QUINOID PIGMENTS OF Echinodermata

III. MINOR PIGMENTS OF THE SEA URCHIN Strongylocentrotus nudus*

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The quinoid pigments of the test and needles of the sea urchin Strongylocentrotus nudus (Agas) consist mainly of spinochromes A, B, and C and echinochrome A [2]. We have established that, in addition to the components mentioned, these animals produce a number of minor quinones of the same type. The chromatographic mobilities of the pigments are given below (the R_f values were determined in a nonfixed layer of KSK–ShshK silica gel in system 1):

Pigment	R_f
Pigment I	0.67
Pigment II	0.60
Spinochrome A	0.56
Spinochrome C	0.42
Pigment III	0.40
Echinochrome A	0.38
Pigment IV	0.37
Spinochrome B	0.34
Pigment V	0.30
Pigment VI	0.25
Pigment VII	0.24

The least polar minor component I, with a violet color, has an absorption spectrum that is typical for naphthazarin derivatives [3]. The mass spectrum contains the peaks of the molecular ion m/e 278 and of fragmentary ions with m/e 236 (M–42) and 235 (M–43). These fragmentation pathways are confirmed by the presence in the spectrum of the peaks of metastable ions at 200.3 and 198.5 and show the presence of an acetyl group in the molecule of this fragment [4]. Since the splitting out of CO from the molecular ion precedes the loss of the methyl radical of the acetyl group, the latter is most probably located in the quinoid part of the molecule. The ion (M – 28) also splits out water, which is confirmed by a metastable ion at 215.5, showing the adjacent positions of the acetyl and hydroxy groups [4]. The known quinoid pigments of sea urchins are either unsubstituted polyhydroxynaphthoquinones or compounds of this type containing one two-carbon substituent, and therefore the observation in the fragment under investigation of, in addition to the acetyl group, another carbon-containing substituent, a methyl group, was all the more unexpected and interesting. Its presence is shown by an ion with m/e 263 (M – 15) in the mass spectrum, and by a signal at 2.17 ppm (ar. CH_3) in the PMR signal.

On methylation with diazomethane, pigment I formed a dimethyl ether, which was accompanied by an increase in the molecular weight by 14 × 2 units and by the appearance in the PMR spectrum of a signal at 4.08 ppm (6H, 2 ar. CH_3O). The signals of the protons of the two peri-hydroxyls of the naphtharizin system at 12.70 and 13.04 ppm remained unchanged.

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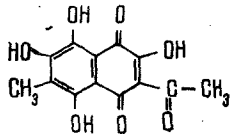
On the basis of these results it may be assumed that the pigment is 3-acetyl-2,7-dihydroxy-6-methylnaphthazarin, or 2-acetyl-3,7-dihydroxy-6-methylnaphthazarin. The C-methylation of natural spinochrome A with acetyl peroxide according to Fieser gave 3-acetyl-2,7-dihydroxy-6-methylnaphthazarin. The chromatographic behavior, melting point, and absorption and mass spectra of the substance synthesized were identical with those of the natural pigment, and this has permitted us to assign the following structure to it:



Another minor violet pigment, IV, and R_f values very close to those of echinochrome A. Absorption and mass spectra and the melting points of the pigment and its dimethyl ether agree well with those given in the literature for mompain (2,7-dihydroxynaphthazarin) and its dimethyl ether, respectively [5]. A confirmation of this structure is the complete coincidence of the chromatographic behavior, melting point, and absorption spectra of pigment IV and the product obtained by the deacetylation of natural spinochrome A.

In individual years, considerable, sometimes predominating, amounts of the highly polar pigment VII appear among the total pigments of the sea urchin, this substance corresponding in all its indices to the spinochrome E described in the literature [6]. The product of its methylation with diazomethane likewise corresponds to spinochrome E tetramethyl ether. Consequently, pigment VII is spinochrome E.

The other minor pigments were isolated in amounts not exceeding 1-1.5 mg. From the nature of their absorption spectra, three of them (II, V, and VI) are naphthazarin derivatives [3]. They were methylated with diazomethane, forming methyl ethers the absorption spectra of which were characteristic for the spectra of methyl ethers of hydroxynaphthazarins [3].

The PMR spectrum of the methyl ether of pigment V contains signals at 12.92 and 13.03 ppm showing the presence of two peri-hydroxyls, and at 3.95, 4.07, and 4.15 ppm, confirming the presence of three methoxyls in the ether molecule. However, neither the initial pigment or its methyl ether showed under electronic impact the fragmentation characteristic for the naphthazarin derivatives usually found in sea urchins and the methyl esters of these compounds. A similar uncertain picture was found in the case of the mass spectrum of pigment II and its methyl ether, and therefore further investigations are necessary to establish the complete structure of these pigments.

