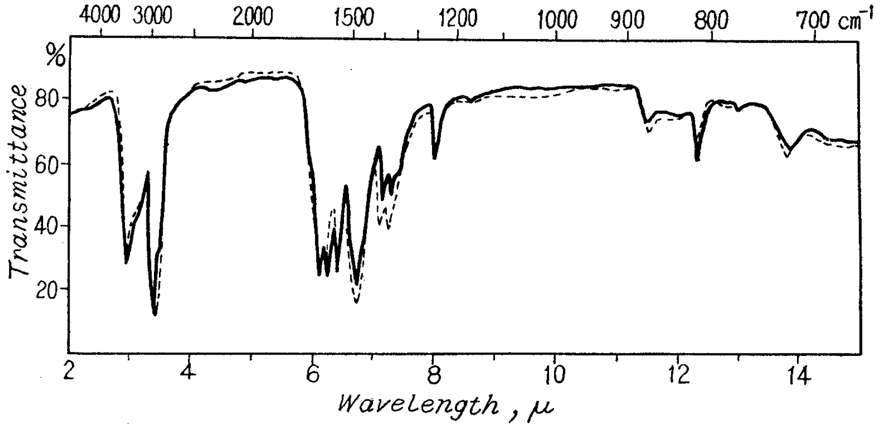
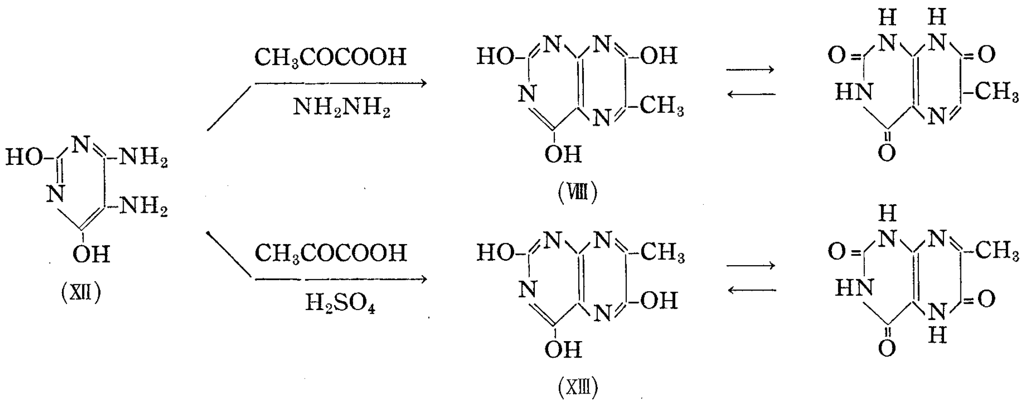


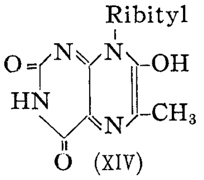
Fig. 6. Comparison of Infrared Absorption Spectra
—— Na-salt of synthetic 2,4,7-trihydroxy-6-methylpteridine
------ Na-salt of photodecomposition product of V-compound

As the analytical data of the photodecomposition product were similar to the theoretical value of 2,4,7-trihydroxy-6-methylpteridine (VIII) or its isomer, 2,4,6-trihydroxy-7-methylpteridine (XIII), (VIII) was synthesized by reacting 2,4-dihydroxy-5,6-diaminopyrimidine (diaminouracil) (XII) with pyruvic acid in the presence of hydrazine hydrate, and (XIII) by the same method but in dil. sulfuric acid and without hydrazine, and they were compared with the photodecomposition product. This result showed that (XIII) is in complete agreement with (VIII) in Rf value, and ultraviolet and infrared spectra. The sodium salt of (VIII) was also in accord with the sodium salt of the photodecomposition product in infrared spectrum (See Table V, Figs. 4∼6).



In addition, the photodecomposition product was in complete agreement with (VIII) in various other properties. For example, they have the same decomposition point, solubility in water, purple fluorescence, and behavior to 5% $AgNO_3$ and $NaHCO_3$ solution.

