Structures of the Pigments from the Spines of *Heterocentrotus Mamillatus* (LINNÉ)

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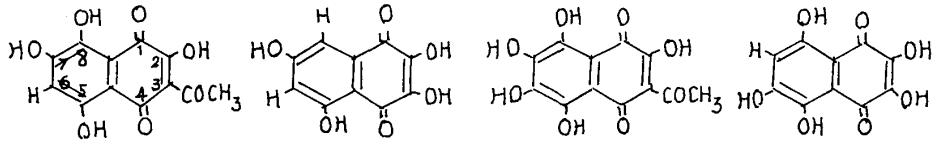
1. Introduction

Lederer and Glaser (1938)[1] first obtained a dark violet pigment from the spines of the sea urchin, *Strongylocentrotus lividus* (LAMARCK), and named it "spinochrome." Since then, a large number of papers on the pigments from the spines and test (shell) of sea urchins have been published, and more than twenty authentic spinochromes have been known.[2] The well-known are spinochrome A* (3-acetyl-2, 7-dihydroxynaphthazarin),[3] spinochrome B (2, 3, 7-trihydroxyjuglone),[4] spinochrome C (3-acetyl-2, 6, 7-trihydroxynaphthazarin)[5] and spinochrome D (2, 3, 6-trihydroxynaphthazarin);[6],[7] their constitutional formulas are shown below.



The separation of spinochromes is difficult because they are obtained as mixtures of compounds with very similar structures.

Kuroda and Ohshima (1940)[10] obtained a crystalline red pigment (m.p. 229°C) from the spines of the Japanese sea urchin, *Heterocentrotus mamillatus* (LINNÉ) (Japanese name,Paipu-uni) and named it "spinochrome F." Later, Kuroda and Okajima (1960)[11] renamed the pigment "spinochrome F_1" (m.p. 245°-247°C) because another pigment was detected in the species; they suggested a molecular formula of $C_{12}H_8O_8$ for sp. F_1.

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* The alphabetical system of nomenclature was proposed by Goodwin et al. The semi-trivial system shown in the parentheses was proposed by Moore et al., by which the pigments are named as substituted juglones or naphthazarins. See the references 8) and 9). The two systems are used in this paper.

** An abbreviation for spinochrome.

However, the structure of sp. F_1 had remained unknown until Chang et al. (1964)[5] reported that sp. F_1 was identical with sp. C (III), one of the spinochromes isolated from the Hawaiian sea urchin, *Echinometra oblonga* (BLAINVILLE).

Therefore, the pigments from the spines of *Heterocentrotus mamillatus* were examined and the separation of the pigments was effected by means of thin-layer chromatography on deactivated silica gel. As a result, three spinochromes were obtained from the species for the first time, and by spectroscopic investigations they were proved to be spinochromes A, B and C, respectively. Kuroda's spinochrome F_1 was proved to be identical with spinochrome C. This paper gives the details of their separation and structure elucidation.

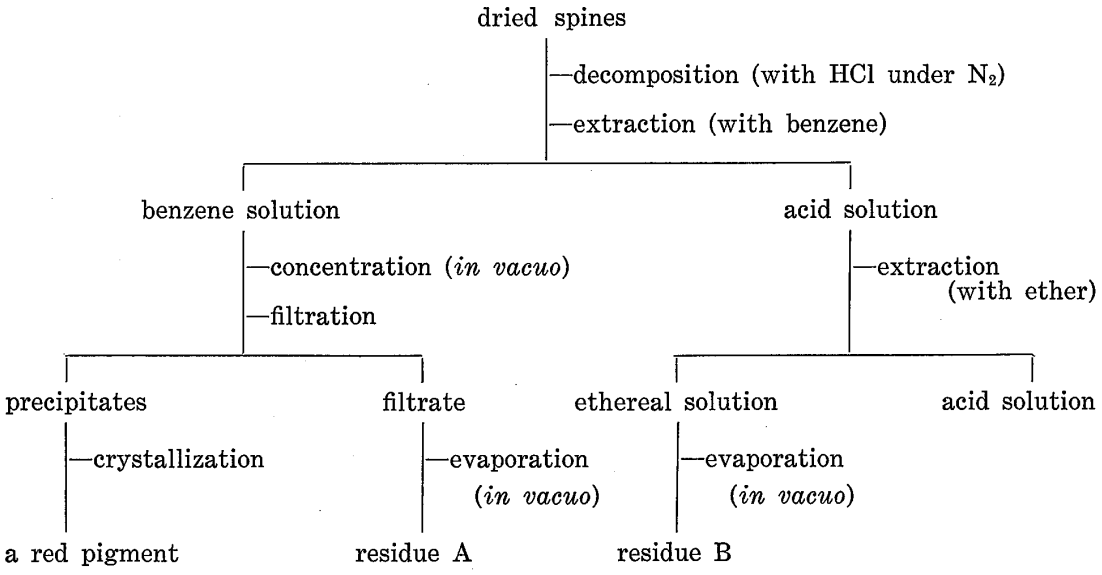
Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus, and uncorrected. The electronic spectra were recorded with a Hitachi 124 double-beam spectrophotometer, and the infrared spectra with a Jasco IR-G spectrophotometer. The nuclear magnetic resonance spectra were recorded with a Varian HA-100 spectrometer or a JEOL JNM-C-60HL spectrometer, tetramethylsilane being used as an internal reference. The mass spectra were obtained on a JEOL JMS-01SG-2 mass spectrometer.

