136. Pteridines

Part C[1])

Structure and Nonenzymatic Synthesis of Aurodrosopterin

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Dedicated to Prof. Dr. *Ted Taylor,* Princeton, USA, on the occasion of his 70th birthday and in admiration of his important contributions to pteridine chemistry

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The nonenzymatic synthesis of aurodrosopterin (**5**) from 6-acetyl-2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido-[4,5-*b*][1,4]diazepin-4-one (**3**) and 7,8-dihydrolumazine (**4**) at pH 3 (HCl) was performed. The identity of the synthesized compound with the natural eye pigment isolated from drosophila heads was confirmed by thin-layer chromatography on cellulose and by comparisons of the ^{1}H-NMR and UV/VIS spectra. The nonenzymatic synthesis of a neodrosopterin-like red pigment from **3** and 2,4-diamino-7,8-dihydropteridine was also carried out, but its identity could not be established. This pigment, called aminodrosopterin, has an absorption peak at 489 nm, which is very close to that of neodrosopterin.

1. Introduction. – The complexity of eye color phenotypes of *Drosophila melanogaster* has attracted the notice of scientists for many years, and the study for it has played an important role in the development of genetics and biochemistry. Two classes of pigments contribute to the eye color of *Drosophila*. One of which consists of brown pigments called 'ommochromes' and the other one comprises the red-orange pigments known as 'drosopterins'. Location of these pigments was first reported by *Lederer* in 1940 [2] and subsequently by *Viscontini et al.* [3]. *Schwinck* [4] described the two-dimensional separation of five distinct drosopterin components on cellulose thin-layer plates. Commonly drosopterin and isodrosopterin are the major components, and neodrosopterin has to be considered as a minor pigment. The amount of aurodrosopterin is the smallest of all drosopterins in wild-type *Drosophila*.

The structure of the drosopterins seem to be closely related due to similar physical properties. Drosopterin and isodrosopterin are enantiomers, as recognized also for aurodrosopterin and neodrosopterin. Several molecular structures of drosopterin/isodrosopterin were proposed [2] [5] [6] till *Theobald* and *Pfleiderer* showed that the complex constitution consists of a pentacyclic ring system containing a 5,6,7,8-tetrahydropterin (= 2-amino-5,6,7,8-tetrahydropteridin-4(1*H*)-one), a 2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido[4,5-*b*][1,4]diazepin-4-one, and a pyrrole ring [7] [8]. The enzymatic synthesis

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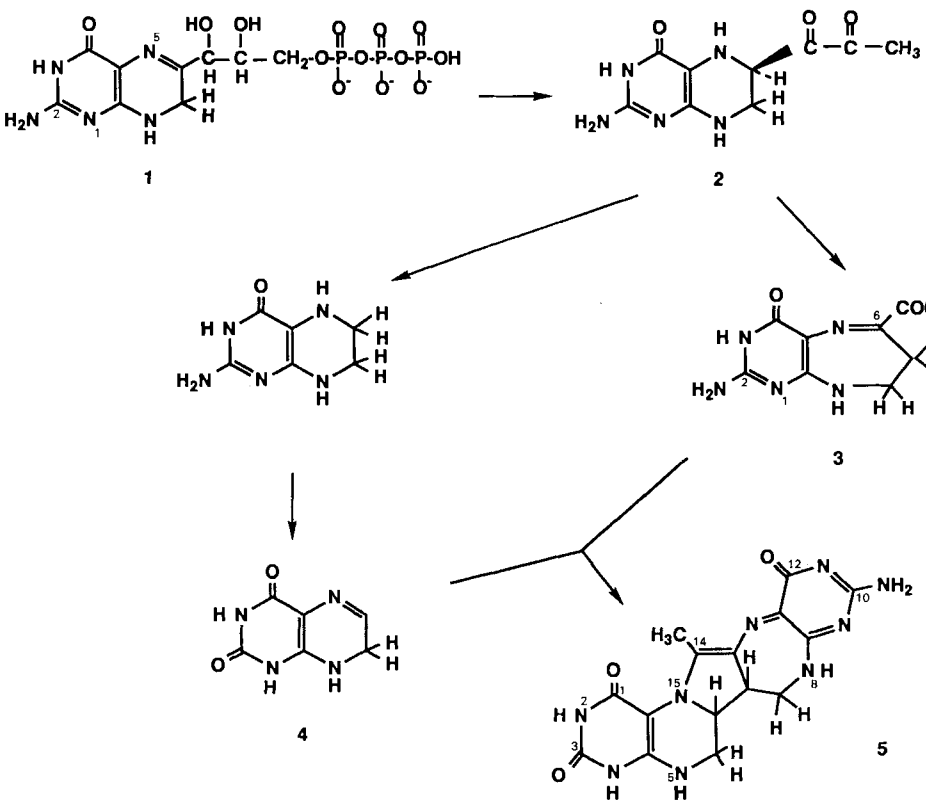
of the drosopterin enantiomers was first achieved with homogenates of *Drosophila* heads, which contain activity for the conversion of radioactive 7,8-dihydroneopterin triphosphate (H_2-NTP; **1**; see *Scheme*) into the eye-pigments [9]. An important advance toward better understanding of the biosynthetic pathway was made in 1981 with the discovery of a new compound [10] which promotes the incorporation of ^{14}C-labeled H_2-NTP into drosopterin [11] [12]. The structure of this compound was elucidated by *Brown* and coworkers [10] and *Dorsett* and *Jacobson* [13] and established to be 6-acetyl-2-amino-3,7,8,9-tetrahydro-4*H*-pyrimido[4,5-*b*][1,4]diazepin-4-one (**3**) which is also known as 6-acetylhomopterin and could arise from **1** *via* **2** (see below). *Paton* and *Brown* later described the nonenzymatic synthesis of the enantiomers drosopterin and isodrosopterin from **3** and 7,8-dihydropterin (= 2-amino-7,8-dihydropteridin-4(1*H*)-one) in an acid-catalyzed condensation [14]. Based on a kinetic study, they proposed a mechanism in which one molecule of each component reacts to form one molecule of drosopterin or isodrosopterin.

