FATTY OILS OF AQUATIC INVERTEBRATES

XVI. FATTY OILS OF *CYNTHIA RORETZI* AND *PINNA PECTINATA JAPONICA* WITH A PARTICULAR REFERENCE TO THEIR STEROL COMPONENTS

TORU TAKAGI, TAKEO MAEDA and YOSHIYUKI TOYAMA

Department of Applied Chemistry

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Cynthia roretzi, a species of the *Protochordata*, has a comparatively wide distribution in Japanese waters, and its fleshy portion is edible. As regards the lipids of the *Protochordata*, only a few studies have come to the scope of our knowledge. Bergmann[1] reported cholesterol to be the major component of sterols of *Styela plicata*. Recently Kita and Wada[2] separated Δ^{7}-decenol together with two sterols, poriferasterol and clionasterol, from the acetone-extract of *Cynthia roretzi*. In the present study, characteristics of the fatty oil extracted from *Cynthia roretzi* and its fatty acids were determined, and sterol components in the unsaponifiable matter were examined.

The crude sterol mixture from *C. roretzi* was acetylated, and the acetylation product was first recrystallized from methanol and then subjected to chromatographic fractionation on an adsorption column of alumina, by which a less easily elutable fraction of $\Delta^{5,7}$-diene content 35.1% and an easily elutable fraction of $\Delta^{5,7}$-diene content below 5% were separated. Recrystallizing the former fraction repeatedly from acetone, a steryl acetate fraction was eventually obtained, the melting point and specific rotation of which accorded with those of 7-dehydrostigmasteryl acetate. It showed the ultraviolet absorption characteristic to $\Delta^{5,7}$-diene; $\varepsilon_{282}=12{,}330$ (molecular formula being taken as $C_{31}H_{48}O_2$). An inspection of its infrared spectrum indicated the presence of *trans* configuration in the side chain (absorption band at 966 cm^{-1}). The free sterol from this acetate fraction agreed with 7-dehydrostigmasterol in characteristic properties. 7-Dehydrostigmasterol was first prepared by Linsert[3] in 1936 from stigmasterol. Recently Matsumoto and his co-workers[4] separated this sterol from three species of shellfish, *Cristaria plicata spatiosa*, *Corbicula leana* and *Corbicula japonica*, and came to the conclusion that corbisterol[5]* formerly separated from *C. leana* is very likely to be an impure 7-dehydrostigmasterol.

The easily elutable fraction of $\Delta^{5,7}$-diene content below 5% was brominated in ether, and ether-insoluble bromide and ether-soluble bromide were separated.

* While the steric configuration at C-24 in corbisterol ($\Delta^{5,7,22}$-C_{29} sterol) could not been established conclusively, this sterol was tacitly regarded as a C-24 epimer of stigmasterol, since its acetate obtained in a previous study[5] showed a melting point (152°–153°C) considerably lower than the melting point (172°C) of 7-dehydrostigmasteryl acetate reported by Linsert.

Debromination of the ether-insoluble bromide yielded a steryl acetate which was regarded as poriferasteryl acetate. Free sterol and benzoate prepared from this acetate agreed with poriferasterol and its benzoate, respectively, in their properties. The ether-soluble bromide was fractionated further using ethanol, and a major fraction obtained was debrominated to give an acetate which was recognized as clionasteryl acetate. Free sterol and benzoate from this acetate agreed with clionasterol and its benzoate, respectively, in their properties.