1. *Separation and purification*

The spines of *Heterocentrotus mamillatus* collected in the waters of Okinawa Island were used as material.

The spines were washed with water until the washings became colorless and odorless, and dried in the air. The air-dried spines



(1 kg) were decomposed with concentrated hydrochloric acid (800 m*l*) under nitrogen and treated as shown in the accompanying flow chart.

Separation of the pigments from each residue was accomplished by means of thin-layer chromatography under nitrogen. Glass plates (20×10 cm) coated with deactivated silica gel were used. (The deactivated silica gel was prepared by the addition of 36 m*l* of 3% aqueous oxalic acid to 18 g of Merck silica gel G.). Development was effected with a mixture of benzene, chloroform and ethanol (5:10:2), and three bands were obtained. The band with the highest R_f value was purple, which was followed by an orange band, and the one with the lowest R_f value was yellow. After elution with acetone and removal of the eluent, each pigment was extracted with benzene and the benzene was removed *in vacuo*. Spinochrome 1 from the purple band, sp. 2 from the orange band and sp. 3 from the yellow band were obtained.

Spinochrome 1 was recrystallized from methanol as red-purple needles, m.p. 197°–198° C; $\lambda_{max.}^{EtOH}$ 520 nm (log ε, 3.86), 314 nm (log ε, 4.26), 270 nm (sh.), 250 nm (log ε, 4.29); IR (KBr) 1550–1660 cm^{-1}; NMR (DMSO-d_6) δ3.16 (*s*, 3H), δ6.56 (*s*, 1H) ppm; *m/e* 264 (M^+), 218, 124, 69.

Spinochrome 2 was recrystallized from benzene, and then from chloroform as red-orange needles, m.p. 255° C; $\lambda_{max.}^{EtOH}$ 460 nm (log ε, 3.89), 325 nm (sh.), 295 nm (log ε, 4.22), 240 nm (log ε, 4.21); IR (KBr) 1668, 1618, 1600, 1580, 1555 cm^{-1}; NMR (DMSO-d_6) δ 2.59 (*s*, 3H) ppm; *m/e* 280 (M^+), 234, 140. Found: C, 51.59; H, 2.84%. Calcd. for $C_{12}H_8O_8$: C, 51.43; H, 2.86%.

Spinochrome 3 was recrystallized from methanol as red needles, which sublimed at above 300°C without decomposition; $\lambda_{max.}^{EtOH}$ 475 nm (log ε, 3.09), 384 nm (log ε, 3.43), 320 nm (log ε, 3.87), 271 nm (log ε, 4.12).

The precipitates obtained from the benzene extract (cf. the flow chart) were recrystallized from benzene, and then from chloroform to give red-orange needles, m.p. 255°C. The pigment was identical with sp. 2 in all respects.

From 1 kg of the spines, 55 mg of crude sp. 1, 350 mg of crude sp. 2 and a small amount of crude sp. 3 were obtained.

2. *Acetylation of spinochrome* 3

To a mixture of sp. 3 (40 mg) and acetic anhydride (0.4 m*l*), was added a small amount of concentrated sulfuric acid dropwise with stirring. After 55 minutes, the reaction mixture was decomposed with ice to give yellow crystals, which were collected by filtration. The product was recrystallized from methanol as yellow needles. m.p. 154°–154.5°C; *m/e* 390 (M^+), 340, 306, 264, 222. Found: C, 55.37; H, 3.86%. Calcd. for $C_{18}H_{14}O_{10}$: C, 55.39; H, 3.62%.

