Isolation and Characterization of Pteridines from *Pseudomonas ovalis*

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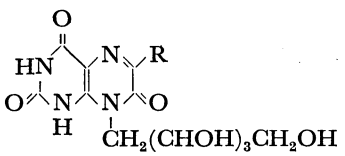
Four ribityllumazine compounds were isolated from the cultured medium of *Pseudomonas ovalis*. Three of them were characterized as 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine, 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine and 6-(*p*-hydroxyphenyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine by comparison with synthetic samples. The fourth compound, named *putidolumazine*, was supposed to be a new compound (2-carboxyethyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine from its chemical and spectroscopic properties. This was confirmed from the identity of both natural and synthetic products.

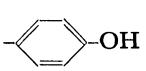
6,7-Dimethyl-8-(1-D-ribityl)-2,4-dioxohexahydropteridine (Compound G)[1] and 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (Compound V) (IV)[2] were isolated from *Eremothecium ashbyii* as a green and a purple fluorescent materials, respectively. Recently, two new ribityllumazines were isolated from *Achromobacter petrophilum* by Takeda and Hayakawa.[3] One of them was identified as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (III)[4] and the other was supposed to be 6-(*p*-hydroxyphenyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (II), the structure being proposed on the basis of biosynthesis.[5]

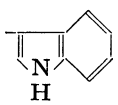
The function of ribityllumazines was recently clarified; *i.e.* Compound G is an intermediate of riboflavin and Compound V functions as an inhibitor.[6] Tyrosine and tryptophan have been found to be effectively incorporated into II and III.[5]

This paper describes the isolation of four ribityllumazine compounds from cultures of *Pseudomonas ovalis*. Three of them have been identified as compounds II, III, and IV. The last compound (*Putidolumadine*) is assumed to be a new ribityllumadine compound, 6-(2-carboxyethyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (I), from spectroscopic and chemical data. This was confirmed by the synthesis from 2,6-dihydroxy-5-nitro-4-D-ribitylaminopyrimidine and α-ketoglutaric acid.



(I) R: $-CH_2CH_2COOH$

(II) R: 

(III) R: 

(IV) R: $-CH_3$

Experimental

Isolation of Ribityllumazines. *Pseudomonas ovalis* (IAM 1506) was cultivated for 10 days in a fermentation jar (capacity: 20 *l*) at 32°C with aeration. The medium used was 0.75% casein enzymatic hydrolysate (10 *l*); the solution was adjusted to pH 7.0 with ammonia. After centrifugation the culture was concentrated to about 100 m*l* *in vacuo* below 40°C. Ribityllumazines were separated by conventional chromatographic methods using a cellulose column (7×25 cm) and isopropyl alcohol-1% ammonia (2:1) as a solvent. The fluorescent compounds were separated into two bands, *i.e.* a band containing green, purple and blue fluorescent compounds (Fraction 1), and a band containing a greenish-blue fluorescent compound (Fraction 2). The two fluorescent bands were eluted and the eluates were evaporated to dryness *in vacuo* at below 40°C. The residues were again purified chromatographically using a cellulose column (5×12 cm; solvent: water) for Fraction 2 and a DEAE-cellulose column (5×22 cm; solvent: 1 *l* of water, followed by 0.003 N hydrochloric acid) for Fraction 1. The greenish-blue fluorescent compound from Fraction 2 was eluted and eluate was evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of water, and acetone was added. The precipitate was washed with cold water and dried over P_2O_5; yield, 3 mg (UV: $\lambda_{max}^{0.1N\ NaOH}$ mμ, 260, 307, 403; $\lambda_{max}^{H_2O}$ mμ, 215, 299, 377; $\lambda_{max}^{0.1N\ HCl}$ mμ, 240, 295, 365). This compound was identified as 6-(*p*-hydroxyphenyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (II) by comparison with synthetic material (see blow) and also with Compound GB, which was isolated from *Achromobacter petrophilum*[3].

