The Structure of the Pteridine Glycoside from *Aspergillus oryzae*

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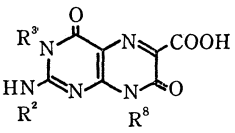
The structures of two blue-fluorescent substances, asperopterin-A and -B, isolated from *Aspergillus oryzae* were investigated. Asperopterin-B was identified as 2-amino-3,4,7,8-tetrahydro-6-hydroxymethyl-8-methyl-4,7-dioxopteridine (**7**) by studying its chemical degradation, pK_a values, and UV spectra; the structure was confirmed by synthesis. Asperopterin-A was shown to be the β-D-ribofuranoside of **7**, 2-amino-3,4,7,8-tetrahydro-8-methyl-4,7-dioxo-6-pteridinylmethyl β-D-ribofuranoside (**10**).

Two blue-fluorescent substances, asperopterin-A (**1**) and -B (**2**), have been isolated as crystals from *Aspergillus oryzae* T-17.[1,2] In a previous communication,[3] **1** was shown to be a D-riboside of **2**, and a tentative approach to the structure of **2** was made. The present paper will record the complete structures of **1** and **2** as well as the synthesis of the latter.

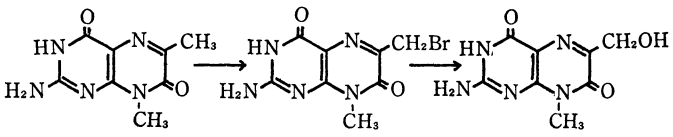
Results and Discussion

Structure of Asperopterin-B (2). It has previously been established[3] that **2** is a derivative of isoxanthopterin bearing a CH_2OH group at the 6-position and an extra methyl group elsewhere in the molecule. In the present work, this was supported by the NMR spectrum of **2**, which showed two singlets, at 3.22 and 4.52 ppm, representing a methyl and methylene group respectively. Moreover, it is known that isoxanthopterin has the 2-amino-4,7-dioxo-form in an aqueous solution with its two pK_a values (7.34 and 10.06) arising from the hydrogen atoms attached to the N-3 and N-8 positions of the ring.[4] Since **2** has only one acidic pK_a value (8.07), one of these hydrogen atoms must be replaced by a methyl group. In order to find the position of this methyl group, **2** was oxidized with potassium permanganate and the oxidation product (**3**)[3] was compared with the three synthetic *N*-methylisoxanthopterin-6-carboxylic acids (**4a**—**c**); **4c** proved to be identical with **3** (Table 1). Thus, asperopterin-B (**2**) was assumed to be 2-amino-3,4,7,8-tetrahydro-6-hydroxymethyl-8-methyl-4,7-dioxopteridine (**7**); this assumption was eventually confirmed by the following synthesis. 6,8-Dimethylisoxanthopterin (**5**)[4] was brominated using bromine in 48% hydrobromic acid; without isolation, the bromo derivative (**6**) was converted by 0.5 N sodium hydroxide into the 6-hydroxymethyl compound (**7**) which was identical with **2** in R_f and in pK_a value, and also in its UV and IR spectra (Table 1, Fig. 1).



4

	R^2	R^3	R^8
4a	CH_3	H	H
4b[5]	H	CH_3	H
4c[5]	H	H	CH_3
4d[5]	H	CH_3	CH_3
4e	CH_3	CH_3	CH_3



5 **6** **7**

Structure of Asperopterin-A (1). Although it has been shown that **1** was a D-riboside of **2**,[3] the position of the ribosyl group has remained unknown. Because **1** has an acidic pK_a and UV spectra closely

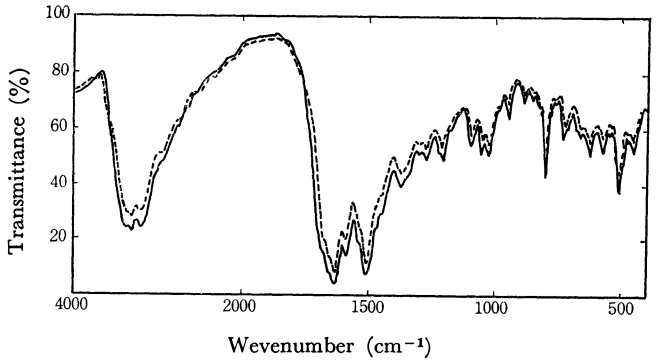


Fig. 1. IR spectra of asperopterin-B (solid line) and **7** (dotted line).