Results and discussion

1. *Spinochrome* 1 (*red-purple needles from methanol, m.p.* 197°-198°C)

The pigment appears as a purple band with the highest R_f value on the deactivated silica gel. The infrared spectrum (KBr) shows the carbonyl absorption of the quinonoid system at 1660-1550 cm^{-1} and agrees with that of an authentic sample of spinochrome A(I).

The n.m.r. spectrum in dimethyl sulfoxide-d_6 shows singlets at δ 3.16 ppm and δ 6.56 ppm, which are in good agreement with those at 3.16 ppm (C_3-$COCH_3$) and δ 6.50 ppm (C_6-H) in the spectrum of an authentic spinochrome A.

The mass spectrum (Fig. 1) shows the same fragmentation pattern as that of spinochrome A, and discloses that the molecular weight is 264, which agrees with that of spinochrome A.

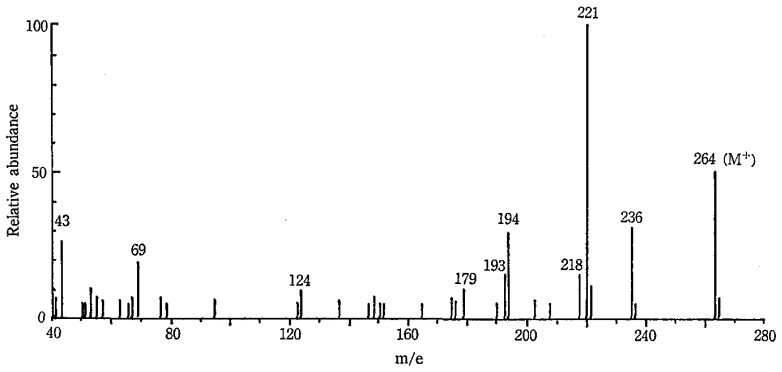
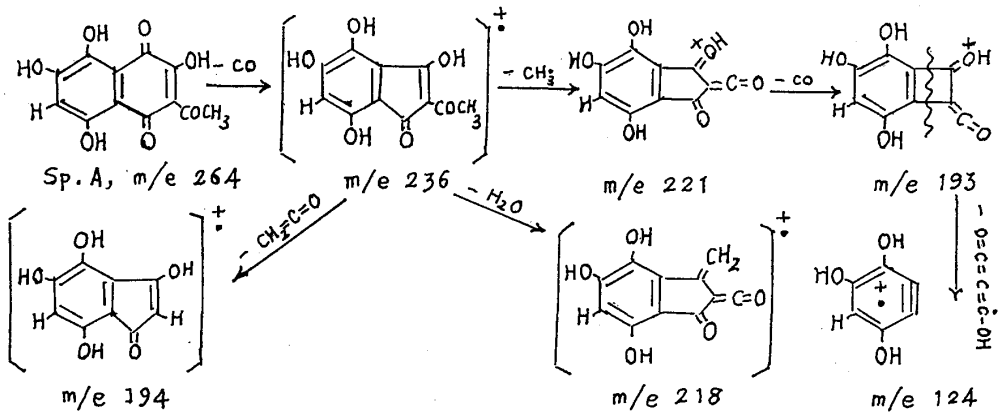


Fig. 1. Mass spectrum of spinochrome A.

The fragmentation pattern indicates the decomposition mode of 3-acetyl-2-hydroxynaphthoquinones, which was generalized by Djerassi et al. and applied successfully to the structure elucidation of spinochrome A.[12),13)] The mode is illustrated as follows.



In the decomposition mode, the elimination of carbon monoxide from the quinoid ring is considered to occur as a first fragmentation step because of the hydrogen bonding between the vicinal acetyl and hydroxyl groups. The peak at *m/e* 236 indicates the presence of the vicinal acetyl and hydroxyl groups and the peak at *m/e* 124 that of three hydroxyl groups on the benzene ring.

Consequently, spinochrome 1 is identical with spinochrome A (I), 3-acetyl-2, 7-dihydroxynaphthazarin.

