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Synthesis of 2-Amino-4-hydroxy-6-acetyl-7-methyl-7,8-dihydropteridine

Katsura SUGIURA and Miki GOTO

Department of Chemistry, Gakushuin University, Toshima-ku, Tokyo

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2-Amino-4-hydroxy-6-acetyl-7-methylpteridine was synthesized by condensation of 2,4,5-triamino-6-hydroxypyrimidine and triketopentane. Reduction of this compound with sodium amalgam yielded a coloring matter, whose UV-spectra and chemical reactions strictly correspond to those of sepiapterin or isosepiapterin. On the basis of NMR spectra, the coloring matter was proved to be 7,8-dihydro-2-amino-4-hydroxy-6-acetyl-7-methylpteridine. Hence, it can be concluded that sepiapterin has the 7,8-dihydro structure as proposed by Nawa.

Sepiapterin is the main pteridine from the mutant *sepia* of the flies, *Drosophila melanogaster* and from the mutant *lemon* of the silkworms, *Bombyx mori*. This compound was proved to be 2-amino-4-hydroxy-6-lactyl-7,8-dihydropteridine by Nawa; evidence to support the 7,8-dihydro structure was obtained by demonstrating the production of dihydroxanthopterin from sepiapterin on treatment with borax solution.[1] Some additional evidences suggested also the 7,8-dihydro structure.[2-5]

The present work was undertaken in order to prove the dihydro structure more definitely by synthesizing a model compound and measuring the NMR spectrum. We have synthesized 2-amino-4-hydroxy-6-acetyl-7-methylpteridine (I) by condensation of 2,4,5-triamino-6-hydroxypyrimidine and triketopentane. Dihydro-2-amino-4-hydroxy-6-acetyl-7-methylpteridine was obtained by reduction of the compound with sodium amalgam. The UV-spectra of the dihydro compound corresponded strictly to those of sepiapterin, and the chemical reactions were similar to those reported for sepiapterin and isosepiapterin.[6,7] On the basis of NMR spectra and UV-spectral change on reduction with sodium borohydride, it was clearly demonstrated that the dihydro compound has the structure *7,8-dihydro*-2-amino-4-hydroxy-6-acetyl-7-methylpteridine. Hence, it can be now definitely concluded that sepiapterin has the 7,8-dihydro structure, as suggested by Nawa.[1]

Experimental

The NMR spectra were obtained with a Varian A-60 instrument with tetramethylsilane or sodium (ω-trimethylsilyl)trimethylenesulfonate as an internal reference. Each sample was dissolved in deuteriodimethylsulfoxide or 1N NaOD at a 10% concentration just before use. The UV-spectra were obtained using a Hitachi EPS-3 spectrophotometer.

2-Amino-4-hydroxy-6-acetyl-7-methylpteridine (I). 2,4,5-Triamino-6-hydroxypyrimidine sulfate mono-

1) S. Nawa, This Bulletin, **33**, 1555 (1960).
2) S. Kaufman, *J. Biol. Chem.*, **242**, 3934 (1967).
3) T. Fukushima and M. Akino, *Arch. Biochem. Biophys.*, **128**, 1 (1968).
4) H. S. Forrest and S. Nawa, *Nature*, **196**, 372 (1962).
5) M. Viscontini, L. Merlini and W. von Philipsborn, *Helv. Chim. Acta*, **46**, 1181 (1963).
6) H. S. Forrest, C. Van Baalen and J. Myers, *Arch. Biochem. Biophys.*, **83**, 508 (1959).
7) H. S. Forrest, D. Hatfield and C. Van Baalen, *Nature*, **183**, 1269 (1959).