As regards *Pinna pectinata japonica* and a form of the same species, *forma lischkeana*, characteristic properties of the fatty oils extracted from these shellfish together with $\Delta^{7,5}$-diene content of the crude sterols separated from these oils were previously reported in the 5th and 7th reports of this series.[6][7] In the present study, the fatty oil extracted from a larger quantity of the viscera from *P. pectinata japonica* was examined with a particular reference to sterol components. The crude steryl acetate mixture was found to contain 13.2% of $\Delta^{5,7}$-diene. After two recrystallizations from acetone, however, the $\Delta^{5,7}$-diene content was found to be lower than that before recrystallization. The steryl acetate mixture recovered from the mother liquors of recrystallization was chromatographed on an adsorption column of alumina, and an easily elutable fraction having a considerably high melting point and small content of $\Delta^{5,7}$-diene and a difficultly elutable fraction of $\Delta^{5,7}$-diene content 95%. The latter fraction gave a steryl acetate of m.p. 120°–122 °C after recrystallization from methanol-acetone. The free sterol from this fraction had m.p. 122°–123°C.* Although this sterol was not closely examined due to the scarcity of material, it is characterized by a low melting point for both free sterol and acetate, and appears to be different from any of the hitherto known sterols of low melting point, such as 7-dehydro-epicholesterol[8] and a $\Delta^{5,7}$-sterol[9] separated from *Modiolus demissus*. From the easily elutable fraction having a high melting point, clionasterol was separated by way of bromide.

Properties of the sterols separated in this study and their derivatives together with those reported in related literature are shown in Table 1.

TABLE 1. Properties of Sterols and Their Derivatives

	Free sterol		Acetate		Benzoate	
	m.p.(°C)	$[\alpha]_D^\circ$	m.p.(°C)	$[\alpha]_D^\circ$	m.p.(°C)	$[\alpha]_D^\circ$
Cynthia roretzi						
7-Dehydro-stigmasterol	154–155	−115	172–173	−78	—	—
〃 [1]	154	−113	172	—	—	—
〃 [4]	153–154	−114	172–173	−79	175–176	—
Poriferasterol	154–155	− 50	146–147	−52	140–141	—
〃 [10]	155–156	− 49.7	146.5–147	−53	139.5–140.5	−21.9
Clionasterol	138–139	− 40	135–137	−42	137–139	−19
〃 [10]	137.5–138.5	− 37	137	−41.9	134.5–135	−16.8
P. pectinata japonica						
$\Delta^{5,7}$-Sterol	122–123	−105	120–122	−75	—	—
7-Dehydro-epicholesterol [8]	124–126	− 70.5	114–115	−35	118–119	+48.5
Sterol from *Modiolus demissus* [9]	125.5–127	−108	137.5–138.5	−72	—	—
Clionasterol	136–137	− 37	136–138	—	137–139	—

* A sterol which appears to be identical with this sterol has recently been separated from *Tonna luteostoma* in this laboratory, and its structure is being studied.

Experimental

1. *Cynthia roretzi* v. Drasche

(i) **Fatty oil and its fatty acids.** Two samples of the ascidian, *Cynthia roretzi*, were used in this study. Sample No. 1 was supplied by the courtesy of Dr. Yamada, Faculty of Fisheries, Hokkaido University in November, 1954. This sample (410 g) was prepared by removing the outer skin from a lot of ascidians (7.3 kg) caught in Hokkaido and then sun-drying the fleshy portion including viscera (1.6 kg) to some measure. The material received was dried further in an electric oven at a temperature below 80°C to give 297 g of dried material. Sample No. 2 was supplied by the courtesy of Dr. S. Umemoto, Tohokukai Regional Fisheries Research Laboratory in June, 1955. This sample was salted fleshy portion including viscera prepared from fresh ascidians, 110 in number, caught at Watanoha, Miyagi Prefecture. The material received was rinsed with water and then dried in this laboratory. The dried material weighed 1,340 g.

The dried material from each sample was extracted with ether. The ether-extract was refluxed with tenfold acetone for a while and then cooled down to ordinary temperature, the acetone-insoluble matter (phosphatide) was removed by filtration, and the fatty oil was obtained from the acetone-solution. Properties of fatty oil and its fatty acids prepared in the usual way are given in Table 2.

