Photochemistry of Ommochrome Pigments

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Received September 30, 1987

The ommochrome pigment present in the eyes of *Loligo Vulgaris* was extracted with a butanol-acetic acid-hydrochloric acid solution. The extraction mixture was examined before and after irradiation by visible light. A phenoxazinone ring opening together with a photo-induced solvent addition, causing a yellow-red bathochromic shift, were observed and a plausible explanation of the process has been carried out and is reported on the basis of the isolated products.

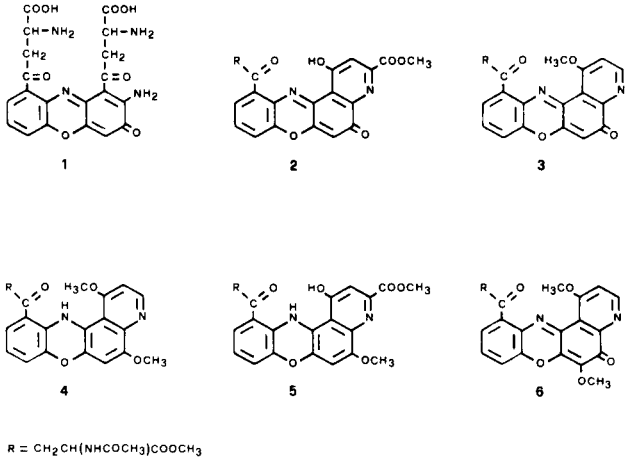
J. Heterocyclic Chem., **25**, 1243 (1988).

Introduction.

An acid-labile ommochrome pigment (λ max 440-420 nm) present in the eyes and skin of cephalopods, *Loligo Vulgaris, Sepia Officinalis,* and *Octopus Vulgaris*, is transformed in the extraction mixture (butanol-acetic acid-hydrochloric acid) into a red pigment (λ max 490-385 nm), identified as dihydroxanthommatin (**7**) [1].

The reported data supported the hypothesis that the yellow extracted pigment is a phenoxazinone derivative **1** [1,2].

In order to obtain further information about the native pigment, insoluble in organic solvents and non-volatile, *i.e.*, non-analysable with the usual spectroscopic (ir, nmr, mass) and chromatographic techniques, we examined the extraction mixture, after aqueous acetic anhydride and methanol-sulphuric acid treatment [3]. Then the photochemical behaviour of the native pigment was compared with the one of the synthetic models [4].



R = $CH_2CH(NHCOCH_3)COOCH_3$

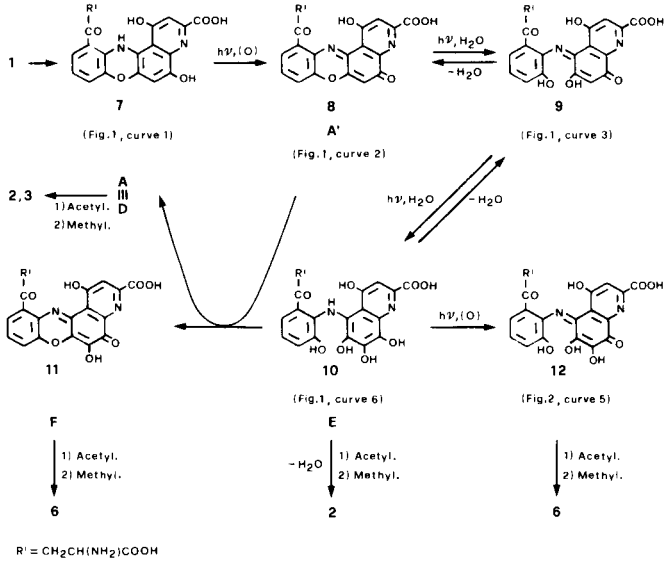
Results.

The extraction of *Loligo Vulgaris* eyes with a butanol-acetic acid-hydrochloric acid mixture, as reported [1], gave a yellow pigment (λ max 440-420 nm) being converted, in 1 hour, into a red one (λ max 490-385 nm). This red solution **A**, containing dihydroxanthommatin **7**, concentrated *in vacuo*, purified on a carboxymethylcellulose column, eluted with a butanol-acetic acid-water mixture, gave two coloured fractions: a red **B** (λ max 485-385 nm) and a yellow **C** (λ max 450-400 nm).