hydrate (2.8 g) was dissolved in 60 ml of 1N hydrochloric acid at 70—90°C. Triketopentane (1.3 g) was added drop by drop and the mixture was heated for 15 min at 100°C. The solution was neutralized with 10N sodium hydroxide and left standing at room temperature. The precipitate was dissolved in 20 ml of 1N sodium hydroxide and filtered. Addition of a few drops of 10N sodium hydroxide gave yellow needles of the sodium salt of I, yield, 1.2 g. The salt (200 mg) was dissolved in 10 ml of water, acidified with acetic acid, and the resulting precipitate was filtered and washed with water, ethanol and ether. The pale yellow material was dried *in vacuo* over P_2O_5 at 120°C, yield, 160 mg, mp>250°C. NMR (1N NaOD, 40°C): τ 7.45 (3H, singlet, $-COCH_3$); UV: $\lambda_{max}^{0.1NNaOH}$ mμ (ε), 273 (1.78×10^4), 310 (7.0×10^3), 366 (1.45×10^4); $\lambda_{min}^{0.1NNaOH}$ mμ (ε), 239 (5.5×10^3), 302 (6.9×10^3), 324 (6.6×10^3); $\lambda_{max}^{0.1NHCl}$ mμ (ε), 269 (9.8×10^3), 321 (1.37×10^4), 380 (*infl.*, 1.2×10^3); $\lambda_{min}^{0.1NHCl}$ mμ (ε), 249 (6.5×10^3), 290 (5.8×10^3). Found: C, 49.02; H, 4.14; N, 31.11%. Calcd for $C_9H_9N_5O_2$: C, 49.31; H, 4.40; N, 31.95%.

2-Amino-4-hydroxy-6-(1-hydroxyethyl-7-methylpteridine (II). The sodium salt of I (482 mg) in 25 ml of water was added with 740 mg of sodium borohydride in 20 ml of 0.01N sodium hydroxide. The mixture was stirred for 6 hr at room temperature and acidified slightly with 6N hydrochloric acid. The pale brown precipitate thus obtained was crystallized from dimethylformamide and the material further purified by dissolving in 1/25N sodium hydroxide and precipitating with acetic acid. The faint yellow product (300 mg) was dried at 120°C over P_2O_5 for 6 hr, mp> 250°C. NMR (1N NaOD, 40°C): τ 8.45 (3H, doublet, J=7 cps, CH_3), 7.44 (3H, singlet, CH_3), 4.80 (1H, quartet, J=7 cps, CHOH). UV: $\lambda_{max}^{0.1NNaOH}$ mμ (ε), 253 (2.42×10^4), 358 (9.0×10^3); $\lambda_{min}^{0.1NNaOH}$ mμ (ε), 220 (9.2×10^3), 297 (1.4×10^3); $\lambda_{max}^{0.1NHCl}$ mμ (ε), 216 (2.18×10^4), 253 (1.04×10^4), 322 (1.03×10^4); $\lambda_{min}^{0.1NHCl}$ mμ (ε), 240 (8.7×10^3), 276 (2.4×10^3). Found: C, 46.88; H, 5.20; N, 30.51%. Calcd for $C_9H_{11}N_5O_2\cdot\frac{1}{2}H_2O$: C, 46.96; H, 4.81; N, 30.43%. II was also obtained by reduction of I with zinc powder in 0.5N sodium hydroxide at 40°C or by hydrogenation in 0.1N sodium hydroxide over PtO_2 (1 mole of hydrogen was absorbed per mole of I). Hydrogenation over palladised black in 0.1N sodium hydroxide did not give II.

2-Amino-4-hydroxy-6-(1-hydroxyethyl)-7-methyl-5,6,7,8-tetrahydropteridine (III). II (90 mg) was dissolved in 11 ml of 6N hydrochloric acid and hydrogenated over Adams' catalyst (60 mg). Hydrogenation was stopped, when 2.3 mol of hydrogen was absorbed per mole of II. The catalyst was filtered off and the solution was concentrated to dryness *in vacuo* at 40°C. Recrystallization of the product from a mixture of water, ethanol and ether (3 : 5 : 1) gave faint orange needles (hygroscopic), yield, 58 mg, mp>250°C. For analyses the material was dried *in vacuo* for 6 hr over P_2O_5 at 100°C. UV: $\lambda_{max}^{0.1NNaOH}$ mμ (ε), 255 (*infl.*, 6.3×10^3), 288 (8.5×10^3); $\lambda_{min}^{0.1NNaOH}$ mμ (ε), 235 (4.3×10^3); $\lambda_{max}^{0.1NHCl}$ mμ (ε), 218 (*infl.*, 1.20×10^4), 265.5 (1.79×10^4); $\lambda_{min}^{0.1NHCl}$ mμ (ε), 237 (5.4×10^3). Found: C, 39.98; H, 6.44; N, 26.71%. Calcd for $C_9H_{15}N_5O_2\cdot HCl\cdot\frac{1}{2}H_2O$: C, 39.93: H, 6.28; N, 25.87%.