TABLE 1. PAPER CHROMATOGRAPHY AND ELECTROPHORESIS OF PTERIDINES

Substance	Solvents[a]				Electrophoresis[b]
	A	B	C	D	
Greenish-blue fluorescent compound	0.23	0.19	0.11	0.10	7
After KIO_4 oxidation and $NaBH_4$ reduction	0.37	0.26	0.09	0.22	6
Compound GB	0.23	0.19	0.11	0.10	7
II	0.23	0.19	0.11	0.10	7
VI	0.37	0.26	0.09	0.22	6

a) Solvents: A. isopropyl alcohol: 2% ammonium acetate (1:1)
B. isopropyl alcohol: 1% ammonia (2:1)
C. 3% aqueous ammonium chloride
D. *n*-butyl alcohol: acetic acid: water (4:1:1)

b) Distance (in mm) to anode after paper electrophoresis at pH 4.65 (sodium acetate buffer) for 60 min at 18V/cm.

1) T. Masuda, *Pharm. Bull. Jap.*, **5**, 375 (1958).
2) T. Masuda, T. Kishi, and M. Asai, *ibid.*, **6**, 291 (1958).
3) I. Takeda and S. Hayakawa, *Agr. Biol. Chem.*, **32**, 873 (1968).
4) W. S. McNutt and I. Takeda, *Biochemistry*, **8**, 1370 (1969).
5) I. Takeda, *Hakko Kyokaishi*, **27**, 305 (1969).
6) G. W. E. Plaut, *J. Bio. Chem.*, **238**, 2225 (1963).

The compound thus obtained (*ca.* 1 mg) was dissolved in 0.2 m*l* of water and 4 mg of potassium periodate was added. After one hour the product was purified by paper chromatography using water as a solvent. The oxidation product was then reduced with a small amount of sodium borohydride in 0.1 m*l* of 1% ammonia. The solution was kept standing at room temperature for 30 min, then adjusted to pH 1 with hydrochloric acid. The product was again purified by paper chromatography using water as a solvent. The final product was identified as 6-(*p*-hydroxyphenyl)-8-(2-hydroxyethyl)-2,4,7-trioxohexahydropteridine (VI) by comparison with synthetic material (see Table 1).

The material from Fraction 1 was separated into two purple fluorescent and one greenish-yellow fluorescent bands on a DEAE-cellulose column. The greenish-yellow compound was further purified by column chromatography using a cellulose column (7×25 cm) and isopropyl alcohol-1% ammonia (2:1) as solvent; yield, *ca.* 30 mg. The material was recrystallized twice from 50% acetic acid to give yellow crystals; yield, 15.3 mg, mp 285—286°C (decomp.) (UV: $\lambda_{max}^{0.1N\ NaOH}$ mμ, 271, 308, 401; $\lambda_{max}^{H_2O}$ mμ, 213, 312, 398; $\lambda_{max}^{0.1N\ HCl}$ mμ, 216, 317, 390).

Found: C, 50.40; H, 4.39; N, 14.50%. Calcd for $C_{19}H_{19}O_7N_5 \cdot H_2O$: C, 51.00; H, 4.73; N, 15.65%.

The compound was identified as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (III) by comparison with synthetic material (see below) and also with Compound GY, which was isolated from *Achromobacter petrophilum* by McNutt and Takeda[4] (Table 2). The purple fluorescent compound was identified as 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (IV) by comparison with Compound V of Masuda[2] (Table 2). The yield was *ca.* 2 mg.

Table 2. Paper chromatography and electrophoresis of pteridines

Substance	Solvents[a]				Electrophoresis[b]
	A	B	C	D	
Greenish-yellow fluorescent compound	0.13	0.13	0.02	0.11	2
Purple fluorescent Compound	0.57	0.35	0.62	0.14	23
Compound GY	0.13	0.13	0.02	0.11	2
III	0.13	0.13	0.02	0.11	2
Compound V	0.57	0.35	0.62	0.14	23

a) and b): See the footnote in Table 1.

Another purple fluorescent compound (putidolumazine) was further purified by conventional chromatographic methods using the following column and solvent: 1. a cellulose column (7×25 cm); developer: isopropyl alcohol-1% ammonia (2:1), and 2. a Sephadex G-25 column (5.5×25 cm); developer: water. The crude product (30 mg) from 30 *l* of the cultured medium was recrystallized twice from water to give colorless needles; yield, 8 mg; mp >265°C (decomp.).