In this communication, the nonenzymatic syntheses of aurodrosopterin (**5**) from 7,8-dihydrolumazine (= 7,8-dihydropteridine-2,4(1*H*,3*H*)-dione; **4**) and 6-acetylhomopterin (**3**) and of a neodrosopterin-like red pigment from 2,4-diamino-7,8-dihydropteridine and **3** are reported.

Scheme



2. Results and Discussion. – The condensation of various 7,8-dihydropteridine derivatives with 6-acetylhomopterin (**3**) was performed at pH 3 (HCl). The pigments were formed at acidic pH in the range 2–7, showing an optimum at pH 3. Above pH 7, very little conversion was detected, and in stronger acidic medium, **3** was instable. The identity of the synthetic pigment obtained from 7,8-dihydrolumazine (**4**) and **3** with natural aurodrosopterin (**5**) was established first by thin-layer chromatogaphy (TLC) on cellulose sheets. The R_f values were very close or identical when measured in 4 different systems (*Table*). The two-dimensional TLC confirmed the identity of both materials (*Fig. 1*), especially by an increase of fluorescence strength of the aurodrosopterin spot, when the synthesized pigment **5** was added to fly-head extracts.

Table. R_f *Values on Cellulose Plates (Avicel) of Drosopterins Isolated from Head Extracts of* Drosophila

Solvent system[a])	Aurodroso-pterin (**5**)	Isoaurodroso-pterin	Droso-pterin	Isodroso-pterin	Neodroso-pterin	Isoneodroso-pterin
1	0.14	0.14	0.15	0.18	0.15	0.17
2	0.20	0.23	0.14	0.18	0.16	0.16
3	0.25	0.25	0.17	0.20	0.19	0.19
4	0.10	0.10	0.13	0.17	0.12	0.15
5	0.17	0.17	0.17	0.21	0.15	0.17
6	0.23	0.24	0.14	0.16	0.13	0.17

[a]) See *Exper. Part, General.*

a) Oregon-R head extract only: b) Oregon-R head extract plus synthesized pigment:

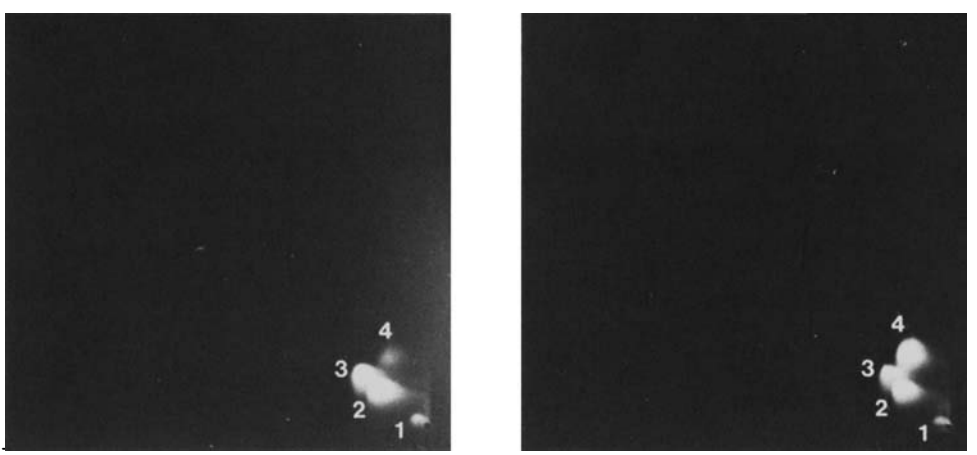


Fig. 1. *Two-dimensional TLC of drosopterins from Oregon-R head extracts in the presence or absence of synthetic aurodrosopterin* (**5**). The identities of the spots are as follows: *1*) neodrosopterin, *2*) drosopterin, *3*) isodrosopterin, *4*) aurodrosopterin.