From the results mentioned above the photodecomposition product was established as 2,4,7-trihydroxy-6-methylpteridine (VIII) and from the conclusion the structure of V-compound is presumed to be (XIV). When thus considered, the conversion of V-com-



(XIV)

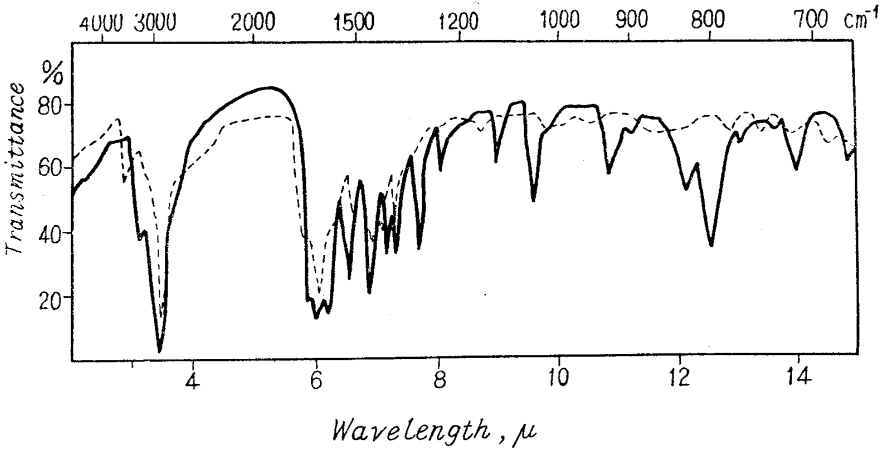


Fig. 7. Comparison of Infrared Absorption Spectra
—— 2,4,6-Trihydroxy-7-methylpteridine
------ Photodecomposition product of V-compound

pound($C_{12}H_{16}O_7N_4$) to its photodecomposition product ($C_7H_6O_3N_4$)[14] by elimination of $C_5H_{10}O_4$ can be explained without thinking of a simultaneous dehydrogenation.

The authors formerly reported that reaction *in vitro* of G-compound (2,4-dihydroxy-6,7-dimethyl-8-ribitylpteridine)[15] with diacetyl or acetoin readily produced riboflavin, and Katagiri, *et al.*[16] also confirmed the formation of riboflavin from G-compound by the action of an enzyme solution or washed cells of *E. coli*, microörganisms for acetone-butanol fermentation, etc. The authors therefore consider the process of the formation of V-compound in the mycelium and its relation with G-compound to be as shown in Chart 1.

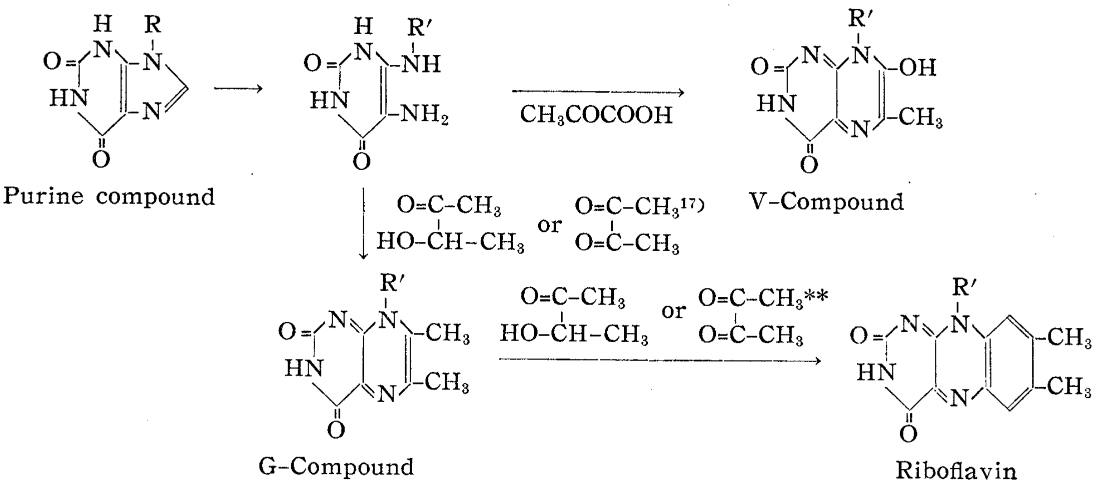


Chart 1.

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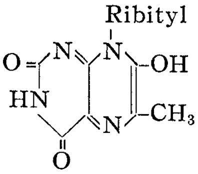
Summary

Basing on various experimental results the authors corrected the conclusion made in previous papers.[1,2] In the present work the photodecomposition product of V-compound produced by *Er. ashbyii* was established to be 2,4,7-trihydroxy-6-methylpteridine, and therefore the structure of V-compound was presumed to be as shown below.



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[14] The empirical formula was reported as $C_7H_4O_4N_4$ in the previous paper but it is now corrected to $C_7H_6O_3N_4$ according to the analytical values of the preparation purified by repeated crystallizations from dil. HCl.

[15] T. Masuda : This Bulletin, **5**, 136(1957).

[16] H. Katagiri, K. Imai, I, Takeda, H. Yamada : Vitamins (Kyoto), **12**, 480(1957).

[17] It is a well known fact that acetoin or diacetyl is reversible with pyruvic acid in the living body.