TABLE 2. Fatty Oil and Its Fatty Acids from *Cynthia roretzi*

	Sample No. 1	Sample No. 2
Yield of ether-extract (g)	32.7	100
Yield of ether-extract (%)	11.0	7.5
Yield of fatty oil (g)	27.5	77
Yield of fatty oil (%)	9.3	5.7
d_4^{30}	0.9414	0.9512
n_D^{30}	1.4820	1.4879
Acid value	39.7	41.9
Saponification value	152.2	154.5
Iodine value	179.9	194.7
Unsaponifiable matter (%)	15.57	15.60
Fatty acids		
n_D^{30}	1.4708	1.4740
Neutralization value	—	191.9
Iodine value	193.0	210.8
Ether-insol. bromide (%)	80.2	—
Solid acids (%)	20.4	21.2
Iodine value of solid acids	10.6	12.4

Notes: Both oils were dark reddish orange and deposited some solid at ordinary temperature. Percentage yields of ether-extract and fatty oil were expressed on the basis of dried material. Unless stated otherwise, all iodine values were determined by the Wijs method. Solid acids were determined by the lead salt ethanol method.

The fatty acids from No. 2 oil were isomerized under the condition of 21% KOH-ethylene glycol, 180°C and 15 min. with a current of nitrogen, and the ultraviolet absorption for the isomerization product was measured. The composition of polyethenoid acids was calculated from the absorption data by assuming the formula given by Hammond and Lundberg[11] to be applicable to this case. The results are shown in Table 3.

TABLE 3. Composition of Polyethenoid Acids of *Cynthia roretzi* Oil (No. 2 Oil)

Wave length (mμ)	Sp. extinct. coefficient	Polyethenoid acids (%)		
			A	*B*
235	22.78	Diethenoid ·····	3.7	6.0
269	20.72	Triethenoid ·····	−2.4	1.6
316	24.13	Tetraethenoid ·····	6.6	10.9
347	18.12	Pentaethenoid ·····	26.5	16.3
376	4.12	Hexaethenoid ·····	14.7	14.7

Notes: The figures in the column *A* were obtained by taking pentaethenoid acids as C_{22}, while those in the column *B* were obtained by taking pentaethenoid acids as C_{20}. The iodine value of total fatty acids can be calculated from the data in Table 3 and the percentage and iodine value of solid acids in Table 1 on the assumptions that monoethenoid acids contained in total fatty acids consist entirely of oleic acid and that the iodine value of solid acids is due solely to oleic acid. The calculated iodine value thus obtainable is 225.6 for the case *A* (triethenoid acids being taken as zero) and 218.4 for the case *B* as compared with the observed iodine value 210.8.

(ii) **Sterol.** The unsaponifiable matter from No. 1 oil had sterol-content 68.2% (digitonin method). The crude sterol mixture obtained from the unsaponifiable matter by recrystallization from about thirtyfold methanol melted at 127°–129°C and its acetate had m.p. 117°–120°C and $\Delta^{5,7}$-diene content 8.4%*. Recrystallization of this acetate brought out rise in melting point and decrease in $\Delta^{5,7}$-diene content. After four recrystallizations, the acetate showed m.p. 133°–135°C and $\Delta^{5,7}$-diene content 4.15%.

The unsaponifiable matter from No. 2 oil had sterol-content 54.0%. The crude sterol obtained from the unsaponifiable matter by recrystallization from about fiftyfold methanol had m.p. 126°–128°C, and its acetate, recrystallized from methanol, had m.p. 127°–129°C and $\Delta^{5,7}$-diene content 8.1%. The acetate (2.5 g) was dissolved in hexane, and the solution was gradually poured into an adsorption column of alumina, 1.8 cm in diameter and 28 cm in height. After developing with 100 cc of hexane, the adsorption column was eluted with hexane containing 0.5% of ethanol. Eight fractions were separated as shown in Table 4.

