FATTY OILS OF AQUATIC INVERTEBRATES

XIX. NON-CONJUGATED STEROLS AND OTHER UNSAPONIFIABLE COMPONENTS IN THE FATTY OIL FROM *TONNA LUTEOSTOMA*

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In a previous study[1] of this series, oils were extracted from the shellfish *Tonna luteostoma* (Küster); both flesh and viscera oils from the shellfish sample *A* and viscera oil alone from the shellfish sample *B*. Characteristics of these oils were determined. The sterol mixture from viscera oil was found to contain a large amount of $\Delta^{5,7}$-conjugated components from which a fraction regarded as $\Delta^{5,7,22,25}$-cholestatetraenol was separated. In a continuation of the previous study, the non-conjugated sterol components of the flesh oil and also the non-conjugated sterol components and unsaponifiable components other than sterol of the viscera oil have been studied. The present paper records the results of these studies.

The crude sterol mixture obtained by recrystallization of the unsaponifiable matter of flesh oil from the shellfish sample *A* was acetylated to give its crude steryl acetate mixture. After bromination of this acetate, the bromide was fractionated using ether at about $-10°C$ into the ether-insoluble fraction I-a and the ether-soluble fraction I-b. The bromide I-a was fractionally crystallized and the main fraction obtained was debrominated to give a steryl acetate fraction which, on fractional crystallization, gave cholesteryl acetate as a chief component. The main fraction obtained from the bromide I-b by fractional crystallization was debrominated. The steryl acetate obtained was purified by recrystallization to give eventually a fraction which was recognized as clionasteryl (γ-sitosteryl) acetate. Considering from the yield of each bromide fraction, cholesterol appeared to be most predominant among the non-conjugated sterol components of flesh oil.

The crude sterol mixture from each viscera oil of the shellfish samples *A* and *B* was acetylated and the crude steryl acetate mixture was chromatographed to separate the conjugated steryl acetate (cf. the previous report[1]). The more easily eluted fractions obtained thereby, containing little conjugated steryl acetate, were united and the united material was brominated. The bromide was separated into the ether-insoluble fraction II-a and ether-soluble fraction II-b as in the case of flesh oil. After fractionally crystallizing the bromide II-a, the main fraction separated was debrominated to give cholesteryl acetate. The bromide II-b, after the same treatment, provided clionasteryl acetate. Considering from the yield of each bromide fraction, it appears that clionasterol rather than cholesterol is predominant among the non-conjugated sterol components of viscera oil. Although the presence of cholesterol in the sterols of gastropods has been reported by many authors, the occurrence of clionasterol has been known only in the case of *Littorina littorea*,[2] *Nassa obsoleta*,[3] and *Viviparus japonicus*[4] besides *T. luteostoma* of the present

study.

Properties of the sterols which were obtained by debromination of each bromide fraction are summarized in Table 1.

TABLE 1. Properties of the Sterols

	Free sterol		Acetate		Benzoate	
	m.p. (°C)	$[\alpha]_D^\circ$	m.p. (°C)	$[\alpha]_D^\circ$	m.p. (°C)	$[\alpha]_D^\circ$
Sterol from bromide I-a	148.5–149	−38.5	114 –115	−47.0	145.5–146.5; 178	−14.4
Sterol from bromide II-a	148 –149	−38.9	113.5–114.5	−46.8	—	—
Cholesterol	148	−39.5	114–115	−47.5	145.5; 178	−14
Sterol from bromide I-b	137 –138	−36.2	134.5–135.5	−41.1	—	—
Sterol from bromide II-b	137.5–138.5	−36.8	135.5–136.5	−41.5	139–140	−16.2
Clionasterol[5]	137.5–138.5	−37	137	−41.9	134.5–135	−16.8
Clionasterol[6]	139	−35	137	−40	140	−16

The filtrate separated from the crude sterol mixture in the recrystallization of the unsaponifiable matter of viscera oil yielded on further concentration some precipitate. The precipitate from the shellfish sample *A* was converted to its acetate, and the acetate was subjected to chromatography (Table 3), by which an eluate fraction composed of batyl acetate was obtained in a relatively large quantity. The precipitate from the shellfish sample *B* was freed from sterol using digitonin, the non-sterol fraction was recrystallized, and batyl alcohol was obtained in a pure state. The separation and identification of batyl alcohol from gastropods appeared not to have been reported before the present study.