2-Amino-4-hydroxy-6-acetyl-7-methyl-7,8-dihydropteridine (IV). The sodium salt of I (600 mg) was dissolved in 200 ml of 0.03N sodium hydroxide, 33 g of 4% sodium amalgam was added and the mixture was stirred for 2 hr. Mercury was removed and the mixture stirred for 2 hr at room temperature. The solution was neutralized with 6N hydrochloric acid and concentrated *in vacuo* at below 40°C. The concentrate was placed on a 7.0×24.0-cm column of Avicel (microcrystalline cellulose, FMC Corp.) and the column was eluted with 2-propanol, 1% ammonia (2 : 1). A yellow band was eluted and the eluate was acidified with acetic acid. The solution was placed on a 4.0×15.0 cm column of Florisil (100—200 mesh); the yellow compound was adsorbed at the top of the column. The column was washed well with water and then the compound was eluted with 20% aqueous acetone. The eluate was concentrated to dryness *in vacuo*, and the residue was recrystallized from water to give yellow needles (hygroscopic), yield, 102 mg, mp 200—201°C. UV- and NMR-spectra are given in Figs. 1 and 4. For analyses the sample was dried *in vacuo* over P_2O_5 at 100°C. Found: N, 29.51%. Calcd for $C_9H_{11}N_5O_2\cdot H_2O$: N, 29.28%.

Results and Discussion

The product obtained by condensation of 2,4,5-triamino-6-hydroxypyrimidine and triketopentane in acidic media was reduced with 4% sodium amalgam to give a coloring matter with a yellow fluorescence (λ_{max} 513 mμ; exciting wave length: 365 mμ). The UV-spectra of this compound is shown in Fig. 1.

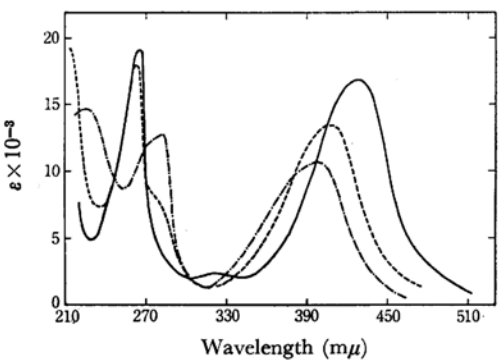


Fig. 1. UV-Absorption spectra of the coloring matter, 2-amino-4-hydroxy-6-acetyl-7-methyl-7,8-dihydropteridine.
—— in 0.1N sodium hydroxide
------ in M/30 KH_2PO_4-Na_2HPO_4 (pH 7.0)
—·—· in 0.1N hydrochloric acid

The reduced product gave the following reactions:[6]

1) Oxidation with alkaline permanganate solution gave 2-amino-4-hydroxy-7-methylpteridine-6-carboxylic acid (V).[8]

2) Oxidation with hydrogen peroxide in 0.1N sodium hydroxide gave 2-amino-4,6-dihydroxy-7-

8) F. Weygand, H. Simon, K. D. Keil and H. Millauer, *Chem. Ber.*, **97**, 1002 (1964).

methylpteridine (VI).[9]

3) Treatment with excess sodium borohydride in 0.1N sodium hydroxide gave quantitatively 2-amino-4-hydroxy-6-(1-hydroxyethyl)-7-methyl-7,8-dihydropteridine (VII), whose UV-spectra (Fig. 3) are superimposable on those of 2-amino-4-hydroxy-6-methyl-7,8-dihydropteridine;[10] UV-spectral change on reduction is given in Fig. 2. VII on heating in dilute acetic acid gave the original coloring matter (IV) and 2-amino-4-hydroxy-6-(1-hydroxyethyl)-7-methylpteridine (II).