TABLE 4. Chromatography of Steryl Acetate Mixture from *Cynthia roretzi* Oil (No. 2 Oil)

Fraction No.	Yield (g)	m.p. (°C)	$\Delta^{5,7}$-Diene content (%)
1	0.42	127–130	0
2	0.30	128–133	0
3	0.34	131–133	0
4	0.22	128–131	0
5	0.18	120–123	5
6	0.25	119–121	33
7	0.22	117–119	38
8	0.38	120–123	6

Another 2.7 g of steryl acetate mixture was subjected to chromatographic fractionation in a similar way as described above. Putting several fractions of relatively large contents of $\Delta^{5,7}$-diene together, there was obtained a combined

* Calculated from the ultraviolet absorption data at 277 mμ, 282 mμ and 290 mμ in ethanol.

fraction (0.83 g from 5.2 g of steryl acetate mixture in total) which had m.p. 117°-120°C and $\Delta^{5,7}$-diene content 35%. Recrystallization of this fraction from acetone brought out rise in melting point and increase in $\Delta^{5,7}$-diene content, and after eight recrystallizations, a steryl acetate (80 mg) of m.p. 172°-173°C and $[\alpha]_D^{20} = -78°$ was eventually obtained; saponification value 126.5 (calcd. for $C_{31}H_{48}O_2$, 123.9), iodine value (perbenzoic acid method) 165.0 (calcd. for $C_{31}H_{48}O_2F_3$, 168.2). Uniformity of this steryl acetate was demonstrated by its constant melting point unaltered by further recrystallization and also by the same melting point for the fraction remaining in the mother liquor of recrystallization. Ultraviolet and infrared spectra for this substance are shown in Figs. 1 and 2, respectively, characteristic absorption values in ultraviolet region being $k_{271}=25.97$, $k_{282}=27.24$, $k_{294}=15.01$ or $\varepsilon_{271}=11{,}760$, $\varepsilon_{282}=12{,}330$, $\varepsilon_{294}=6{,}800$ on the basis of mol. wt. 452.7 ($C_{31}H_{48}O_2$). Free sterol from this acetate, recrystallized from acetone, had m.p. 154°-155°C; $[\alpha]_D^{20} = -115°$; $k_{271}=26.79$, $k_{282}=28.42$, $k_{294}=15.68$; $\varepsilon_{271}=11{,}000$, $\varepsilon_{282}=11{,}670$, $\varepsilon_{294}=6{,}440$ (mol. wt. for $C_{29}H_{46}O=410.7$).

Fractions of $\Delta^{5,7}$-diene content below 5%, obtained by the chromatographic fractionation of steryl acetate mixture, were united, and about 3 g of the united fraction was brominated in ether. The ether-insoluble bromide (0.6 g) formed was fractionally crystallized from chloroform-methanol. After removing the first crop of m.p. 206°C (decomp. with darkening) obtained in a small amount, the second crop was subjected to a further solvent fractionation by which 0.25 g of a bromide fraction of m.p. 192°C and Br-content 41.85% (calcd. for $C_{31}H_{50}O_2Br_4$, 41.28%) was obtained. Debromination of this bromide gave a steryl acetate

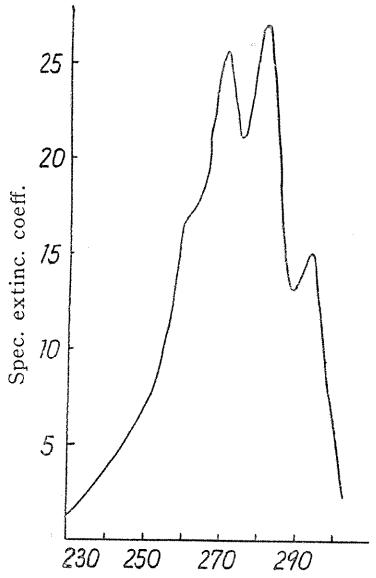

FIG. 1. Ultraviolet absorption spectrum of steryl acetate fraction; m.p. 172°-173°C.