2. *Spinochrome* 2 (*red needles from benzene and chloroform, m.p.* 255°C)

The pigment appears as an orange band following the purple band of spinochrome 1 on the deactivated silica gel. It is also obtained from the benzene extract as a red pigment (cf. the flow chart). The analytical value and the molecular weight (280 from the mass spectrum) suggest a molecular formula of $C_{12}H_8O_8$, which agrees with the formula of spinochrome F_1 reported by Kuroda and Okajima. Examinations by means of thin-layer chromatography showed the identity of spinochrome 2 with spinochrome F_1. The electronic spectral data on spinochrome 2 agree with those on spinochrome C reported by Chang et al.[5]

The infrared spectrum (KBr) shows the carbonyl absorption of the acetyl group at 1668 cm^{-1}, that of the quinonoid system at 1618 cm^{-1} and 1600 cm^{-1}, and the C=C absorption of the benzene ring at 1580 cm^{-1} and 1555 cm^{-1}.

The n.m.r. spectrum in dimethyl sulfoxide-d_3 shows a singlet at δ 2.59 ppm, which is in good agreement with the value δ 2.58 ppm (C_3-$COCH_3$) for spinochrome C reported by Chang et al.[5]

The mass spectrum is shown in Fig. 2.

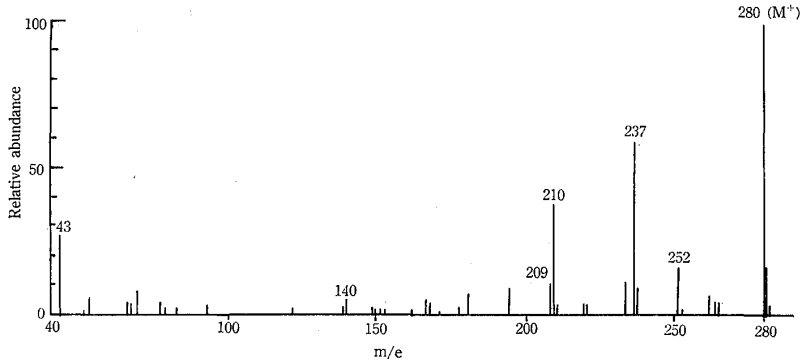
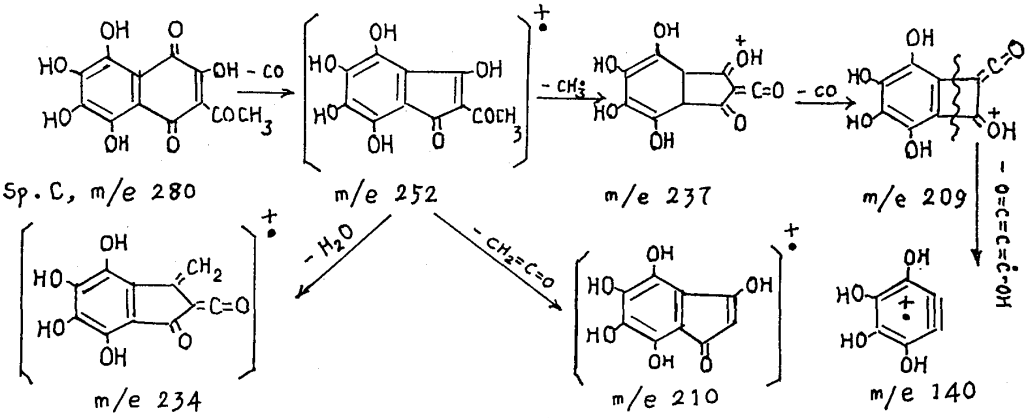


Fig. 2. Mass spectrum of spinochrome C.

On the basis of the fragmentation mechanism for spinochrome A, that for spinochrome C can be assumed as follows.



The fragmentation pattern of spinochrome 2 can be interpreted in terms of the mechanism assumed for spinochrome C.

In the mass spectrum of spinochrome 2, the presence of the peak at *m/e* 234 indicates that one molecule of water is lost from the vicinal hydroxyl and acetyl groups (C_2-OH and C_3-$COCH_3$); that of the peak at *m/e* 140 shows the presence of four hydroxyl groups on the benzene ring.

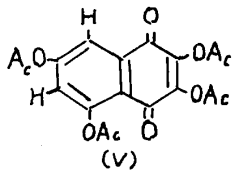
These results described above prove that spinochrome 2 is identical with spinochrome C (III), 3-acetyl-2, 6, 7-trihydroxy naphthazarin.

3. *Spinochrome* 3 (*red needles from methanol, subl.* >300°C)[14]

The pigment appears as a yellow band with the lowest R_f value on the deactivated silica gel. The electronic spectral data agree with those on spinochrome B reported by Anderson et al.[15] The infrared spectrum (Fig. 3) is in good agreement with that of an authentic sample of spinochrome B (II).