The liquid portion, which was obtained in a yield of about 10% of the unsaponifiable matter of viscera oil from the shellfish sample *B* by removing sterols and other solid substances such as batyl alcohol as far as possible, was acetylated and the acetylated product was subjected to a fractional distillation (Table 4). The free alcohol obtained by saponification of the fraction 2 gave on hydrogenation a crystalline solid of m.p. 58°-61°C which appeared to be impure batyl alcohol. The free alcohol obtained by saponification of the fraction 3 was fractionated by chromatography, which yielded a liquid eluate fraction which appeared to be impure selachyl alcohol. These results indicate the occurrence of selachyl alcohol in the liquid unsaponifiable matter. However, since the acetylated product of the liquid unsaponifiable matter has a saponification value exceedingly lower than that of selachyl acetate and an iodine value higher than that of selachyl acetate, it is inferred that other components than selachyl alcohol may also be present in the liquid unsaponifiable matter.

Experimental

The oils from *Tonna luteostoma* used in this study are the same as described in the previous report.[1] Properties of their unsaponifiable matter are recorded in Table 2.

1. Non-conjugated sterol of flesh oil

The unsaponifiable matter (4.5 g), separated in the usual way from the flesh

TABLE 2. Unsaponifiable Matter of the Oils from *T. luteostoma*

	Shellfish sample *A*		Shellfish sample *B*
	Flesh oil	Viscera oil	Viscera oil
Unsaponifiable matter (%)	50.21	15.30	14.72
Iodine value*	93.5	122.4	90.4
Sterol (%)	64.7	37.1	13.5
$\Delta^{5,7}$-Conjugated sterol (%, on the basis of total sterol)	5	31	32

* Iodine values recorded in this paper are determined by the pyridine sulphate dibromide method.

oil of the shellfish sample *A*, was recrystallized from methanol giving two crops of crude sterol mixture; the first crop (2.5 g) of m.p. 136°-139°C and the second crop (0.4 g) of m.p. 129°-133°C. Both crops were united, and the united material was acetylated. A crude steryl acetate mixture of m.p. 107°-110°C and I.V. 77.6 was obtained. This was subjected to the fractional crystallization from methanol which, however, failed to give a uniform steryl acetate fraction. The fractions of the fractional crystallization were then recovered and the united fraction was brominated as follows: 1.9 g of the united fraction was dissolved in 25 cc of ether, a slight excess of bromine in glacial acetic acid (1 : 3) was added dropwise to the solution under cooling at about −10°C, and after standing at the same temperature for 5 hr the resulting insoluble bromide I-a was filtered off. The bromide I-a was dissolved in somewhat large quantity of ether at ordinary temperature, and the bromide was fractionally precipitated with addition of methanol to the solution to give 0.18 g of the first crop of m.p. 116°-118°C, 0.88 g of the second and third crops of m.p. 115°-117°C, 0.37 g of the fourth crop of m.p. 113°-115°C and 0.06 g of the fifth crop of m.p. 102°-105°C. After removal of excess bromine from the filtrate separated from the bromide I-a, the bromide I-b in the filtrate was fractionally precipitated with addition of methanol to give 0.09 g of the first crop of m.p. 116°-118°C and 0.56 g of the second crop of m.p. 115°-117°C.

(i) *Sterol from the bromide I-a*

The second to fourth crops from the bromide I-a were united and the united material was debrominated by refluxing with 20 cc of glacial acetic acid and 8 g of zinc dust for 4 hr. The steryl acetate obtained was separated into five fractions by fractional crystallization from acetone-methanol. The melting point and yield for each fraction were as follows: m.p. 118.5°-119.5°C, 0.03 g; m.p. 116°-117°C, 0.10 g; m.p. 114.5°-115.5°C, 0.20 g; m.p. 114°-115°C, 0.41 g; m.p. 113°-114°C, 0.02 g; respectively. The melting point of the fourth fraction was not altered after recrystallization from ethanol. It showed $[\alpha]_D^{15} = -47.0°$ (in chloroform), S.V. 130.2 and I.V. 59.8 (Calcd. for $C_{29}H_{48}O_2F_1$: S.V. 130.9 and I.V. 59.2). The free sterol prepared by saponification of this fraction showed, after recrystallization from methanol, m.p. 148.5°-149°C and $[\alpha]_D^{15} = -38.5°$. No depression of melting point was observed on admixture with cholesterol. The benzoate prepared from the free sterol had, after recrystallization from ethanol-benzene, $[\alpha]_D^{21} = -14.4°$ and melted to a turbid liquid at 145.5°-146.5°C and became clear at 178°C.