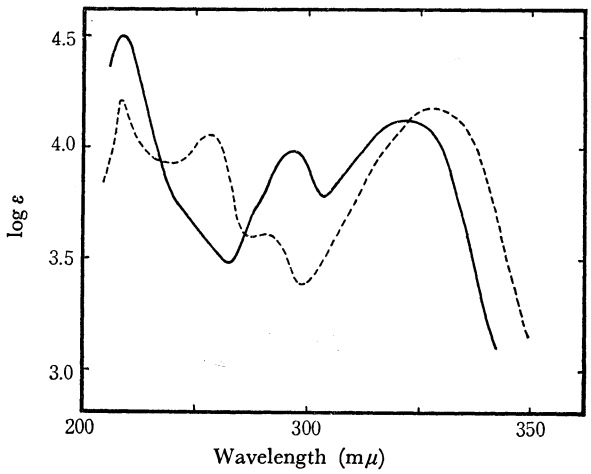


Fig. 2. UV spectra of asperopterin-A.
—— pH 5.5 (neutral molecule)
---- pH 10.5 (anion)

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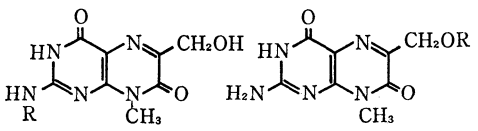
1) Y. Kaneko, *Agr. Biol. Chem.*, **29**, 965 (1965).
2) Y. Kaneko, *Nippon Nogei Kagaku Kaishi*, **40**, 227 (1966).
3) Y. Kaneko and M. Sanada, *J. Ferment. Technol.*, **47**, 8 (1969).
4) W. Pfleiderer and M. Rukwied, *Chem. Ber.*, **94**, 1 (1961).
5) W. Pfleiderer and M. Rukwied, *ibid.*, **95**, 1591 (1962).

TABLE 1. IDENTIFICATION OF PTERIDINES

	R_f[a]			pK_a[b]	UV Spectra				
	a	b	c		pH	Ionic species[c]	λ_{max} (log ε)		
3[d] (Oxidation product of **2**)	0.17	0.69	0.52	3.46 8.17	1.0 5.5 11.0	○ — ——	221 (4.53), 264 (3.79), 290 (3.79), 379 (4.34) 219 (4.51), 293 (3.93), 352 (4.17) 218 (4.25), 260 (4.03), 365 (4.20)		
4c[e]	0.17	0.69	0.52	3.45 8.18	1.0 5.5 11.0	○ — ——	221 (4.53), 264 (3.88), 291 (3.82), 379 (4.37) 219 (4.54), 293 (3.96), 352 (4.20) 218 (4.30), 261 (4.06), 365 (4.24)		
Asperopterin-A (**1**)[f]				8.05	5.5 10.5	○ —	218 (4.57), 292 (4.02), 346 (4.22) 217 (4.27), 259 (4.11), 280 (3.56), 359 (4.26)		
Asperopterin-B (**2**)[d]	0.20	0.46	0.47	8.07	5.5 11.0	○ —	218 (4.57), 292 (4.00), 342 (4.13) 217 (4.22), 257 (4.05), 279 (3.61), 354 (4.17)		
7	0.20	0.46	0.47	8.09	5.5 11.0	○ —	218 (4.53), 292 (4.00), 342 (4.13) 217 (4.21), 257 (4.05), 280 (3.61), 355 (4.16)		
$\mathbf{a}_1$	0.20	0.46	0.47	8.07	5.5 11.0	○ —	217 214	292 256	342 281 354
$\mathbf{b}_2$	0.16	0.75	0.73		1.0 8.5		220 258	253 293	268 355 375
4d[e]	0.16	0.75	0.74	3.74	1.0 8.5	○ —	221 (4.40), 253 (3.90), 269 (3.92), 375 (4.28) 258 (3.75), 292 (3.91), 356 (4.22)		
$\mathbf{c}_2$	0.29	0.75	0.74		1.0 8.5		265 295	292 356	386
4e	0.29	0.75	0.74	3.69	1.0 8.5	○ —	266 (4.02), 293 (3.78), 387 (4.39) 296 (4.00), 358 (4.26)		