The two fractions **B** and **C** were treated with aqueous acetic anhydride and, subsequently, with methanol-sulphuric acid. From the red **B** fraction, four coloured products were obtained: **2, 3, 4** and **5**. The compounds **3, 4** and **5** were identified, by comparison of their spectral (ir, nmr, uv, mass) and chromatographic properties, with authentic samples of 1-methoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)-5*H*-pyrido[3,2-*a*]phenoxazin-5-one (**3**), the 1,5-dimethoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)pyrido[3,2-*a*]phenoxazine (**4**), the 1-hydroxy-3-carbomethoxy-5-methoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)-pyrido[3,2-*a*]phenoxazine (**5**).

The compound **2** was identified as the 1-hydroxy-3-carbomethoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)-5*H*-pyrido[3,2-*a*]phenoxazin-5-one, in fact the uv spectrum, in methanol, showed a shoulder at 380 nm and a maximum at 438 nm, characteristic of a carbomethoxypyrido[*a*]phenoxazinone system [5].

SCHEME A



R' = $CH_2CH(NH_2)COOH$

The ^{1}H nmr showed the characteristic signals attributed to a β-aspartoyl-*N*-acetyl-methyl ester chain [2]. The NH proton signal appeared as a doublet at δ 6.75 coupled with the multiplet of the methine proton at δ 4.85 that was also coupled with the multiplet of the two methylenic protons at δ 3.80. The *N*-acetyl proton signal appeared as a singlet at δ 2.1. The aromatic protons at C-8, C-9 and C-10, respectively, appeared as coupled doublet-triplet-doublet δ 7.50-7.40-7.75, as it would for the proton signals for a phenoxazinone structure. The C-6 proton appeared as a singlet at δ 6.70, at a value characteristic of a quinone structure [6]. The C-2 proton appeared as a singlet at δ 8.1, reasonable for the spectral data of a carbomethoxypyrido-[*a*]phenoxazinone system. Furthermore the two carbomethoxy-groups appeared as two singlets at δ 3.80 and 3.85.

The electron impact measurement did not yield a consistent mass spectrum for the compound **2**; but its reduced form (after ascorbic acid treatment) showed a mass peak at 495.

From the yellow fraction **C**, the just reported **2**, **3** and **4** were obtained.

The red solution **A** containing dihydroxanthommatin (**7**), with λ max at 490 and 385 nm, was transformed into a yellow solution **A'** (λ max 450-385 nm) after 1 hour of uninterrupted visible light irradiation, and into a red solution **A''** (λ max 485-385 nm) after 5 hours of irradiation (Figure 1).

The solution **A**, kept in darkness, for the same period of time, is stable. From the transformed solution **A''**, analysed on carboxymethylcellulose column, two red fractions, **D** and **E**, and an orange fraction **F** were obtained.

The fractions **D**, **E** and **F** were analysed after aqueous acetic anhydride and methanol-sulphuric acid treatments. They gave the products **2** and **3**, **2**, and the 1,6-dimethoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)-5*H*-pyrido[3,2-*a*]-phenoxazin-5-one (**6**), respectively. The untreated fractions **D**, **E** and **F** were also photoirradiated separately and the phototransformation products were analysed. The orange fraction **F** (λ max 477-385 nm) is stable under irradiation.

The red fraction **D**, stable in darkness, with λ max at 485 and 385 nm, has the same photochemical behaviour as the red solution **A** containing **7**. The irradiated solution **D**, subsequently treated with acqueous acetic anhydride and methanol-sulphuric acid, gave the products **2**, **3**, **4** and small amount of **6**.

The red fraction **E**, stable in darkness, with λ max at 490 and 375 nm,, after 4 hours of irradiation under the above reported conditions, showed two maxima at 460 and 375 nm (Figure 2) and, after treatment with aqueous acetic anhydride and methanol-sulphuric acid, afforded **6** together with small amounts of unidentified coloured products.