(ii) *Sterol from the bromide I-b*

The steryl acetate, obtained by debromination of the second crop from the bromide I-b with glacial acetic acid and zinc dust, had m.p. 134.5°–135.5°C, $[\alpha]_D^{15}=-41.1°$ and S.V. 123.5 (Calcd. for $C_{31}H_{52}O_2$, 122.8) after recrystallization from acetone-methanol. The free sterol prepared by saponification of the acetate showed m.p. 137°–138°C, $[\alpha]_D^{21}=-36.2°$ and I.V. 63.0 (Calcd. for $C_{29}H_{50}OF_1$, 61.2) after recrystallization from methanol.

2. Non-conjugated sterols of viscera oil

The crude sterol mixture obtained by recrystallization of the unsaponifiable matter of viscera oil from each of the shellfish samples *A* and *B* from methanol was acetylated, and the acetate was chromatographed using alumina as adsorbent and ethanol-hexane or methanol-hexane as developer and eluant to separate $\Delta^{5,7}$-conjugated steryl acetate (cf. the previous report[1]). The relatively easily eluted fractions obtained thereby were found to contain mainly non-conjugated steryl acetate. Therefore, these eluate fractions were recovered; 1.2 g of an eluate fraction of $\Delta^{5,7}$-conjugated steryl acetate content 0.1% and 1.0 g of an eluate fraction free from $\Delta^{5,7}$-conjugated steryl acetate from the shellfish samples *A* and *B*, respectively. These fractions were united, and the united fraction was brominated as in the case of the crude steryl acetate mixture of flesh oil. The bromide was separated into a fraction II-a difficultly soluble in ether and a fraction II-b easily soluble in ether. The bromide II-a was further fractionally crystallized to separate into 0.15 g of the first crop of m.p. 117°–119°C, 0.32 g of the second and third crops of m.p. 112°–114°C and 0.11 g of the fourth crop of m.p. 110°–112°C. The bromide II-b was separated into 0.20 g of the first crop of m.p. 118°–120°C and 0.77 g of the second crop of m.p. 115°–117°C.

(i) *Sterol from the bromide II-a*

The second to fourth crops from the bromide II-a were united and the united material was debrominated to give steryl acetate. This was fractionally crystallized from acetone-methanol giving 0.13 g of a main fraction having m.p. 113.5°–114.5°C, $[\alpha]_D^{15}=-46.8°$ and S.V. 130.1. The free sterol obtained by saponification of this acetate fraction had, after recrystallization from methanol, m.p. 148°–149°C, $[\alpha]_D^{21}=-38.9°$ and I.V. 66.8 (Calcd. for $C_{27}H_{46}OF_1$, 65.6) and showed no depression of melting point on admixture with cholesterol.

(ii) *Sterol from the bromide II-b*

The steryl acetate obtained by debromination of the second crop from the bromide II-b was recrystallized from methanol-ethanol giving an steryl acetate of m.p. 134°–135°C. After a further recrystallization from acetone-methanol, it showed a constant melting point 135.5°–136.5°C, $[\alpha]_D^{15}=-41.5°$, S.V. 123.3 and I.V. 56.7 (Calcd. for $C_{31}H_{52}O_2F_1$, 55.6). Saponification of this acetate yielded a free sterol which, after recrystallization from methanol, had m.p. 137.5°–138.5°C and $[\alpha]_D^{15}=-36.8°$. The benzoate prepared from the free sterol showed, after recrystallization from ethanol, m.p. 139°–140°C and $[\alpha]_D^{21}=-16.2°$. The melted benzoate showed a bluish green color on its solidification.

3. Non-sterol components of the unsaponifiable matter of viscera oil

(i) *Solid components of the unsaponifiable matter of viscera oil from the shellfish sample A*

The filtrate, which was separated from the crude sterol mixture by recrystallizing the unsaponifiable matter (30 g) of viscera oil from the shellfish sample *A* from methanol (cf. the previous paper[1]), gave a further quantity of crystalline solid on concentration. This solid had m.p. 94°–98°C and the acetate prepared from it had m.p. 38°–42°C, S.V. 224.7 and I.V. 45.0. The acetate (3.7 g) was fractionated by chromatography, using alumina as adsorbent and ethanol-hexane (1 : 200–1) as developer and eluant, with the results shown in Table 3.