a) Descending method was used. Solvent: a: *n*-butanol : 50% acetic acid (2 : 1). b: 4% ammonium chloride. c: 4% sodium citrate. b) Spectroscopic determination. c) ○; neutal molecule. —; monoanion. ——; dianion. d) UV Spectra; *cf.* Kaneko and Sanada.[3] e) UV Spectra and pK_a; *cf.* Pfleiderer and Rukwied.[5] f) UV Spectra; *cf.* Kaneko.[1,2]

resembling those of **2** (Table 1, Figs. 2, 3), either of the two structures (**8** and **9**) seems possible for **1**.



8 **9**

R=D-ribosyl

In order to distinguish between these possibilities, methylation with dimethyl sulfate was carried out. It had previously been reported[6] that isoxanthopterin in an alkaline solution was methylated at N-8, N-3, and the 2-amino group on treatment with dimethyl sulfate; moreover, when **2** was treated similarly, the hydroxyl group of the side chain was methylated together with the ring nitrogen atoms. Therefore, if **1** is **8**, the hydroxyl group of the side chain should be methylated; on the other hand, if **1** is **9**, no methylation could take place at that position, since the hydroxyl group would be protected by the ribosyl group. Using conditions under which no hydrolysis of the glycosidic link occurred, the methylation of **1** in an alkaline solution gave a mixture of products. When this mixture was boiled with 2 N hydrochloric acid, three products, $\mathbf{a}_1$, $\mathbf{b}_1$, and $\mathbf{c}_1$, were obtained. These were then chromatographically separated; $\mathbf{a}_1$ was identified as **2** from its R_f, its pK_a value, and its UV spectrum (Table 1); the permanganate oxidation of $\mathbf{b}_1$ and $\mathbf{c}_1$ gave two acids, $\mathbf{b}_2$ and $\mathbf{c}_2$, which were identical with **4d** and **4e** respectively (Table 1). Since 6-methoxymethylisoxanthopterin[7] was unaffected under the conditions

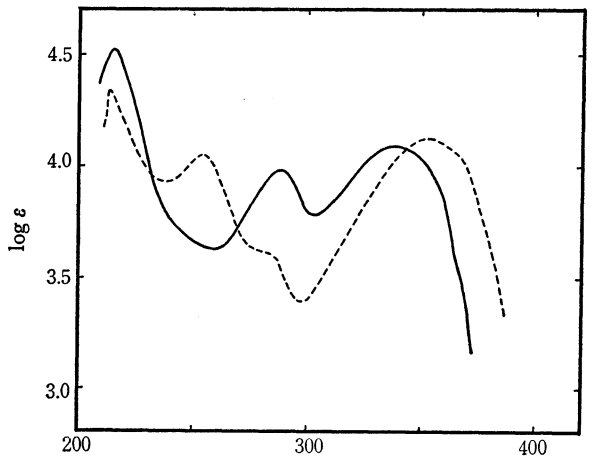


Fig. 3. UV spectra of asperopterin-B.
—— pH 5.5 (neutral molecule)
---- pH 11.0 (anion)

6) T. Sugimoto and S. Matsuura, *Research Bulletin* (Dept. of General Educ., Nagoya Univ.), **11**, 94 (1967).

7) S. Matsuura, S. Nawa, H. Kakizawa, and Y. Hirata, *J. Amer. Chem. Soc.*, **75**, 4446 (1953).

used for the oxidation of **2**, $\mathbf{b}_1$, and $\mathbf{c}_1$, both $\mathbf{b}_1$ and $\mathbf{c}_1$ could be seen to have a hydroxymethyl group at the 6-position, and it could be seen that the glycoside was not methylated at this position, even in the presence of a large excess of dimethyl sulfate. Accordingly, the formula **9**, in which the hydroxymethyl group was protected by a ribosyl group, was confirmed to be that for asperopterin-A.