Found: C, 42.27; H, 4.44; N, 14.08%. Calcd for $C_{14}H_{18}O_9N_4 \cdot H_2O$: C, 41.58; H, 4.95; N, 13.86%, The compound contained neither S nor P. The sample was dried over P_2O_5 for 10 hr at 100—110°C and 3 Torr. *Rf*-values and UV-spectra of putidolumazine are given in Table 3 and Fig. 1.

Reaction of Putidolumazine. Esterification. Putidolumazine (15 mg, recovered from mother liquid) dissolved when it was suspended in absolute ethanol and saturated with dry HCl gas. The reaction mixture was concentrated *in vacuo* below 40°C and purified by chromatography using a cellulose column (5×25 cm; developer: 50% isopropyl alcohol). The eluate was evaporated to dryness *in vacuo* below 40°C, and pale brown precipitate was obtained by recrystallization from a ethanol - ether; yield, 12.4 mg. Recrystallization of the product from ethanol gave a faint yellow product; yield, 5.4 mg, mp 117—120°C; additional crops (*ca.* 5 mg) were also obtained from the mother liquid.

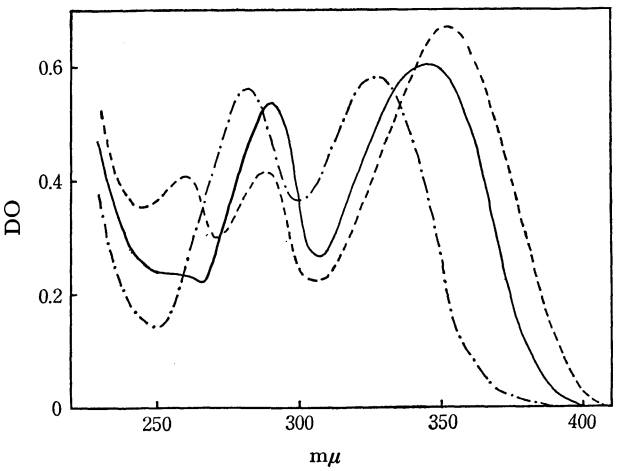


Fig. 1. Ultraviolet absorption spectrum of putidolumazine.
------: In 0.1N NaOH
——: In water
- · -: In 0.1N HCl

Found: N, 14.06%. Calcd for $C_{16}H_{22}O_9N_4$: N, 13.52%.

Acetylation of the Esterified Product: To a mixture of 1 m*l* of pyridine and 1 m*l* of acetic anhydride, 10 mg of the esterified product was added, and the whole was left standing at room temperature. After 2 days the reaction mixture was concentrated to dryness *in vacuo* below 40°C. Pale brown material was obtained by recrystallization from ethyl acetate-ether; yield, 6 mg. Mass spectrum of this compound is shown in Fig. 2.

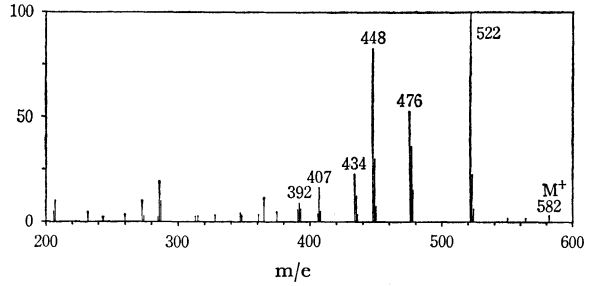


Fig. 2. Mass spectrum of acetylated putidolumazine-ester.

Periodate Oxidation: One milliliter of aqueous solution of putidolumazine (3.85×10^{-5} M) was treated with 2 m*l* of aqueous potassium metaperiodate solution (12.9×10^{-5} M, pH 4.2) at room temperature. At intervals the amount of understroyed periodate was estimated from UV-absorption (223 mμ) by the method of Dixon and Lipkin.[7] The compound (M, 386) consumed 4.70 mol of periodate per mole. For the sake of comparison, an authentic sample of Compound V of Masuda was subjected to periodate oxidation; it consumed 4.25 mol of periodate per mole.