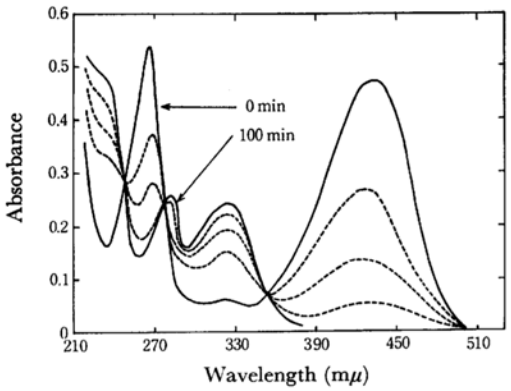


Fig. 2. UV-Spectral change of the coloring matter IV on reduction with sodium borohydride.
The compound (17 μg) was dissolved in 2.5 ml of 0.1N sodium hydroxide and 5 mg of sodium borohydride added (22°C). The UV-absorption spectra were determined before (——) and after 20, 40, 60 (------) and 100 min reduction. The spectrum at 100 min (——) remained unchanged even after 150 min.

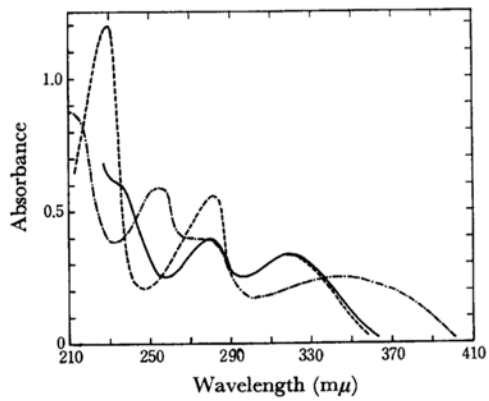


Fig. 3. UV-Spectra of the reduction ($NaBH_4$)product of the coloring matter IV.
The yellow compound IV in 0.1N sodium hydroxide was treated with excess sodium borohydride; the yellow solution became colorless. Excess sodium borohydride was destroyed with acetone. The solution was neutralized and the compound purified using a Sephadex G-25 fine column (developer: water) and an Avicel column (developer: 2-propanol, 1% ammonia=2:1). The UV-spectra of the product are quite in agreement with those of 2-amino-4-hydroxy-6-methyl-7,8-dihydropteridine.
——— in 0.1N sodium hydroxide
-------- in M/30 KH_2PO_4-Na_2HPO_4 (pH 7.0)
-·-·-· in 0.1N hydrochloric acid

4) Treatment with bromine in water or in 0.1N hydrochloric acid gave the original compound, 2-amino-4-hydroxy-6-acetyl-7-methylpteridine (I).

5) Reduction with sodium borohydride at pH 7.0 gave 2-amino-4-hydroxy-6-(1-hydroxyethyl)-7-methyl-5,6,7,8-tetrahydropteridine (III).

6) Action of iodine in 0.1N sodium hydroxide gave 2-amino-4-hydroxy-7-methylpteridine-6-carboxylic acid.[8]

Identification of the products described above was carried out by paper chromatographic and UV-spectral comparison with authentic materials. R_f-values of pteridines are given in Table 1.

Table 1. R_f-Values of pteridines

Compound	R_f-values			
	A	B	C	D*
I	0.54	0.48	0.55	0.50
II	0.49	0.55	0.48	0.52
V	0.14	0.27	0.29	0.58
VI	0.18	0.28	0.34	0.33
IV	0.67	0.67	0.64	0.46
IV, after $KMnO_4$ oxidation in 0.1N NaOH	0.14	0.27	0.29	0.58
IV, after H_2O_2 oxidation in 0.1N NaOH	0.18	0.28	0.34	0.33
IV, after Br_2 treatment in acidic or neutral medium	0.54	0.48	0.55	0.50
IV, after I_2 treatment in 0.1N NaOH	0.14	0.27	0.29	0.58
IV, after $NaBH_4$ reduction and reoxidation	0.49	0.55	0.48	0.52
	0.67	0.67	0.64	0.46