The acid-catalyzed conversion of **3** and **4** to **5** was monitored by recording UV/VIS spectra at various time intervals (*Fig. 2*). The absorption at 477 nm, characteristic for purified **5**, did no longer increase after 24 h, indicating the completion of the reaction. The synthesized pigment was stable and no conversion to another compound was detected after prolonged reaction time. Isolation of **5** by lyophilization gave chromatographically

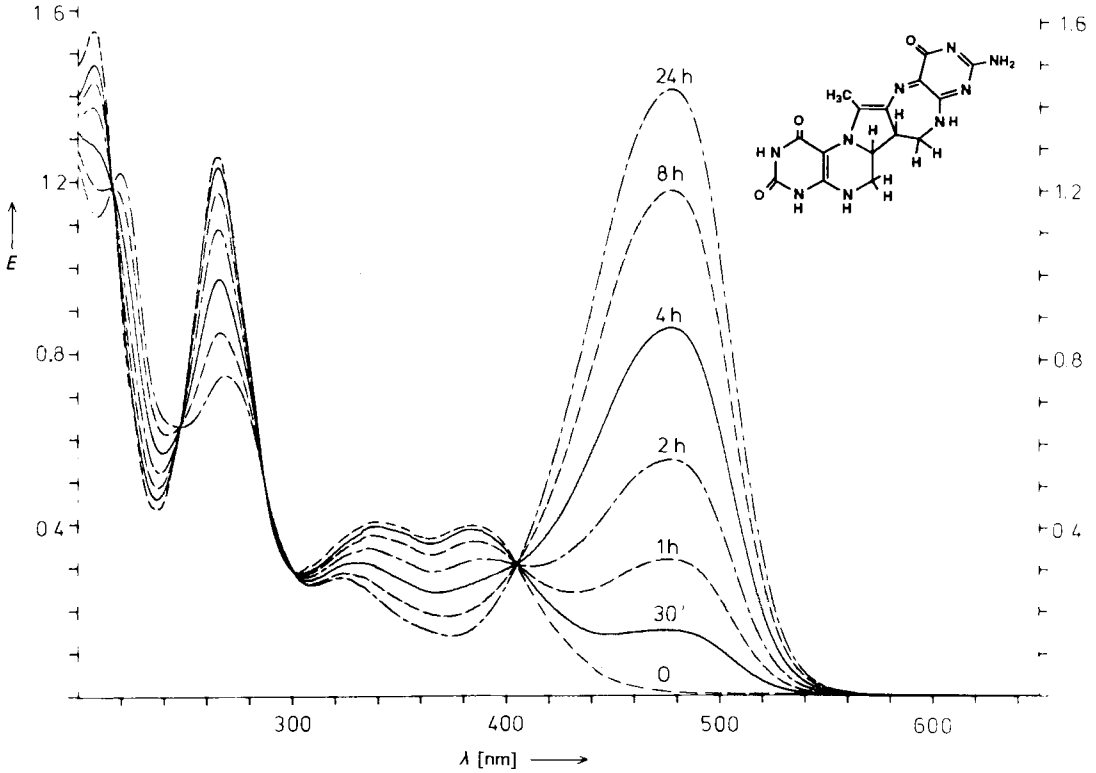


Fig. 2. *UV/VIS Monitoring of the formation of aurodrosopterin* (**5**) *from 7,8-dihydrolumazine* (**4**) *and acetylhomopterin* (**3**) *at pH 3*

pure material. A comparison of the characteristic CH signals of this synthetic **5** between 2 and 5 ppm in the ^{1}H-NMR spectrum (*Fig. 3*) with those of natural **5** confirmed again their identity.

In an acid-catalyzed condensation, a neodrosopterin-like red pigment was formed from 2,4-diamino-7,8-dihydropteridine [20] and acetylhomopterin (**3**) [15], but its chromatographical behavior indicated that it was not identical with natural neodrosopterin isolated from *Drosophila*. This synthetic pigment, which we call aminodrosopterin, has an absorption at 489 nm, which increased with reaction time (see *Fig. 4*) and coincided with the long-wavelength absorption of natural neodrosopterin. But purified neodrosopterin exhibits a quite different absorption pattern in the UV range of the spectrum, since its absorbances around 220 and 270 nm [18] are relatively low compared to those of other drosopterins. It was also detected that the chromatographic behavior of neodrosopterin changed during isolation. Neodrosopterin in fly-head extracts moved primarily little on TLC, but the mobility of this pigment increased after it was eluted from cellulose columns. There are the possibilities that the character of neodrosopterin changes while undergoing isolation, or isolated neodrosopterin is an artifact formed during the various isolation steps. Thus, it will be necessary to isolate the native form of neodrosopterin as a prerequisitive to elucidate the structure of this natural eye pigment.