TABLE 3. Chromatography of the Acetate, m.p. 38°–42°C, from Viscera Oil of the Shellfish Sample *A*

Eluate fraction	Yield (g)	m.p. (°C)	$\Delta^{5,7}$-Conjugated sterol content (%)
1	0.03	—	—
2	0.42	115–116	0
3	0.27	108–111	40
4	0.31	48–53	25
5	0.66	34–38	2
6	1.17	32–35	0
7	0.12	—	0
8	0.51	—	0
9	0.10	—	—

The eluate fractions 2 and 3 in Table 3 showed Liebermann-Burchard reaction indicating the presence of steryl acetate. The eluate fraction 6 did not show Liebermann-Burchard reaction and had S.V. 260.5 (Calcd. for $C_{25}H_{48}O_5$, 261.8) and I.V. 0. On saponification, it yielded free alcohol which, after recrystallization from ethanol, was lustrous plates of m.p. 70°–71°C, and showed no depression of melting point on admixture with batyl alcohol.

(ii) *Solid components of the unsaponifiable matter of viscera oil from the shellfish sample B*

The filtrate, which was separated from the crude sterol mixture by recrystallizing the unsaponifiable matter (about 50 g) of viscera oil from the shellfish sample *B* from methanol (cf. the previous paper[1]), gave a precipitate (4.3 g) of m.p. 67°–69°C and I.V. 4.4 on concentration. This precipitate was treated with digitonin in 90% ethanol to remove sterol completely. The non-sterol fraction obtained had m.p. 69.5°–70.5°C after recrystallization from acetone and a constant melting point 70.5°–71°C after a further recrystallization from ethanol. The acetate showed m.p. 34°–35°C and S.V. 261.2.

(iii) *Liquid components of the unsaponifiable matter of viscera oil from the shellfish sample B*

The methanol filtrate, separated from the above mentioned crude sterol mixture and a solid of m.p. 67°–69°C, from the unsaponifiable matter from the shellfish sample *B* was concentrated further and then cooled, giving 17.8 g of a precipitate of m.p. 63°–66°C and acetyl V. 250.0. Since the material recovered from

the filtrate of this precipitate was suspected to be contaminated more or less with saponifiable matter which escaped saponification in the saponification of the initial viscera oil, it was re-saponified and the unsaponifiable matter was separated. The unsaponifiable matter (23 g) thus obtained was dissolved in acetone and the solution was cooled at about −10°C. After separation of the precipitate formed, 5.4 g (about 10% of the initial unsaponifiable matter) of liquid material was obtained from the acetone filtrate. The acetate (5.6 g) of S.V. 195.8 and I.V. 95.2 prepared by acetylation of the liquid material was fractionally distilled with the results recorded in Table 4. During fractional distillation there was an indication of decomposition with evolution of smoke, leaving a remarkable amount of residue.

TABLE 4. Fractional Distillation of the Acetate of Liquid Unsaponifiable Matter from Viscera Oil of the Shellfish Sample *B*

Fraction	b.p. (°C/6 mm Hg)	Yield (g)	n_D^{25}	S.V.	I.V.
1	-228	0.2	1.4601	186.8	71.5
2	228-235	1.2	1.4629	212.1	66.1
3	235-241	1.7	1.4726	207.3	81.6
Residue (difference)	—	2.5	—	—	119.1

The free alcohol obtained by saponification of the fraction 2 in Table 4 had n_D^{25} 1.4735 and I.V. 77.3. This was hydrogenated with Raney nickel as a catalyst to give a hydrogenation product which, after recrystallization from ethanol, showed m.p. 58°-61°C and acetyl V. 255.2. The free alcohol obtained by saponification of the fraction 3 had n_D^{25} 1.4823 and I.V. 90.3. A portion (0.75 g) of this alcohol was chromatographically fractionated (adsorbent: alumina; developer and eluant: ether-hexane and hexane-methanol) into seven eluate fractions. The first and second eluate fractions were liquid and the former had I.V. 156.4. The third and fourth eluate fractions were crystalline solid of m.p. 115°–120°C and showed Liebermann-Burchard reaction. The fifth eluate fraction was a soft solid. The sixth and seventh eluate fractions were liquid. The sixth eluate fraction was obtained in the largest yield (0.47 g) and had n_D^{25} 1.4728, acetyl V. 252.1 and I.V. 62.8 (Calcd. for $C_{21}H_{42}O_3F_1$: acetyl V. 263.0 and I.V. 74.1).

Summary

1. The non-conjugated sterol components of both the flesh and viscera oils from the shellfish *Tonna luteostoma* were examined. They were found to consist mainly of monoethenoid sterols, among which cholesterol and clionasterol (γ-sitosterol) were identified. It appeared that for the flesh oil cholesterol is present in a larger proportion than clionasterol whereas the latter is predominant for the viscera oil.

2. The unsaponifiable components other than sterol of the viscera oil were examined. Batyl alcohol was separated as the main solid component. The liquid components appeared to contain selachyl alcohol among others.

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