4. *The acetyl derivative of spinochrome* 3 (*yellow needles from methanol, m.p.* 154°-154.5°C)[16]

The analytical value and molecular weight (390 from the mass spectrum) of the derivative suggest a molecular formula of $C_{18}H_{14}O_{10}$. The infrared spectrum (Fig. 4) is in good agreement with that of an authentic sample of 2, 3, 5, 7-tetraacetyl spinochrome B (V).



(V)

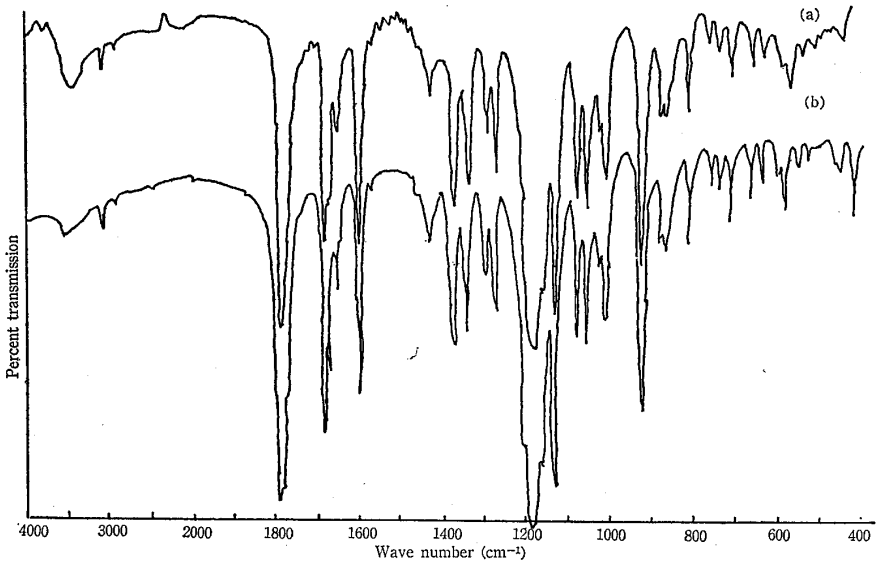


Fig. 3. Infrared spectra (KBr) of sp. 3 (a) and sp. B(b).

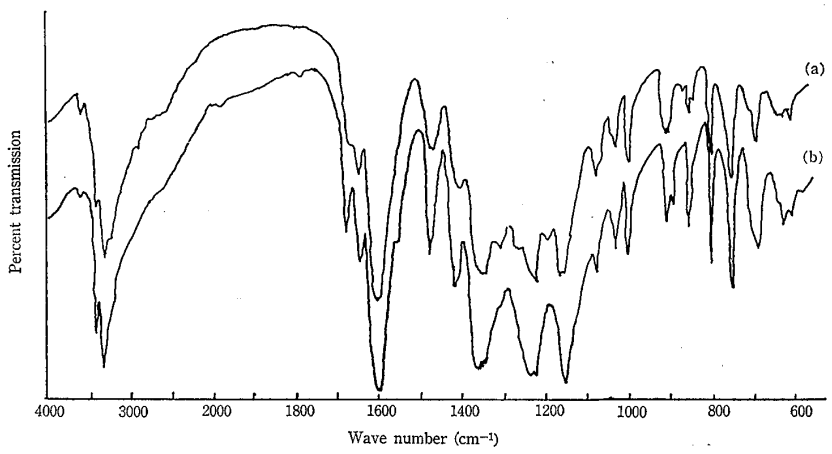


Fig. 4. Infrared spectra (KBr) of tetraacetyl sp. B(a) and the acetyl derivative of sp. 3(b).

The derivative gives no melting point depression when mixed with an authentic tetraacetyl spinochrome B.

Consequently, it can be concluded that the three spinochromes from *Heterocentrotus mamillatus* are sp.'s A, B and C, respectively, and Kuroda's spinochrome F_1 is identical with spinochrome C.

Summary

1. The separation of three spinochromes from *Heterocentrotus mamillatus* was effected by means of thin-layer chromatography on silica gel deactivated with oxalic acid.

2. The structures of the spinochromes were elucidated by spectroscopic methods. As a result, they were proved to be spinochromes

A, B and C, respectively, and the identity of spinochrome F_1 with spinochrome C was confirmed.

3. The fragmentation mechanism for spinochrome C was assumed on the basis of the generalized decomposition mode of 3-acetyl-2-hydroxynaphthoquinones and proved to be effective in the structure elucidation.

Acknowledgments

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