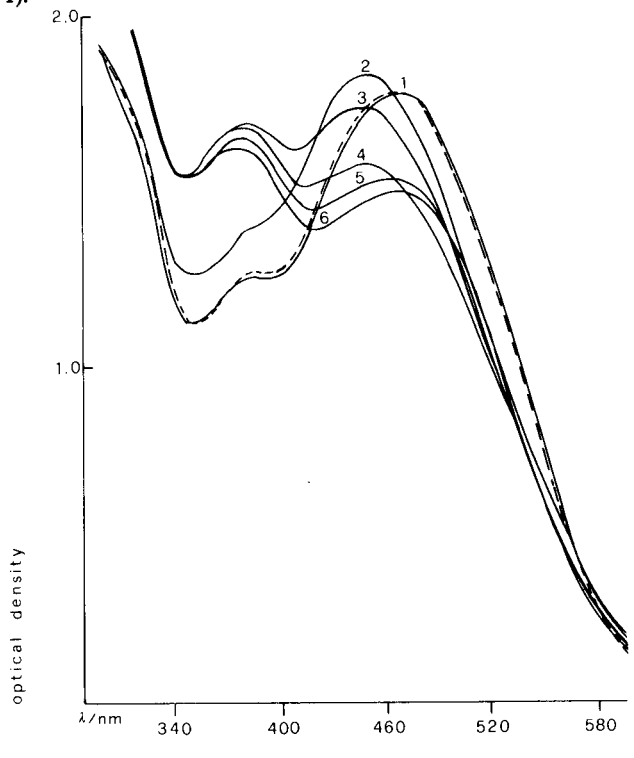


Figure 1. Spectral changes of the native pigment in the extraction solution caused by visible light irradiation; curve 1: first spectrum; curve 6: after 5 hours of irradiation; dashed curve: standard sample.

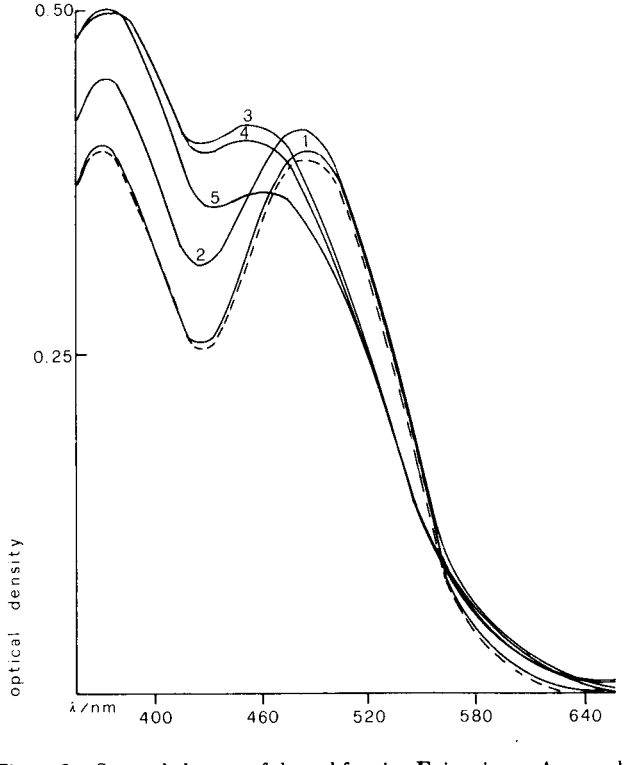


Figure 2. Spectral changes of the red fraction **E**, in mixture A, caused by visible light irradiation; curve 1: first spectrum; curve 5; after 4 hours of irradiation; dashed curve: standard sample.

Discussion.

We reported the presence, in the eyes and skin of some cephalopods, of a yellow phenoxazinone ommatin **1** that cyclizates to dihydroxanthommatin (**7**), oxidizing itself to xanthommatin (**8**) [1]. In no way the product **1** arises from the opening, during the acid extraction, of the pyrido[*a*] ring.

Figure 1 shows the uv absorption curves of the solution **A** irradiated by visible light. After 1 hour of irradiation the starting solution (curve 1, Figure 1), containing dihydroxanthommatin, is transformed into the yellow solution **A'** containing xanthommatin (curve 2, Figure 1) as shown by the maximum shifts from 390-485 nm to 385-450 nm. The subsequent rise in absorption at 380 together with the decrease of the maximum at 450 nm, in the red solution **A''**, showed that the xanthommatin phenoxazinonic system is opening (curve 3, Figure 1), just as Schäfer reported regarding the opening of the phenoxazinonic systems in methanol [7] and as was recorded by us on the light-induced opening of **4** in methanol [4].

After 5 hours of photoirradiation, solution **A''**, which has become photostable, showing two maxima at 385 nm and at 480 nm, is concentrated and analysed on a carboxymethyl-cellulose column. The coloured fractions **D**, **E**, and **F** are recovered.