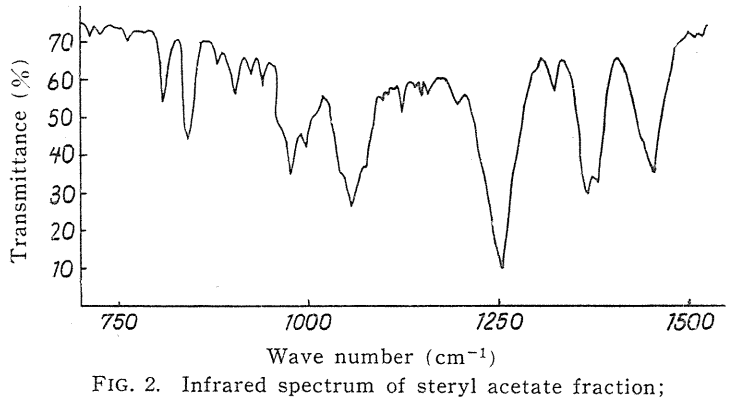

FIG. 2. Infrared spectrum of steryl acetate fraction; m.p. 172°-173°C.

which had m.p. 146°–147°C, $[\alpha]_D^{22} = -52°$ and saponification value 124.0 (calcd. for $C_{31}H_{50}O_2$, 123.4) after recrystallization from methanol. Free sterol from this acetate had m.p. 154°–155°C and $[\alpha]_D^{22} = -50°$ after recrystallization from methanol. Benzoate, recrystallized from methanol, had m.p. 140°–141°C.

The filtrate separated from the ether-insoluble bromide was freed from excess bromine, and then the ether was distilled off. The residue (ether-soluble bromide) was fractionally crystallized from ethanol to give a bromide fraction (2.9 g) of m.p. 123°–124°C. Debromination of this bromide gave a steryl acetate which, recrystallized from methanol, had m.p. 135°–137°C, $[\alpha]_D^{22} = -42°$, saponification value 124.6 (calcd. for $C_{31}H_{52}O_2$, 122.9) and iodine value (pyridine sulfate dibromide method) 61.2 (calcd. for $C_{31}H_{52}O_2$, 59.5). Free sterol from this acetate, recrystallized from methanol, had m.p. 138°–139°C and $[\alpha]_D^{22} = -40°$. Benzoate, recrystallized from methanol, had m.p. 137°–139°C and $[\alpha]_D^{23} = -19°$, and developed a bright blue color in the course of solidification.

2. *Pinna pectinata japonica* Reeve

(i) **Fatty oil.** The material used in this study was fresh viscera (about 6 kg) of the shellfish, *Pinna pectinata japonica*, procured at Ohno, Aichi Prefecture, in March, 1955. The material was dried in an electric oven at a temperature below 80°C to give 1,413 g of dried material. The dried material yielded 110 g or 7.8% of ether-extract, from which 59 g or 4.2% of fatty oil (acetone-soluble oil) was obtained in the same way as described for *Cynthia roretzi*.

The fatty oil was a dark reddish orange liquid with some solid at ordinary temperature, and had d_4^{30} 0.9226, n_D^{30} 1.4720, acid value 76.5, saponification value 146.2, iodine value 171.1 and unsaponifiable matter 19.98%. The fatty acids from this oil had n_D^{30} 1.4716, neutralization value 186.2 and iodine value 193.6.

(ii) **Sterol.** The unsaponifiable matter was found to contain 65.2% of sterol by the digitonin method. Recrystallization of unsaponifiable matter (11.4 g) from methanol (300 cc) gave a crude sterol mixture; 6.5 g, m.p. 115°–125°C. The acetate from this crude sterol mixture had m.p. 116°–120°C and $\Delta^{5,7}$-diene content 13.2%. Two recrystallizations of this acetate from acetone gave 0.8 g of an acetate fraction of m.p. 129°–133°C and $\Delta^{5,7}$-diene content 9.1%.