$NaBH_4$ Reduction of the Periodate Oxidation Product: Putidolumazine (*ca.* 2 mg) was dissolved in 0.4 m*l* of water and 4 mg of KIO_4 was added. After 1 hr, the oxidation

7) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

product was purified by paper chromatography using *n*-butyl alcohol-acetic acid-water (4:1:1) and isopropyl alcohol-water (2:1) as solvents. The periodate oxidation product was dissolved in 0.3 m*l* of warm water. The solution was cooled and a small amount of $NaBH_4$ was added. After standing at room temperature for 30 min, the solution was adjusted to pH 1 with hydrochloric acid and chromatographed on paper with a solvent of isopropyl alcohol-1% ammonia (2:1); the product was indistinguishable from synthetic 6-(2-carboxyethyl)-8-(2-hydroxyethyl)-2,4,7-trioxohexahydropteridine (V) (see below) (Table 3).

Photodecompositon: Putidolumazine (2.5 mg) was dissolved in 3 m*l* of 0.6 N NaOH. After addition of 0.3 m*l* of 30% H_2O_2, the solution was irradiated in a glass tube with an electric lamp of 100 W from a distance of 15 cm for 20 hr. The decomposition product was purified by paper chromatography using *n*-butyl alcohol-acetic acid-water (4:1:1) and isopropyl alcohol-1% ammonia (2:1) as solvents. The product was identified as 6-(2-carboxyethyl)-2,4,7-trioxohexahydropteridine (VII) by comparison with synthetic material (Table 3).

Table 3. Paper chromatography and electrophoresis of pteridines

Substance	Solvents[a]				Electrophoresis[b]
	A	B	C	D	
Putidolumazine	0.45	0.31	0.73	0.12	34
After KIO_4 oxidation and $NaBH_4$ reduction	0.55	0.37	0.68	0.23	39
After photoirradiation	0.36	0.28	0.47	0.21	32
After esterification	0.76	0.75	0.73	0.43	17
I	0.45	0.31	0.73	0.12	34
V	0.55	0.37	0.68	0.23	39
VII	0.36	0.28	0.47	0.21	32
Hydrolysis product of VIII—IX	0.39	0.21	0.61	0.04	68
Al-Hg reduction product of IX	0.52	0.60	0.65	0.12	38

a) and b): See the footnote in Table 1.

6-(2-Carboxyethyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (I). 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine[8] (0.4 g) in water (50 m*l*) was hydrogenated over a platinum oxide catalyst (0.2 g). After the theoretical amount of hydrogen was taken up, the catalyst was removed by filtration. The solution was acidified with acetic acid. (1 m*l*). α-Ketoglutaric acid (0.4 g) was added and the mixture was heated for 30 min on a steam bath. The solution was concentrated *in vacuo* to about 10 m*l* below 40°C, and acetone was added to the residue. The resulting pale yellow precipitate (280 mg) was purified by chromatography using a DEAE-cellulose column (3.5×18 cm; developer: 0.01 N HCl). A purple fluorescent band was eluted and the eluate was concentrated *in vacuo* below 40°C. The residue was recrystallized twice from water-ethanol to give colorless needless, yield, 130 mg (25.8%), mp >265°C (decomp.).

Found: C, 40.96; H, 4.73; N, 14.03%. Calcd for $C_{14}H_{18}O_9N_4 \cdot H_2O$: C, 41.58; H, 4.95; N, 13.86%.

6-(p-Hydroxyphenyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (II). 2,6-Dihydroxy-5-nitro-4-ribitylaminopyrimidine[8] (0.4 g) in water (50 mg) was hydrogenated over platinum oxide (0.2 g). After the theoretical amount of hydrogen was absorbed, the catalyst was removed. After acidification with acetic acid (1 m*l*), *p*-hydroxyphenylglyoxlic acid[9] (0.4 g) was added to the solution and the mixture was heated for 30 min on a steam bath. The solution was filtered and the filtrate was left standing overnight at 4°C. The yellow precipitate (250 mg) was recrystallized twice from water to give pale yellow crystals, yield, 140 mg (26.4%), mp 272—273°C (decomp.).

Found: C, 47.75; H, 4.77; N, 12.64%. Calcd for $C_{17}H_{18}O_8N_4 \cdot H_2O$: C, 48.11; 4.75; N, 13.20%.