The red fraction **D**, containing dihydroxanthommatin, showed the same photochemical behaviour as the red fraction **A**, recording the uv curves of Figure 1 under irradiation. The red fraction **E**, showing an uv spectrum with maxima at 375 and 480 nm, yielded **2** after acetylation and methylation. Differently from the fraction **D**, the fraction **E**, under visible light irradiation, showed an hypsochromic shift from 480 to 460 nm (curve 5, Figure 2) and yielded **6**, after acetylation and methylation. The fraction **F**, stable under irradiation, yielded **6** by acetylation and methylation.

The reported data suggest that the xanthommatin adds water on 6a position under the influence of the light and undergoes the phenoxazinone ring opening **9** (curve 3, Figure 1). Subsequently, on the quinoline *p*-quinonimine system a reductive photoinduced water addition takes place and compound **10** (curve 6, Figure 1), obtained in this way, reduces the xanthommatin, present in the mixture, yielding dihydroxanthommatin and oxidizing itself to **11** [4] (Scheme A).

When the solution **E**, containing **10**, is concentrated *in vacuo*, this intermediate product undergoes oxidative dehydratation to **9** yielding **2** after acetylation and methylation.

Moreover **E** under visible light irradiation, showed a hypsochromic shift from 480 to 460 nm in consequence of a photooxidation yielding **12** and, from this, **6**. Compound **6**, obtained from substitution of the hydroxy-group with a methoxy group during the acid methylation, is isolated only from the fraction **F** and **E**, *after irradiation*, and never from the non-photoirradiated red fractions **A**, **D**, **E** or from other fractions.

The photochemical behaviours of the synthetic models of **3** and **4** [3] also showed a bathochromic shift associated with a reversible solvent photoaddition on the C-6 of the phenoxazinonic system [4]. On the contrary, the photochemical reactions reported here are not reversible in darkness overnight, because they involve the opening of the phenoxazinone or phenoxazine system as observed for the photochemical behaviour of **4** in methanol [4].

Moreover, it is known that the acid environment gives rise to the decarboxylation reaction on the pyrido[*a*] system [8] and in this way the finding of carboxylated and decarboxylated products is justified.

While th primary process of vision is based on a transduction mechanism and the rodopsine turnover is well known the secondary process of photoinduced excitation is not at all known [9].

Colour changes depending on *p*H values as well as on spatial structure transformation of photopigments under visible light irradiation and the redox properties put the ommochromes in a crucial position with respect to the complex phenomen of the photoinduced excitation response [10].

EXPERIMENTAL

The uv spectra were recorded with a Perkin-Elmer 550-S spectrophotometer. The ir spectra were detected in chloroform with a Perkin-Elmer 399 spectrophotometer. The ^{1}H nmr were recorded with a Bruker 270 MHz spectrometer in deuteriochloroform using tetramethylsilane as an internal reference, chemical shifts are given in δ (ppm), s = singlet, d = doublet, t = triplet, m = multiplet; signal attributions were confirmed with the homonuclear de-coupling technique.

The natural pigment was purified on CM23 carboxymethylcellulose columns eluted with a buthanol-acetic acid-water 60:15:25 v/v mixture (mixture A).

General Procedure for Acetylation and Methylation.

The products, evaporated *in vacuo*, were treated with 300 ml of an acetic anhydride-water 50:50 v/v mixture at room temperature for 18 hours. The mixture, evaporated *in vacuo*, was treated with 250 ml of a methanol-sulphuric acid conc. 99:1 v/v mixture and heated to reflux for 2 hours. The solution, cooled to room temperature and neutralized with sodium acetate, was extracted three times with 150 ml of chloroform. The products, evaporated *in vacuo*, were purified on 0.5 mm Whatman PK6F silica gel layers eluted with a benzene-methylene chloride-methanol 45:45:10 v/v mixture (mixture B). The chromatographic purity and the Rf were checked on 0.25 mm Whatman PK6F silica gel analytic layers eluted with mixture B.

Extraction and Purification of Native Pigment.

The eyes of *c.a.* 10 Kg of *Loligo Vulgaris* were treated five times with 300 ml of acetone, filtered *in vacuo* and extracted six times with 300 ml of a butanol-acetic acid-0.5*N* hydrochloridric acid 60:15:25 v/v mixture in darkness at a temperature of 5°. The extracted mixture **A** (*c.a.* 14 mg of pigment), concentrated *in vacuo*, was purified on a CM23 column eluted with mixture A. From the column two coloured fractions were eluted: a red fraction **B** (λ max 485-385 nm, 8 mg) and a yellow fraction **C** (λ max

450-400 nm, 6 mg). From the acetylation and methylation, as described above, for the red fraction **B**, four coloured products were obtained: **2** (2.7 gm), **3** (1.2 mg), **4** (1.7 mg) and **5** (3.2 mg), identified by comparison of their spectral (ir, uv, nmr) and chromatographic properties with authentic samples.