* Solvent: A, 2-propanol, 1% ammonia (2:1)
B, 2-propanol, 2% ammonium acetate (1:1)
C, 1-butanol, acetic acid, water (4:1:1)
D, 3% aqueous ammonium chloride

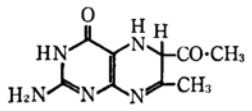
These results are in accordance with the observation for sepiapterin[1] and isosepiapterin[6] and suggest that the yellow coloring matter is a dihydropteridine with an acetyl group at the 6-position and a methyl group at the 7-position and not its isomer. Moreover, the absorption spectra of the dihydropteridine in acidic, neutral and alkaline media were superimposable on the corresponding spectra of sepiapterin and isosepiapterin; this evidence suggests that the dihydropteri-

9) G. B. Elion, G. H. Hitchings and P. B. Russel, *J. Am. Chem. Soc.*, **72**, 78 (1950).

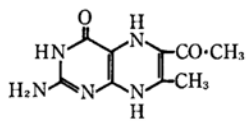
10) W. Pfleiderer and H. Zondler, *Chem. Ber.* **99**, 3008 (1966).

dine has the same chromophore as in sepiapterin and isosepiapterin. Proof of the dihydro structure of the colored reduction product of the ketone was obtained from a study of the nuclear magnetic resonance spectrum of the coloring matter.

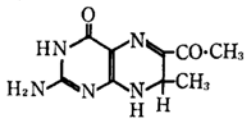
The following three dihydro structures come into consideration for dihydro-2-amino-4-hydroxy-6-acetyl-7-methylpteridine:



(VIII)



(IX)



(X)

The NMR spectrum of dihydro-2-amino-4-hydroxy-6-acetyl-7-methylpteridine (Fig. 4) has a singlet at $\tau=7.66$, a doublet at $\tau=9.03$, a quartet at $\tau=5.37$ and broad singlets at $\tau=3.22$, 2.25 and -0.28; the proton ratio of each signal is 3 : 3 : 1 : 2 : 1 : 1. In comparison with NMR signals of guanosine in deuteriodimethylsulfoxide,[11] the signals $\tau=3.22$, 2.25 and -0.28 can be assigned to the amino protons at the position 2, the imino protons at the positions 8 and 3, respectively. The signal at $\tau=7.66$ is assigned to the acetyl protons at the position 6 and the doublet signal at $\tau=9.03$ to the methyl protons at the position 7. The quartet signal at $\tau=5.37$ is assigned to the proton at the position 7; this is coupling with the signal ($\tau=9.03$) of the neighboring methyl protons ($J=7$ cps) and *vice versa* ($J=7$ cps). This evidence supports the structure X for the dihydro coloring matter and the 5,6- and 5,8-dihydro structures (VIII and IX) are ruled out. It can be now definitely concluded that the dihydro-2-amino-4-hydroxy-6-acetyl-7-methylpteridine, and also, by analogy, sepiapterin has the 7,8-dihydro structure.

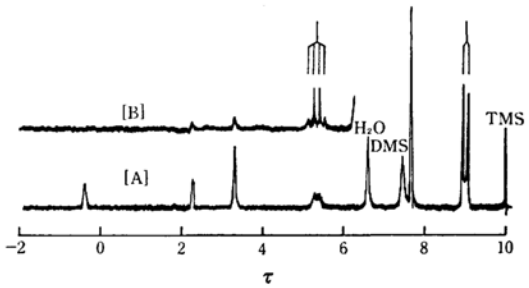


Fig. 4. NMR spectra of the yellow coloring matter IV.

The spectra were obtained with tetramethylsilane as an internal reference (60 Mc, 40°C). After spectrum A was measured in deuteriodimethylsulfoxide, a small amount of D_2O was added to exclude the interaction between the ring protons at the positions 7 and 8, and spectrum B was taken again.

The authors wish to express their thanks to Dr. M. Kondo and Mr. K. Ono, Sankyo Co., Ltd., for measuring NMR spectra and for carrying out microanalyses.

11) L. Gatlin and J. C. Davis, ibid., **84**, 4464 (1962).