Pigment VI has a mass spectrum coinciding, in the main, with that of the binaphthoquinone isolated from Stronglyocentrotus intermedius and Strongylocentrotus dröebachiensis, but possesses greater mobility on TLC and a lower melting point of 221-222°C (for the binaphthoquinone mentioned, R_f =0.22, system 1, mp > 310°C). Pigment VI and its methyl ether have absorption spectra close to those of the binaphthoquinone and its hexamethyl ether, respectively. Furthermore, the ether of VI has mp 66-68°C, which is close to the melting point of the hexamethyl ether of the binaphthoquinone isolated from Stronglyocentrotus dröebachiensis (71-73). The mass spectrum of the methyl ether of pigment VI differs not only from the spectrum of the methyl ether of the binaphthoquinone but also from the spectra of the methyl ethers of monomeric polyhydroxynaphthoquinones.

Another pigment, III, present in trace amounts, has been found in this species of sea urchin. On KSK silica gel in all solvent systems used it has R_f values very close to those of spinochrome C. It was possible to separate it on silica gel LCh of Czech origin containing 15% of 1 N oxalic acid solution. Pigment III is yellow but on storage in the crystalline state and in solution a substance with the R_f values and absorption and mass spectra of spinochrome C appears. The mass spectrum of pigment II is also identical with that of spinochrome C [2].

When a methanolic solution of spinochrome C was stored for a long time, again the appearance of a yellow pigment chromatographically indistinguishible from pigment III took place. We have also observed such mutual transitions of one colored form of a minor pigment into another in the case of other species of sea urchin (for example, in Scaphechinus mirabilis. It is possible that the mutual conversions of these pigments are connected with the tautomerism of the naphthalene system upon which they are based.

EXPERIMENTAL

The pigments were separated first by column chromatography and then by repeated preparative TLC in a nonfixed layer of KSK silica gel containing 15% of a 1 N aqueous solution of oxalic acid (KSK-ShchK), using the

following solvent system (by volume): 1) benzene–methanol–1 N oxalic acid (5 : 1 : 0.05); 2) benzene–methanol –1 N oxalic acid (10 : 1 : 0.005); 3) chloroform–methanol–1 N oxalic acid (20 : 1 : 0.01); 4) chloroform.

The methyl ethers of the pigments were obtained by treating ethereal solutions of them with an ethereal solution of diazomethane until the evolution of bubbles of gas ceased. The main methylation product was separated by preparative TLC on KSK-ShchK silica gel in chloroform; the R_f values of the ethers are given for these conditions.

The absorption spectra were taken on a Shimadzu MPS-5000 spectrophotometer, the PMR spectra on a Brüker HX-90E instrument with a working frequency of 90 MHz (δ, ppm; 0 – TMS), and the mass spectra on an LKB-9000S instrument with the direct introduction of the sample into the ion source at an ionizing voltage of 70 eV. The melting points were determined on a Kofler block.

3-Acetyl-2,7-dihydroxy-6-methylnaphthazarin (pigment I). This pigment was isolated from the first colored fraction eluted from the column. It was purified first by preparative TLC (system 4) and then by vacuum sublimation. On heating to 130°C, the crystals changed into thin elongated needles melting at 179-181°C. Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 261, 324, 506 sh., 535, 568 sh. (log ε 4.24, 4.02, 3.71, 3.47, 3.66). Mass spectrum: m/e 278 (M^+, 100%), 263(5), 250(45), 236(9), 235(72), 232(18), 209(5), 208(26), 189(5), 179(5), 138(5), 137(5), 109(2), 93(5), 83(10), 77(4), 69(5), 55(5), 43(5); PMR spectrum ($CDCl_3$) : 2.17 (ar. CH_3), 2.86 (ar. Ac); 12.0 and 15.6 (ar. OH); (CD_3OD): 2.15 (ar. CH_3), 2.47 (ar. Ac).

After chromatographic purification, 3-acetyl-2,7-dimethoxy-6-methylnaphthazarin was recrystallized from methanol, mp 116-118°C. Mass spectrum: m/e 306 (M^+). Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 247, 312, 490 sh., 513 sh. (log ε 3.56, 3.36, 3.24, 3.26, 3.18). PMR spectrum ($CDCl_3$): 2.15 (3H, ar. CH_3), 2.54 (3H, ar. $COCH_3$), 4.08 (6H, 2 ar. CH_3O), 12.71 (1H, ar. OH), 13.05 (1H, ar. OH).

Synthetic 3-Acetyl-2,7-dihydroxy-6-methylnaphthazarin. A solution of 20 mg of spinochrome A (3-acetyl-2,7-dihydroxynaphthazarin) in acetic acid was treated with an acetic acid solution of acetyl peroxide, and the mixture was heated in an atmosphere of argon on the steam bath for 2 h. After the end of the reaction, the mixture was diluted with benzene and evaporated in vacuum. Dilution followed by evaporation was repeated twice more. The main reaction product was purified by preparation TLC (chloroform). Yield 3.6 mg (R_f 0.67 (system 1). On heating to 130°C, the crystals changed into thin elongated needles melting at 179-181°C. Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 260, 323, 507 sh., 532, 565 sh. (log ε 4.26, 4.06, 3.72, 3.77, 3.68). The mass spectrum corresponded in detail with the mass spectrum given for the natural sample.