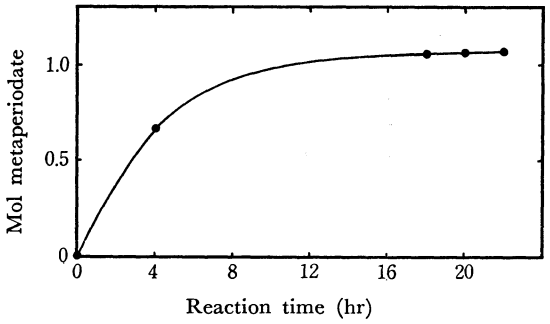
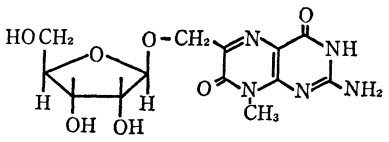


Fig. 4. Periodate oxidation of asperopterin-A.

The structure of the sugar moiety of **1** was determined by periodate oxidation according to the method of Lythgoe[8] and Jackson;[9] **1** consumed one molar equivalent of the reagent and produced no formic acid (Fig. 4). It was clear, therefore, that **1** was a ribofuranoside. The anomeric configuration of **1** was deduced to be β by a comparison of its molecular rotation (−223°) with those of known D-ribofuranosides.[10] Thus, the formula **10** was given to asperopterin-A:



10

Experimental

All the melting points are uncorrected. The NMR spectrum was measured at 60 MHz with a JEOL Model JNM-3 spectrometer, using a 0.5 M KOD solution and TMS as the external reference. The UV spectra were measured on a Shimadzu SV-50 spectrometer and checked on a Shimadzu QR-50 manual instrument. The IR spectra were recorded on a Hitachi Model EPI-G spectrometer. Optical rotations were determined with a Shimadzu Lippich-type polarimeter. The ionization constants were determined spectroscopically.

Permanganate Oxidation of Asperopterin-B (2). 0.1 M Potassium permanganate was added, drop by drop, to **2** (77.6 mg) in 0.01 M sodium hydroxide (100 m*l*) at 60°C until a purple color remained. The solution was then set aside at this temperature for 20 min, and the excess of permanganate was decomposed with 0.05 M potassium sulfite. The manganese dioxide was filtered off and washed with hot water. The combined filtrate and washings were adjusted to pH 2 with hydrochloric acid and passed through a florisil column (1.5×10 cm). The pteridine, adsorbed on the top of the column, was then eluted with water; a small amount of the starting material remained on the column.[13] The eluate was concentrated to a volume of 50 m*l* and adjusted to pH 2. The repeated recrystallization of the precipitate from 1 M potassium hydroxide and finally from an aqueous buffer at pH 2 gave pale yellow needles (45 mg) of the product; mp>300°C.

Found: C, 39.86; H, 3.37; N, 29.02%. Calcd for $C_8H_7N_5O_4 \cdot 1/4H_2O$: C, 39.76; H, 3.13; N, 28.98%.
This product was identical with authentic **4c**.

2,5-Diamino-4-hydroxy-6-methylaminopyrimidine Hydrochloride.[4] 2-Amino-4-hydroxy-6-methylanino-5-nitrosopyrimidine[14] (35 g) and 10% palladized charcoal (5 g) in 400 m*l* water was shaken under hydrogen. After 3 min, 1 M sodium hydroxide (100 m*l*) was added and shaking was continued until the absorption of hydrogen ceased (9700 m*l* within 100 min). After the subsequent acidification of the mixture with 10 M hydrochloric acid (100 m*l*), the catalyst was removed by filtration. The concentration of the filtrate under reduced pressure gave the product in an 85% yield. The catalyst could be used repeatedly without any depression of its activity providing it was washed with ethanolic sodium hydroxide (4 g of sodium hydroxide in 100 m*l* of 20% aqueous ethanol).

In a similar way, 4,5-diamino-6-hydroxy-2-methylaminopyrimidine hydrochloride[4] was prepared from the corresponding 5-nitrosopyrimidine[15] in an 85% yield.