From the acetylation and methylation of the yellow fraction **C**, three coloured products were isolated and identified: **2** (3.5 mg), **3** (1.9 mg) and **4** (1.3 mg).

General Procedure for Photoirradiation.

The solution was exposed to a 650 W OSRAM lamp at 10 cm of distance at a temperature of 5° and was spectrophotometrically controlled every hour. A small amount of solution was preserved in darkness at a temperature of 5° as standard sample. The irradiated solution was kept in darkness at a temperature of 5° overnight and was again irradiated the day after.

Photochemistry of the Native Pigment in the Extraction Mixture.

The extracted solution **A**, with λ max at 490-385 nm, was photoirradiated. After 1 hour of irradiation, the uv spectrum showed two maxima at 450 and 385 nm. After 5 hours of irradiation the uv spectrum showed two maxima at 485 and 385 nm and the reaction was not reversible in darkness overnight (Figure 1). The irradiated mixture, concentrated *in vacuo*, was purified on a CM23 column eluted with mixture A. From the column two red fractions **D** (4.1 mg) **E** (3.7 mg), and an orange fraction **F** (3.5 mg) were eluted. The three fractions were separately concentrated *in vacuo* to 200 ml. Two hundred ml of each fraction was irradiated and 200 ml was acetylated and methylated as above described. The acetylated and methylated fraction **D** yielded: **2** (2.0 mg), **3** (1.2 mg). Many coloured products in small amounts were obtained from the acetylated and methylated fraction **E**, among which **2** (2.7 mg) was isolated and identified. From the orange fraction **F** (λ max 477-385), after acetylation and methylation **6** (3.4 mg) was isolated and identified.

Photoirradiation of the Fraction **D**.

The red fraction **D**, stable in darkness, with λ max 485-385 nm, was irradiated in mixture A. After 1 hour of irradiation, the uv spectrum showed a maximum of absorption at 450 nm and a shoulder at 385 nm. After 5 hours of irradiation, the uv spectrum showed two maxima at 485 and 385 nm, and the reaction was not reversible in darkness overnight (Figure 1). The irradiated mixture, evaporated *in vacuo*, was acetylated and methylated as above described. Four coloured products were obtained: **2** (1.8 mg), **3** (0.8 mg), **4** (1.3 mg) and small amounts of **6**.

Photoirradiation of the Fraction **E**.

The red fraction **E**, stable in darkness, with λ max 490 and 375 nm, was irradiated in mixture A. After 4 hours of irradiation, the uv spectrum showed two maxima at 460 and 375 nm (Figure 2), and the reaction was not reversible in darkness overnight. The irradiated mixture, evaporated *in vacuo* and acetylated and methylated as above described, yielded many coloured products in small amounts among which **6** (3.3 mg) was isolated and identified.

1-Hydroxy-3-carbomethoxy-11-(β-aspartoyl-*N*-acetyl-methyl ester)-5*H*-pyrido[3,2-*a*]phenoxazin-5-one (**2**).

From the reaction mixture yellow crystals of Rf 0.28 (mixture B) were isolated; ir (chloroform): cm^{-1} 3400-3300 (NH, OH), 1740 (COOCH), 1660-1650 (CO); uv (methanol): λ max (log ε) 438 (3.9), 380 (s); ^1H nmr (deuteriochloroform): δ 2.1 (s, 3H, CH_3CO-), 3.80 (m, 2H, -CH_2-CO-), 3.80 (s, 3H, CH_3O-), 3.85 (s, 3H, CH_3O-), 4.85 (m, 1H, -C*H*-NH-), 6.70 (s, 1H, quinone), 6.75 (d, 1H, -N*H*-CH-), 7.40 (t, 1H, aromatic), 7.50 (d, 1H, aromatic), 7.75 (d, 1H, aromatic), 8.1 (s, 1H, aromatic).

Anal. Calcd. for $C_{24}H_{19}N_3O_9$: C, 58.42; H, 3.88; N, 8.52. Found: C, 58.50; H, 3.96; N, 8.82.

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