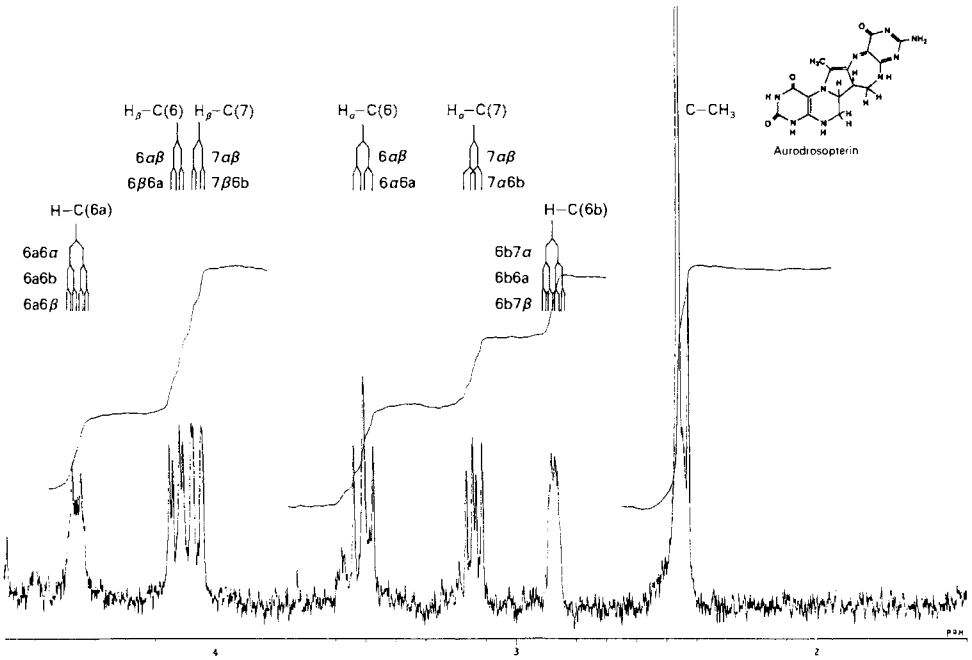


Fig. 3. *^{1}H-NMR Spectra of natural and synthetic aurodrosopterin* (**5**) *in D_2O DCl*

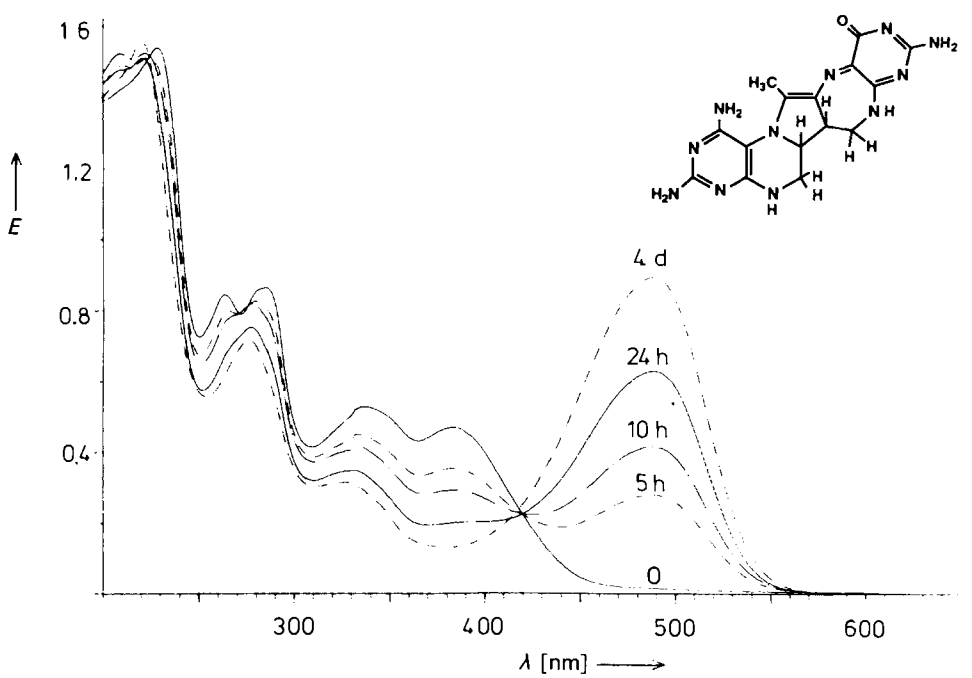


Fig. 4. *UV/VIS Monitoring of the formation of aminodrosopterin from acetylhomopterin* (**3**) *and 2,4-diamino-7,8-dihydropteridine at pH 3*

The kinetics of the pigments syntheses were examined under conditions that kept the concentration of one reactant constant and high and that of the other reactant variant. The order of reaction of substrates with respect to initial concentration was determined by the measurement of the initial velocity of pigment synthesis. In these studies, **3** was pretreated with HCl at pH 3.0 for 1 h following the method of *Paton* and *Brown* [14]. The UV/VIS monitoring of **3** at pH 3 showed a steady decrease in absorbance with time. However, the overall spectrum hardly changed, except for the small decreases. Furthermore, when the pH was elevated to 7 or above, the decreases were reversed. The addition of the second reactant to the pretreated **3**, when the decrease was about maximal, resulted in substantial synthesis of pigment. When **3** was pretreated for 1 h, more pigment was produced than after a 5-min exposure to HCl. *Paton* and *Brown* hypothesized that at mildly acidic pH, a non-destructive change occurs in **3** and this species is probably the active molecule in pigment synthesis.