6-Carboxy-3,4,7,8-tetrahydro-2-methylamino-4,7-dioxopteridine (4a). This compound was prepared in an 80% yield from the above 4,5-diamino-6-hydroxy-2-methylaminopyrimidine hydrochloride using the method for its 3-methyl isomer (**4b**);[5] mp>300°C.

Found: C, 38.59; H, 3.67; N, 27.90%. Calcd for $C_8H_7N_5O_4 \cdot 2/3H_2O$: C, 38.56; H, 3.37; N, 28.11%.

2-Amino-3,4,7,8-tetrahydro-6-hydroxymethyl-8-methyl-4,7-dioxopteridine (7). Bromine (1.00 g) was added to **5**[4] (1.20 g) in 48% hydrobromic acid (100 m*l*) at 60°C. The mixture was heated at 80°C until the initially-formed precipitate had dissolved, and then cooled quickly. It was diluted with water (200 m*l*), and its pH was adjusted to 2 with crystalline sodium acetate. The solution was evaporated to dryness under reduced pressure, and the residue was heated at 80°C with 0.5 M sodium hydroxide (200 m*l*) for 15 min. The separated needles (1.01 g) were purified by repeated recrystallization from 1 M potassium hydroxide until only one spot was observed on a paper chromatogram.

8) B. Lythgoe and A. R. Tood, *J. Chem. Soc.*, **1944**, 592.
9) E. L. Jackson and C. S. Hudson, *J. Amer. Chem. Soc.*, **61**, 1530 (1939).
10) It has been reported that methyl β-D-ribofuranoside has a molecular rotation of −102° in contrast to that of the α-isomer (+241°).[11] When the aglycon was replaced by a benzyl alcohol residue, the value for the β-isomer moved to −145°.[12] This displacement (43°) caused by the aglycon exchange is much smaller than the difference (343°) between the rotations of the α- and β-isomers.
11) G. R. Barker and D. C. C. Smith, *J. Chem. Soc.*, **1954**, 2151.
12) R. K. Ness, H. W. Diehl, and G. G. Fletcher, Jr., *J. Amer. Chem. Soc.*, **76**, 763 (1954).
13) The florisil column for chromatography was prepared as before.[2] The behavior of the adsorbed pteridines towards elution depends on their pK_a values. A pteridine with an acidic pK_a below 4 was eluted with water; one with a pK_a between 6 and 9 was eluted with 1% aqueous ammonia or, more effectively, with acetone-ammonia (acetone, 30 m*l*; 1.5% aqueous ammonia, 60 m*l*).
14) W. V. Curran and R. B. Angier, *J. Org. Chem.*, **28**, 2672 (1963).
15) R. Roth, J. M. Smith, Jr., and M. E. Hultquist, *J. Amer. Chem. Soc.*, **73**, 2864 (1951).

Finally, the pH value of the solution of the potassium salt in water (50 ml) was adjusted to 5 to give the product as pale yellow needles (270 mg, 22%); mp>300°C.

Found: C, 41.87; H, 4.00; N, 30.24%. Calcd for $C_8H_9N_5O_3 \cdot 1/2H_2O$: C, 41.38; H, 4.34; N, 30.15%.

*Methylation and Subsequent Hydrolysis of Asperopterin-A (**1**).* Dimethyl sulfate was added in portions (0.016 ml; 1 equivalent each) to **1** (56 mg, 0.016 mmol) in 0.1 M potassium hydroxide (20 ml); the mixture was then maintained at pH 10 by adding 10 M potassium hydroxide. After the addition of every five equivalents of the reagent, the mixture was examined by paper chromatography. Additions were continued until no spot of the starting material was visible. Seventy-five equivalents of dimethyl sulfate were added in all. The reaction mixture, after the inorganic salts had been removed using a florisil column, was heated with 2 M hydrochloric acid (5 ml) at 100°C for 10 min. This produced three compounds, $\mathbf{a}_1$, $\mathbf{b}_1$, and $\mathbf{c}_1$ (R_f: 0.20, 0.30 and 0.47 using *n*-butanol-acetic acid), which were separated by a paper-chromatographic method (*n*-butanol-acetic acid as a solvent) and purified using the florisil column.[13]

Permanganate Oxidation of $\mathbf{b}_1$ and $\mathbf{c}_1$. The above $\mathbf{b}_1$ fraction was oxidized with permanganate as has been described before. After the removal of the manganese dioxide, the product was adsorbed on a florisil column (0.3×3 cm) and eluted with 1% aqueous ammonia. The product ($\mathbf{b}_2$), obtained by the evaporation of the eluate to dryness, was identified as **4d**.