2,7-Dihydroxynaphthazarin (pigment IV). This pigment, after column chromatography on KSK-ShchK, was freed from echinochrome A on a column of Sephadex LH-20, being eluted with a mixture of benzene and methanol (8:1). Needles from benzene not melting below 310°C. Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 272, 318, 495 sh., 522, 560 sh. (log ε 3.66, 3.62, 3.45, 3.49, 3.27). Mass spectrum: m/e 222 (M^+, 100%), 206(6), 194(32), 176(10) 166(10), 152(20), 148 (12), 138(6), 137(10), 124(15), 97(10), 96(12), 95(10), 83(12), 69(42).

After preparative TLC, the 2,7-dimethoxynaphthazarin was purified by sublimation, mp 273-275°C (subl.).

Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 266, 290, 311, 490 sh., 523, 564, sh. Mass spectrum: m/e 250 (M^+).

Deacetylation of Spinochrome A. A mixture of 143.5 mg of spinochrome A (3-acetyl-2,7-dihydroxynaphthazarin) and 3 ml of concentrated H_2SO_4 was heated in the boiling-water bath in an atmosphere of argon for 2.5 h. Then it was poured onto ice and extracted with diethyl ether. The deacetylation product was purified by sublimation. Yield 37 mg. Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 272, 318, 495 sh., 522, 560 sh. (log ε 3.66, 3.62, 3.45, 3.49, 3.27). mp >310°C.

Spinochrome E (pigment VII) was isolated from the last fractions issuing from the column, and it was transferred into methanolic solution and was reprecipitated from it with benzene. The brown-orange needles did not melt below 310°C. Absorption spectrum, nm; $\lambda_{max}^{CH_3OH}$ 269, 359, 453 sh., 479, 513 sh. (log ε 4.15, 3.67, 3.47, 3.55, 3.35). Mass spectrum: m/e 254 (M^+, 83%), 226 (100), 197(10), 180 (6), 169 (13), 152 (15), 141 (9), 124 (17), 113 (8), 95(9), (93)(10), 77(13), 69(11), 53(16). After preparative TLC, 2,3,6,7-tetramethoxynaphthazarin (R_f 0.35) was recrystallized from aqueous methanol. mp 183-184°C. Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 243, 325, 475 sh., 503, 537 sh. Mass spectrum: m/e 310 (M^+).

Pigment V. After preparative TLC, this was recrystallized from acetic acid. mp. 183-186°C (decomp.). Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 268, 321, 470, 531 sh. The methyl ether of pigment V, after preparative

TLC, was recrystallized from aqueous methanol. mp 133-134°C. Absorption spectrum; nm: $\lambda_{max}^{CHCl_3}$ 259, 317, 477 sh., 501, 533 sh.

Pigment II. After preparative TLC, this was recrystallized from methanol. On being heated to 131°C it formed needles subliming at 201-203°C. Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 272, 316 sh., 492 sh., 520, 564 sh. The methyl ether of pigment II was prepared, and the melting point of the substance eluted from a preparative plate was 153.5-156°C. Absorption spectrum, nm: $\lambda_{max}^{CHCl_3}$ 242, 282 sh., 310, 496 sh., 515, 548 sh.

Pigment VI. After preparative TLC, this was recrystallized from aqueous acetic acid. mp 221-222°C. Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 265, 339, 470, 490 sh., 528 sh. Mass spectrum m/e 368, 290, 275, 264, 238, 210, 192, 181, 168, 153, 140, 136, 125, 124, 108.

The methyl ether of pigment VI, after preparative TLC, was recrystallized from aqueous methanol. mp 66-68°C. Absorption spectrum; nm: $\lambda_{max}^{CHCl_3}$ 246, 312, 481 sh. 506, 539 sh.

Pigment III. This was freed from spinochrome C by preparative TLC on LCh silica gel containing 15% of 1% aqueous oxalic acid solution in the benzene–acetone–1 N oxalic acid (3 : 1 : 0.03) system. Under these conditions the R_f value of pigment III was 0.04 and that of spinochrome C 0.34. Absorption spectrum, nm: $\lambda_{max}^{CH_3OH}$ 271, 295, 415. Mass spectrum: m/e 280, 265, 262, 252, 237, 234, 210, 185, 140, 139, 43.

SUMMARY

1. 3-Acetyl-2,7-dihydroxy-6-methylnaphthazarin, not previously described, has been isolated from the test and needles of the sea urchin Strongylocentrotus nudus.

2. The pigments of this sea urchin have been shown to include 2,7-dihydroxynaphthazarin and spinochrome E.

3. A number of minor pigments forming naphthazarin derivatives has been isolated. One of them is connected with spinochrome C by mutual transformations.

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