When the studies were performed with isolated **3** and 7,8-dihydrolumazine (**4**) and 2,4-diamino-7,8-dihydropteridine the double-log plots of initial reactant concentration and initial velocity gave nearly straight lines with good regression coefficients, and the slopes of lines which show the order of reaction, are approximately one (*Figs. 5–8*). It can be said that the reactions follow the pseudo-first-order kinetics with respect to the concentration under these conditions. Thus, the reactions are second order and one molecule of pigment is made from one molecule of each **3** and **4** or 2,4-diamino-7,8-dihydropteridine.

We propose that the structure of aurodrosopterin (**5**) is closely related to that of drosopterin, and the presence or absence of the amino group in the pteridine moiety determines the characteristic of each pigment molecule. The molecule containing dihy-

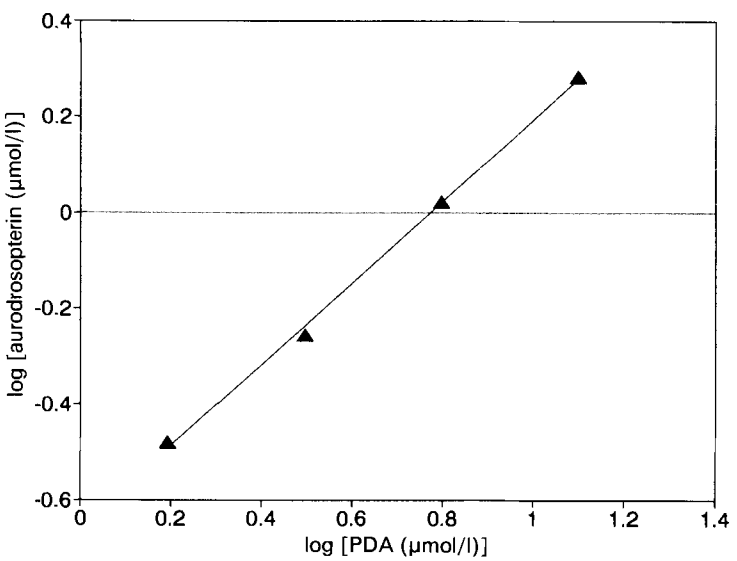


Fig. 5. *Order of reaction for the acid-catalyzed condensation of* **3** *with 7,8-dihydrolumazine* (**4**). Varying concentrations of **3** (1.5–12.5 µM) were pre-incubated at pH 3 for 1 h. 150 µM of dihydrolumazine was added to initiate the pigment synthesis. Slope: 0.85, regression coefficient: 0.998.

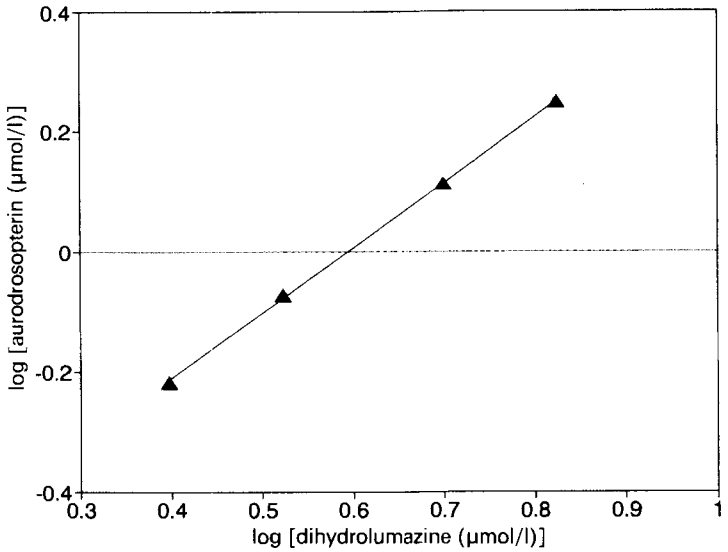


Fig. 6. *Order of reaction for the acid-catalyzed condensation of 7,8-dihydrolumazine* (**4**) *with* **3**. 150 μM **3** was pre-incubated at pH 3 for 1 h. Varying concentrations of 7,8-dihydrolumazine (2.5–6.7 μM) were added. Slope. 1.09, regression coefficient. 0.999.