Similarly, the $\mathbf{c}_1$ fraction was oxidized; the product, $\mathbf{c}_2$, was identified as **4e**.

5-Formamido-4-hydroxy-2,6-bismethylaminopyrimidine. Formic acid (98—100%; 60 ml) was added, drop by drop, to a vigorously-stirred solution of 4-hydroxy-2,6-bismethylaminopyrimidine[14] (27 g) and sodium nitrite (15 g) in formamide (200 ml). The mixture was heated to 90°C, and sodium dithionite (1.2 g) was added. An exothermic reaction occurred, and the nitroso compound dissolved. After 3 min, the formamido compound separated as needles (30.5 g, 90%); mp 282—283.5°C (recrystallized from water).

Found: C, 40.70; H, 6.32; N, 33.94%. Calcd for $C_7H_{11}N_5O_2 \cdot 1/2H_2O$: C, 40.77; H, 5.84; N, 33.97%.

5-Formamido-1,6-dihydro-1-methyl-2,4-bismethylamino-6-oxopyrimidine. Dimethyl sulfate (16.5 g) was added in portions to the above formamidopyrimidine (16 g) in 1 M sodium hydroxide (200 ml). The mixture was stirred for 1 hr at 25°C, during which time the pH value of the solution was maintained at 13 by the addition of 10 M sodium hydroxide throughout the reaction. After chilling overnight, the precipitate was collected and recrystallized from water to give colorless needles (9.0 g, 53%) of the product; mp 291—292°C (dec.).

Found: C, 45.23; H, 6.39; N, 33.20%. Calcd for $C_8H_{13}N_5O_2$: C, 45.49; H, 6.39; N, 33.16%.

*6-Carboxy-3,4,7,8-tetrahydro-3,8-dimethyl-2-methylamino-4,7-dioxopteridine (**4e**).* The above 5-formamido-1-methylpyrimidine (2.2 g) was dissolved in 12 M hydrochloric acid (10 ml) and set aside at room temperature overnight. Ethanol (20 ml) was then added to the mixture, and the precipitated 5-aminopyrimidine was used for condensation without further purification. The 5-aminopyrimidine was added to diethyl oxomalonate (3.2 g), sodium acetate (3 g), and potassium metabisulfite (100 mg) in water (50 ml), and the mixture was boiled for 10 min. The yellow needles (2.7 g) were collected and boiled in 1 M sodium bicarbonate (150 ml) for 55 min. The precipitate, dissolved in a 0.5 M potassium hydroxide solution (300 ml), was added, drop by drop, to boiling 20% acetic acid (300 ml) to give yellow needles (2.4 g, 87%); mp>300°C.

Found: C, 44.82; H, 4.19; N, 26.13%. Calcd for $C_{10}H_{11}N_5O_4 \cdot 0.1H_2O$: C, 44.98; H, 4.23; N, 26.23%.

*Periodate Oxidation of Asperopterin-A (**1**).* Sodium metaperiodate (0.113 M, 7.50 ml) was added to **1** (57.0 mg) in water (100 ml) and diluted to exactly 120.0 ml. The solution was incubated at 25°C. Samples (2.0 ml) were taken out at intervals, and the excess of metaperiodate in each was estimated iodometrically.[8,9] 0.68, 1.06, 1.07, and 1.07 molar equivalents of periodate were consumed after 4, 18, 20, and 22 hr respectively. After 22 hr, 10 ml of the solution was diluted with water and titrated with 0.02 M sodium hydroxide;[8] the diluted solution consumed no sodium hydroxide.

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