6-(3-Indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (III). 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine[8] (0.2 g) in water (25 m*l*) was hydrogenated over platinum oxide (0.1 g). After the theoretical amount of hydrogen was taken up, the catalyst was removed. The solution was acidified with acetic acid (0.2 m*l*) and warmed. 3-Indolylglyoxylic acid[10] (0.12 g) in ethanol (3 m*l*) was added drop by drop, and the mixture was heated on a steam bath for 30 min. The solution was left standing overnight at 4°C. The brown product (96 mg) was recrystallized twice from 2 N HCl to give a pale yellow substance, yield, 60 mg (21.4%), mp 285—286°C (decom.).

Found: C, 50.77; H, 4.46; N, 15.61%. Calcd for $C_{19}H_{19}O_7N_5 \cdot H_2O$: C, 51.00; H, 4.73; N, 15.65%.

6-(2-Carboxyethyl)-8-(2-hydroxyethyl)-2,4,7-trioxohexahydropteridine (V). 5-Nitroso-6-(β-hydroxyethylamino)-2,4-dihydroxypyrimidine[11] (300 mg) was suspended in 5 m*l* of water, and sodium hydrosulfite (0.8 g) was added. When the solution became pale yellow, acetic acid (1 m*l*) and α-ketoglutaric acid (0.3 g) were added; the reaction mixture was heated on a steam bath for 1 hr. The solution was filtered and the filtrate was left standing overnight at 4°C. The needles (200 mg) thus obtained were further recrystallized from 50% acetic acid, yield, 125 mg (28.2%), mp 262—265°C.

Found: C, 45.07; H, 4.25; N, 19.17%. Calcd for $C_{11}H_{12}O_6N_4$: C, 44.60, H, 4.08; N, 18.91%.

6-(p-Hydroxyphenyl)-8-(2-hydroxyethyl)-2,4,7-trioxohexahydropteridine (VI). 5-Nitroso-6-(β-hydroxyethylamino)-2,4-dihydroxypyrimidine[10] (70 mg) was suspended in 1 m*l* of water and sodium hydrosulfite (150 mg) was added. When the solution became pale yellow, acetic acid (1 m*l*) and *p*-hydroxyphenylglyoxylic acid[9] (85 mg) were added and the solution was heated on a steam bath for 1 hr. The solution was filtered and the filtrate was left standing overnight at 4°C. The pale yellow product (79 mg) was recrystallized twice from 70% ethanol, yield, 32 mg (28.9%), mp>300°C.

Found: C, 50.74; H, 3.95; N, 16.54%. Calcd for $C_{14}H_{12}O_5N_4 \cdot H_2O$: C, 50.30; H, 4.22; N, 16.76%.

6-(2-Carboxyethyl)-2,4,7-trioxohexahydropteridine (VII). 4,5-Diaminouracil sulfate (250 mg) was dissolved in 20 m*l* of 1N NaOH by warming. α-Ketoglutaric acid (150 mg) was added and the mixture was heated on a steam bath for 1 hr. The solution was adjusted to pH 1 with concd. HCl and left standing at room temperature. The yellow precipitate (200 mg) was recrystallized from 1 N HCl to give pale yellow plates, yield, 50 mg (20.9%), mp 259—263°C (decomp.).

Found: C, 42.82; H, 2.68; N, 22.15%. Calcd for $C_9H_8O_5N_4$: C, 42.86; H, 3.20; N, 22.22%.

6-Ethoxycarbonyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (VIII). Zinc powder (150 mg) was added to a suspension of

8) R. M. Cresswell and H. C. S. Wood, *J. Chem. Soc.*, **1960**, 4768.

9) M. Businelli, *Farm. Sci. e tec. (Paria)*, **5**, 522 (1950); *Chem. Abstr.*, **45**, 3819f (1951).

10) K. N. F. Shaw, A. McMillan, A. G. Gudmondson, and M. D. Armstrong, *J. Org. Chem.*, **23**, 1171 (1958).

11) W. S. McNutt, *J. Amer. Chem. Soc.*, **82**, 217 (1960).