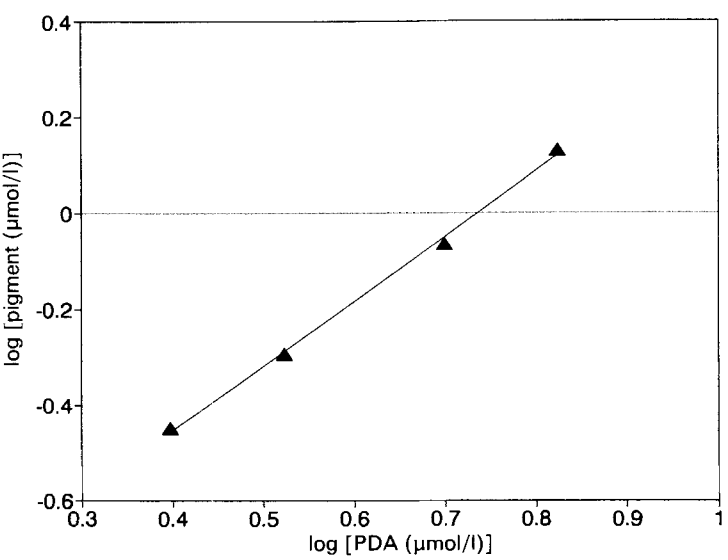


Fig. 7. *Order of reaction for the acid-catalyzed condensation of* **3** *with 2,4-diamino-7,8-dihydropteridine.* Varying concentrations of **3** (2.5–6.7 μM) were pre-incubated at pH 3 for 1 h. 150 μM 2,4-diamino-7,8-dihydropteridine was added. Slope: 1.35, regression coefficient: 0.998.

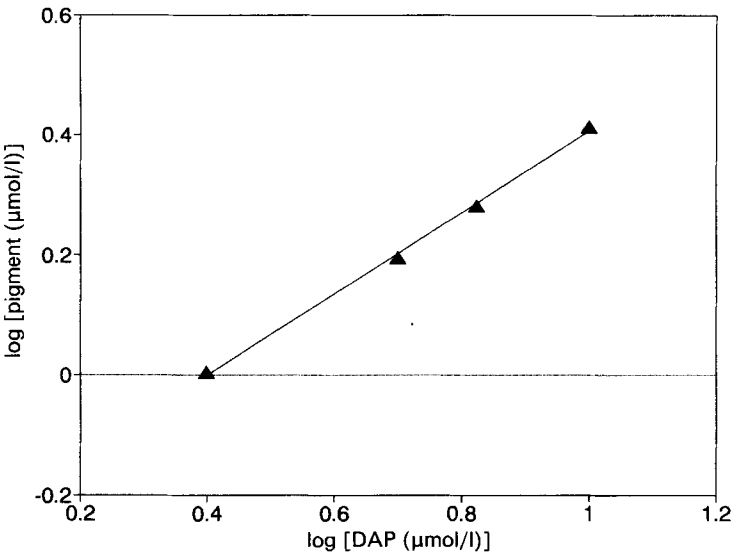


Fig. 8. *Order of reaction for the acid-catalyzed condensation of 2,4-diamino-7,8-dihydropteridine with* **3**. 150 μM **3** were pre-incubated at pH 3 for 1 h. Varying concentrations of 2,4-diamino-7,8-dihydropteridine (2.5–10 μM) were added. Slope: 0.68, regression coefficient: 0.998.

dropterin should be drosopterin or its enantiomer isodrosopterin, and the molecule containing 7,8-dihydrolumazine (**4**), the deaminated counterpart of 7,8-dihydropterin, should be aurodrosopterin or isoaurodrosopterin. Recently, we demonstrated the activity of 5,6,7,8-tetrahydropterin deaminase in the head extract of *Drosophila*. This enzyme uses tetrahydropterin as substrate and produces dihydrolumazine **4** [16]. Since tetrahydropterin is known to occur in *Drosophila* heads [17], this deamination can produce one of the reactants for aurodrosopterin synthesis in *Drosophila*.

From the results presented in this communication, along with the new discovery of tetrahydropterin-specific deaminase in *Drosophila*, a probable route for the biosynthesis of aurodrosopterin (**5**) from **1** *via* **2** can be postulated (see *Scheme*). We hope that the structure of the other unknown drosopterins can be established by a combination of chemical and biochemical studies.

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Experimental Part

General. TLC: cellulose thin-layer sheets *F 1440* from *Schleicher & Schüll*. Column chromatography: cellulose powder *Nr. 123* from *Schleicher & Schüll; Sephadex G-25* (*Pharmacia*). Paper chromatogrphy: PC sheets (58 × 60 cm) from *Schleicher & Schüll*. UV/VIS: *Lambda 5* (*Perkin-Elmer*); λ_{max} in nm (log ε). ^{1}H-NMR: *Bruker WM-250* and *Jeol-JNM-G* × *400*; δ in ppm rel. to TMS (= 0 ppm). Solvent systems: *1* = 2% aq. sodium citrate; *2* = 3% aq. NH_4Cl soln.; *3* = 1M AcOH; *4* = i-PrOH/2% aq. NH_4OAc soln. 1:1; *5* = PrOH/1% aq. NH_4Cl soln. 1:1; *6* = PrOH/1% aq. NH_3 soln.