The steryl acetate remaining in the mother liquors of recrystallization was recovered, and 5.4 g of this acetate was chromatographically fractionated, using

TABLE 5. Chromatography of Steryl Acetate Mixture from *Pinna pectinata japonica*

Fraction No.	Yield (g)	m.p. (°C)	$\Delta^{5,7}$-Diene content (%)
1	0.84	121–133	0
2	0.60	131–133	0
2	0.56	131–133	0
4	0.60	127–129	0
5	0.44	127–129	3
6	0.08	125–129	14
7	0.15	119–121	33
8	0.06	116–120	86
9	0.07	116–119	95
10	0.10	118–119	65
11	0.08	126–129	15

an adsorption column of alumina, 2.5 cm in diameter and 50 cm in height, hexane as solvent and developer, and hexane containing 0.5% of ethanol as eluant. The results are shown in Table 5.

Recrystallization of the fraction 9 in Table 5 from methanol-acetone gave a steryl acetate of m.p. 120°–122°C, $[\alpha]_D^{20} = -75°$, saponification value 128.7 and iodine value (perbenzoic acid method) 147.9. The ultraviolet absorption curve for this acetate are shown in Fig. 3; $k_{271} = 26.38$, $k_{282} = 27.63$, $k_{294} = 15.94$. Free sterol from this acetate had m.p. 122°–123°C and $[\alpha]_D^{20} = -105°$ after recrystallized three times from methanol; $k_{271} = 26.74$, $k_{282} = 27.94$, $k_{294} = 16.10$.

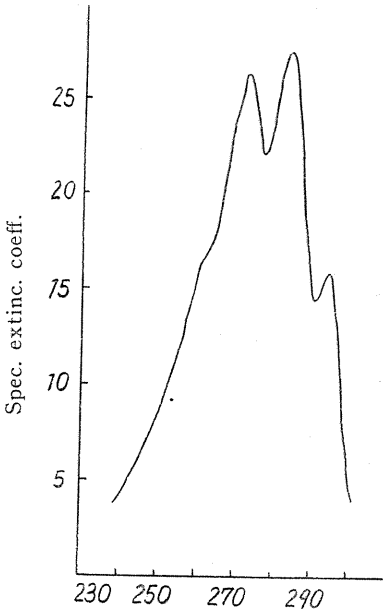

FIG. 3. Ultraviolet absorption spectrum of steryl acetate; m.p. 120°-122°C.

The fractions 1–5 were united, and 2.5 g of united material was brominated in ether. The ether-insoluble bromide formed in a minor amount (0.06 g) was removed by filtration, and the filtrate, after removal of excess bromine, was concentrated. Ethanol was then added to the concentrated filtrate to give a precipitate of solid bromide. This bromide was further fractionated using ethanol, and after removal of high melting fraction and low melting fraction, a bromide fraction (0.4 g) of m.p. 123°–124°C was obtained. Debromination of this fraction gave a steryl acetate which, recrystallized from methanol, had m.p. 136°–138°C, $[\alpha]_D^{25} = -38°$, saponification value 125.3 and iodine value (pyridine sulfate dibromide method) 57.1. Free sterol from this acetate had m.p. 136°–137°C and $[\alpha]_D^{25} = -37°$ after recrystallization from methanol. Benzoate had m.p. 137°–139°C and developed a bright blue color in the course of solidification.

Summary

1. Characteristics of fatty oils extracted from the body except outer skin of *Cynthia roretzi* and from the viscera of *Pinna pectinata japonica* were determined. Both oils had a comparatively high iodine value. The polyethenoid acids from *Cynthia roretzi* oil were found to contain various components in the order of pentaethenoid > hexaethenoid > tetraethenoid > diethenoid > triethenoid by the ultraviolet absorption measurement for the alkali-isomerized fatty acids.

2. Sterols from *Cynthia roretzi* oil were found to contain a $\Delta^{5,7}$-sterol identical with 7-dehydrostigmasterol together with poriferasterol and clionasterol.

3. Sterols from *Pinna pectinata japonica* oil were found to contain a $\Delta^{5,7}$-sterol of m.p. 122°–123°C (its acetate, m.p. 120°–122°C) and clionasterol.

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