4-ribitylamino-5-phenylazouracil[12] (150 mg) in water; the mixture was heated at 100°C for 10 min. Five N H_2SO_4 was added until the solution became clear. The solution was filtered once and adjusted to pH 1 with 4N NaOH. Diethyl mesoxalate (300 mg) was added and the solution was heated on a steam bath for 30 min. The solution was left standing at room temperature for 2 days. The pale yellow needles (52 mg, 32.8%) were recrystallized from ethanol to give faint yellow needles, mp 128—129°C.

Found: C, 43.74; H, 4.71; N, 15.30%. Calcd for $C_{14}H_{18}O_9N_4$: C, 43.52; H, 4.70; N, 14.50%.

Table 4. UV absorption maxima (mμ) of synthetic pteridines

Compound	max ($10^{-3}\varepsilon$ in parenthesis)	solvent
I	260(8.12), 288(8.31), 352(13.4)	0.1NNaOH
	289(10.7), 345(12.2)	H_2O
	282(11.2), 328(11.6)	0.1N HCl
II	260(12.7), 307(12.3), 403(22.1)	0.1NHaOH
	215(33.7), 299(12.0), 377(20.0)	H_2O
	240(14.4), 295(10.8), 365(19.7)	0.1N HCl
III	271(11.3), 308(10.3), 401(19.5)	0.1NNaOH
	213(39.1), 312(10.8), 398(18.6)	H_2O
	216(26.1), 317(8.38), 390(16.8)	0.1N HCl
V	258(6.00), 290(9.98), 350(13.9)	0.1NNaOH
	287(11.4), 341(12.8)	H_2O
	282(12.8), 328(13.1)	0.1N HCl
VI	259(12.9), 307(13.5), 401(21.4)	0.1NNaOH
	213(39.7), 298(13.4), 377(22.6)	H_2O
	239(14.6), 295(11.7), 364(20.0)	0.1N HCl
VII	279(9.55), 330(15.3)	0.1NNaOH
	278(10.3), 327(15.0)	H_2O
	278(11.1), 325(13.8)	0.1N HCl

Results and Discussion

Four pteridine compounds were isolated from the culture of *Pseudomonas ovalis*. Three of them were the pteridines already described, *i.e.* a purple fluorescent compound was identified as 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine[2] (Table 2), a greenish-yellow fluorescent compound as 6-(3-Indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine[4] (Table 2) and a greenish-blue fluorescent compound as 6-(*p*-hydroxyphenyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine[5] (Table 1) by comparison with natural and synthetic samples.

The fourth compound, designated as putidolumazine ($C_{14}H_{18}N_4O_9$), was supposed to be a new pteridine. UV-Spectra of this compound suggested that it was a 2,4,7-trioxohexahydropteridine with alkyl side chains at N-8 and C-6 positions (Fig. 1). Putidolumazine was stable against Al-Hg treatment[13] suggesting that it has $-CH_2-$ group at position 6. On photoirradiation in the presence of H_2O_2 it gave 6-(2-carboxyethyl)-2,4,7-trioxohexahydropteridine (VII); This compound was synthesized by condensation of 4,5-diaminouracil and α-ketoglutaric acid (Table 3). On oxidation with periodate followed by reduction with sodium borohydride, putidolumazine yielded 6-(2-carboxyethyl)-8-(2-hydroxyethyl)-2,4,7-trioxohexahydropteridine (V); this was identified by comparison with synthetic material (Table 3). Periodate consumption of putidolumazine suggests the presence of four neighboring hydroxy groups. Finally, acetylated putidolumazine ethyl ester gave a molecular peak of *m/e* 582 on mass spectrum.

From these results the structure of 6-(2-carboxyethyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (I) was postulated for putidolumazine. This compound was synthesized by condensation (reduction) of 2,6-dihydroxy-5-nitro-4-D-ribitylaminopyrimidine and α-ketoglutaric acid. Its identity with the natural product was confirmed (Table 3, 4; Fig. 1).

We thought that putidolumazine was produced by enzymic condensation with 4-ribitylamino-5-aminouracil and α-ketoglutaric acid produced from glutamic acid and the possible formation of putidolumazine has been implicated in the enzymic production of riboflavin.

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12) W. Pfleiderer and G. Nübel, *Chem. Ber.*, **93**, 1406 (1960).

13) S. Matsuura, S. Nawa, M. Goto, and Y. Hirata, *J. Biochem.*, **42**, 413 (1955).