1. *Isolation of Aurodrosopterin* (**5**) *and Neodrosopterin.* Isolation was performed by a modified method described first by *Rokos et al.* [18]. In 4 volumes of 0.1M phosphate buffer (pH 7.0), 30 g of *Drosophila melanogaster*

heads (Oregon-R) were homogenized. The homogenate was centrifuged at 12000 rpm for 30 min to remove proteins, and the precipitate was then suspended in 150 ml of the extracting solvent MeOH/AcOH/H_2O 4:1:5. After centrifugation, the drosopterin pigments were extracted again from the precipitate. Extraction was repeated 3 more times using 150 ml of the same solvent mixture each time. The combined extracts were evaporated at 40°. The dried material was dissolved in 100 ml of dil. NH_4OH soln. (0.1% NH_3 in H_2O) and centrifuged. The supernatant was applied on a column of *Sephadex G-25* (4.0 × 52 cm) equilibrated with H_2O. The column was washed with H_2O until all the yellow pigments were eluted, and the separation of the drosopterins was performed with dil. NH_4OH soln. (flow rate 13 ml/10 min). The pooled fractions containing drosopterins were evaporated and the residues dissolved again in dil. NH_4OH soln. After centrifugation, the supernatant was loaded onto a column of cellulose (4.0 × 30 cm); on washing with H_2O, a yellow-orange pigment (aurodrosopterin (**5**)) migrated relatively fast, while the rest of the pigments stayed near the top of the column. Thus, the column was treated with 0.5% NH_4OAc soln. (14 ml/10 min), and the eluates were monitored by VIS (λ 502 nm). The fractions containing **5** were concentrated and applied to a column of *Sephadex G-10* (1.4 × 30 cm) to remove NH_4OAc. The column was washed with H_2O and eluted with dil. NH_4OH soln. (5 ml/10 min). The mixture of the residual drosopterins obtained from the first cellulose column was concentrated and applied to a second cellulose column (3 × 20 cm). The column was washed with H_2O to remove NH_4OAc and then eluted with dil. NH_4OH soln. (14 ml/10 min). A fast moving pinkish material, neodrosopterin, was separated from drosopterin/isodrosopterin. Neodrosopterin and drosopterin/isodrosopterin were collected separately. R_f Values of the drosopterins were determined in 6 solvent systems using microcrystalline cellulose plates (*Avicel; from Schleicher & Schüll*; see *Table*). The aurodrosopterin/isoaurodrosopterin can be separated into the enantiomers only in solvent **2**, whereas the enantiomers drosopterin/isodrosopterin are separated in most solvent.

2. *Isolation of Neodrosopterin.* In 4 volumes of 0.1M phosphate buffer (pH 7.0), 12 g of *Drosophila melanogaster* (Oregon-R strain) heads were homogenized. The homogenate was centrifuged to remove proteins and the precipitate suspended in 60 ml of extracting solvent (MeOH/AcOH/H_2O 4:1:5). After centrifugation, the supernatant was pooled and the extraction repeated 2 more times from the precipitate. The combined extracts were evaporated to 60 ml at 40°. To remove proteins, the extracts were heated to 60° for 10 min followed by centrifugation. Next, HCl was added to adjust to pH 2 and the extract kept for 2 h at r.t. After another centrifugation, 500-µl aliquots of the extracts were applied to 40 cellulose TLC plates (*Eastman Kodak*, No. *13255* without fluorescent indicator, 20 × 10 cm) which were developed in 1M AcOH. The plates were dried by hot air, and the bands containing neodrosopterin were scraped and extracted with 50 ml of 1% Et_3N/H_2O. After centrifugation, extraction was repeated from the precipitate, the extracts were combined and lyophilized. As the lyophilized material was still contaminated with other drosopterins, it was applied again to cellulose plates and the above described separation and isolation procedure repeated.

3. *Two-Dimensional TLC.* Microcrystalline cellulose plates (10 × 10 cm) were used. On one plate, only the supernatant of fly-head extracts was spotted. On the other one, the pigment **5** synthesized from **3** and **4** was added to the spot. The two plates were developed in the same chamber. i-PrOH/aq. 2% NH_4OAc soln. 1:1 and 3% aq. NH_4Cl soln. were used as first and second developing solvent. Plates were dried using a hair drier, and fluorescent spots were detected by a long-wave UV lamp (model *UVL-21*, *Ultra-Violet Products Inc.*).

4. *UV/VIS Monitoring.* Acetylhomopterin (**3**) was pretreated at pH 3 (HCl) and then used directly or after storage in the dark for 1 h. The UV/VIS spectra of aurodrosopterin (**5**) and aminodrosopterin, respectively, were obtained after incubation at pH 3 at various intervals, under dark conditions for 1 day.

5. *Determination of the Order of Reaction.* The reactions were performed by varying the concentration of one reactant (1.5–12.5 µM), while maintaining the concentration of the other reactant constant and high (150 µM). Acetylhomopterin (**3**) was pretreated with HCl (pH 3) for 1 h. The amount of synthesized pigments was measured after 24 h by a *Schimadzu-UV-160* spectrophotometer at 502 nm. At this wavelength, the reactant **3**, **4**, and 2,4-diamino-7,8-dihydropteridine have no or extremely small absorbance values. As the molar extinction coefficients of racemic aurodrosopterin and neodrosopterin were not known, they were assumed to be the same as that of drosopterin (ε 34700) [6].

6. *7,8-Dihydrolumazine* (= *7,8-Dihydropteridine-2,4(1H,3H)-dione;* **4**). In 40 ml of 1N $NaHCO_3$, 5,6,7,8-tetrahydrolumazine monohydrochloride [19] (1.02 g, 5 mmol) was suspended and then stirred at r.t. for 1.5 days in an open flask to achieve air oxidation. The resulting precipitate was collected, washed with H_2O, MeOH, and Et_2O. Drying in a vacuum desiccator yielded 0.48 g (46%) of a pinkish powder. M.p. > 270° (dec.). UV (pH 0): 263 (4.18), 360 (3.26). ^{1}H-NMR (CF_3COOD): 7.90 (*t*, H–C(6)); 5.20 (*d*, CH_2(7)). Anal. calc. for $C_6H_5N_4NaO_2 \cdot H_2O$ (206.1): C 34.96, H 3.40, N 27.17; found: C 35.35, H 3.40, N 27.59.

7. *2,4-Diamino-7,8-dihydropteridine* [20]. a) According to the procedure of *Taylor* and *Sherman* [20], 1.62 g (0.01 mol) of 2,4-diaminopteridine [21] were heated in 100 ml of DMF to 80° with stirring. A soln. of 4 g of $NaBH_4$ in 40 ml of H_2O was added and the mixture then heated to 100° for 2 h. After evaporation, the residue was extracted by hot i-PrOH to give, on cooling, a brownish precipitate. Recrystallization from i-PrOH with little charcoal gave 0.82 g (82%) of a slightly brownish powder. M.p. 250° (dec.). ^{1}H-NMR: 2,4-diamino-7,8-dihydropteridine.

b) A soln. of 0.42 g (2.5 mmol) of 2,4-diamino-5,6,7,8-tetrahydropteridine [21] in 15 ml of H_2O was stirred in an open *Erlenmeyer* flask for 8 h. A precipitate separated gradually. It was collected by suction, washed with little H_2O, and dried in a vacuum desiccator to give 0.25 g. Recrystallization from H_2O yielded 0.18 g (43%) of a yellowish powder. M.p. 250° (dec.). UV (pH 3): 207 (4.12), 233 (4.36), 289 (4.08), 320 (sh, 3.68). UV (pH 9): 210 (4.33), 289 (3.90), 326 (3.66). ^{1}H-NMR ((D_6)DMSO): 6.89 (*t*, H–C(6)); 6.27 (*s*, H–N(8)); 5.85 (*s*, NH_2); 5.70 (*s*, NH_2); 4.01 (*d*, 2 H–C(7)). Anal. calc. for $C_6H_8N_6$ (164.2): C 43.89, H 4.91, N 51.20; found: C 43.76, H 5.03, N 51.12.

8. *Aurodrosopterin* (= *10-Amino-5,6,6a,6b,7,8-hexahydro-14-methyl-2*H-*pyrimido[4″,5″:2′,3′][1,4]diazepino[6′,5′:3,4]pyrrolo[1,2-f]pteridine-1,3,12(4*H*)-trione*; **5**). To a soln. of 2.2 mg (10 μmol) of acetylhomopterin [15] (**3**) in 10 ml of 0.001N HCl (pH 3) were added 2.1 mg (10 μmol) of **4** and then stirred in the dark for 48 h (TLC: no starting components left; red soln.). Lyophilization gave an orange solid, which was pure on TLC. UV (pH 3): 219, 274, 477. ^{1}H-NMR (D_2O/DCl): 4.46 (*m*, H–C(6a)); 4.13 (*dd*, H_β–C(6)); 4.03 (*dd*, H_β–C(7)); 3.50 (*dd*, H_α–C(6)); 3.14 (*dd*, H_α–C(7)); 2.87 (*m*, H–C(6b)); 2.46 (*s*, MeC(14)).

9. *Aminodrosopterin* (= *1,3,10-Triamino-5,6,6a,6b,7,8-hexahydro-14-methylpyrimido[4″,5″:2′,3′][1,4]diazepino[6′,5′:3,4]pyrrolo[1,2-f]pteridin-12-one*). A soln. of 1.1 mg (5 μmol) of acetylhomopterin [15] (**3**) in 5 ml of 0.001N HCl (pH 3) was kept at r.t. for 1 h and then 1 mg (5 μmol) of 2,4-diamino-7,8-dihydropteridine [20] added with stirring. The soln. was kept 4 days in the dark and the change from colorless to red followed spectrophotometrically (*Fig. 4*).

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