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Sterols of the Horseshoe Crab, the Sea Caterpillar, and the Warf Monkey

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Abstract

The sterol composition of three species of marine invertebrates was investigated in the viewpoint of comparative biochemistry. The horseshoe crab, *Trachypleus tridentus*, contained cholesterol (78.0%) and small amounts of C_{27}-, C_{28}-, and C_{29}-sterols with Δ^0, Δ^5, or $\Delta^{5,22}$-bond besides C_{26}-$\Delta^{5,22}$-sterols. Cholesterol was also the major component in the sea caterpillar, *Chloeia flava* (75.3%) and the warf monkey, *Ligia exotica* (81.5%). The latter two invertebrates possessed C_{27}, C_{28}, and C_{29}-sterols as a minor component besides cholesterol.

During the past decade, the investigation of marine sterols using modern techniques has thrown light upon the detailed profile of sterols occurring in many marine invertebrates[1-3]. As a result, the sterols of marine invertebrates, especially filter feeders, have been shown to be extremely complex mixtures than suspected in earlier studies[4].

As a part of our long-term projects on marine sterols, we intend to clarify the sterol composition of the horseshoe crab, *Trachypleus tridentus* (Arthropoda, class Xiphosura), the sea caterpillar, *Chloeia flava* (Annelida, class Polychaeta), and the warf monkey, *Ligia exotica* (Arthropoda, class Crustacea, order Isopoda). Especially, the sterols of the horseshoe crab is interesting from a phylogenetic viewpoint, because it is the most primitive Arthropoda. Generally, it is recognized that the horseshoe crabs originated from the trilobite-type of arthropods and flourished in the Cretaceus period of Mesozoic era[5]. At the present, only five species (three classes) of the horseshoe crabs are found in the world, and they are under the protection of government as a live fossil in Japan[5].

The present paper deals with these results and discussion.

Materials and Methods

Sample The horseshoe crab was obtained from Okayama, Japan, May 15, 1978. The specimens of sea caterpillar and warf monkey were collected in Kagoshima, June 2 and October 10, respectively, 1978. The invertebrates were dipped in methanol and transported to this laboratory.

Isolation of sterols Lipids were extracted from the horseshoe crab (except

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carapace) and the whole bodies of sea caterpillar and warf monkey according to the method of BLIGH and DYER[6] with chloroform-methanol-water, and saponified with 10% ethanolic potassium hydroxide to yield the unsaponifiable matters. Crude sterols were isolated from the unsaponifiable matters by alumina column chromatography with hexane-benzene[7] and then acetylated with dry pyridine-acetic anhydride (1: 1).

Identification of sterol components Gas-liquid chromatography (GLC)[8] was carried out by using a Shimadzu GC-4BPF equipped with a column (3 m × 3 mm i.d.) of 1.5% OV-17 on 80–100 mesh Shimalite W at 245°C. The mixture of steryl acetates was separated into the components by thin-layer chromatography on 10% (w/w) $AgNO_3$-Kieselgel G ($AgNO_3$-TLC) with chloroform (ethanol-free). The sterol components were preliminarily identified by comparison of relative retention times (RRT) to standards in consideration of the mobilities in $AgNO_3$-TLC. Finally, the fractions from $AgNO_3$-TLC were analyzed by GLC-Mass spectrometry[9] on 3.0% OV-1 on 60–80 mesh Chromosorb W (2 m × 2 mm i.d.) at 260°C to confirm the identity of the sterol components. The following sterols were identified by means of GLC-Mass spectrometry as acetates:

Cholestanyl acetate (**1**): m/e 430 (M^+), 415, 370, 355, 316, 290, 276, 275, 257, 230, and 215. 24-*Methylcholestanyl acetate* (**2**): m/e 444 (M^+), 429, 384, 330, 290, 276, 275, 257, 230, and 215. 24-*Ethylcholestanyl acetate* (**3**): m/e 458 (M^+), 443, 398, 383, 344, 290, 276, 257, 230, and 215. *Cholesteryl acetate* (**4**): m/e 368 (M^+–AcOH), 353, 275, 260, 255, 247, 228, and 213. 24-*Methylcholesteryl acetate* (**5**): m/e 382 (M^+–AcOH), 274, 261, 255, 228, and 213. 24-*Ethylcholesteryl acetate* (**6**): m/e 396 (M^+–AcOH), 288, 275, 255, 228, and 213. 24-*Norcholesta*-5, 22-*dienyl acetate* (**7**): m/e 352 (M^+–AcOH), 337, 282, 267, 255, 253, 228, and 213. *Cholesta*-5, 22-*dienyl acetate* (**8**): m/e 366 (M^+–AcOH), 351, 282, 255, 253, 228, and 213. 24-*Methylcholesta*-5, 22-*dienyl acetate* (**9**): m/e 380 (M^+–AcOH), 351, 282, 267, 255, 228, and 213. 24-*Ethylcholesta*-5, 22-*dienyl acetate* (**10**): m/e 394 (M^+–AcOH), 379, 351, 282, 267, 255, 228, and 213. 24-*E-Ethylidenecholesteryl acetate* (**11**): m/e 394 (M^+–AcOH), 379, 296, 281, 267, 255, 253, 228, and 213. 24-*Z-Ethylidenecholesteryl acetate* (**12**): m/e 394 (M^+–AcOH), 379, 351, 281, 267, 255, 253, 228, and 213. *Cholesta*-5, 24-*dienyl acetate* (**13**): m/e 366 (M^+–AcOH), 351, 342, 255, 253, 228, 213, and 69.

Results

The sterols were isolated from the horseshoe crab, sea caterpillar, and warf monkey (Table 1). The preliminary GLC on 1.5% OV-17 of the acetate derivatives of sterols showed that the sterols from three invertebrates contained at least 7 or more components. The steryl acetates were separated into the several fractions with the different number and position of double bonds by $AgNO_3$-TLC, and the components of each fraction were characterized by GLC-Mass spectrometry. Table 2 indicates the sterol composition of the horseshoe crab, sea caterpillar, and warf monkey. All

Table 1. Yields of lipids, unsaponifiable matters, and sterols from the horseshoe crab, sea caterpillar, and warf monkey.

Fraction	Weight (g)		
	Horseshoe crab	Sea caterpillar	Warf monkey
Fresh weight	80.0	43.0	120.0
Lipids	0.621	1.150	1.060
Unsaponifiable matters	0.096 (15.5%)*1	0.607 (52.7%)	0.136 (12.8%)
Sterols	0.012 (1.9%)	0.132 (11.5%)	0.021 (2.0%)

*1 Percentage of lipids

sterols listed in Table 2 gave the identical behaviour on TLC and GLC, and the same mass spectral pattern with authentic sterols.

The acetate derivatives of sterols isolated from the horseshoe crab gave seven peaks in GLC on 1.5% OV-17 and separated into six bands in $AgNO_3$-TLC. The most less polar band in $AgNO_3$-TLC was composed of three components with RRT of 1.01, 1.28, and 1.60 in GLC on 3.0% OV-1. The three components were confirmed to be cholestanyl (**1**), 24-methylcholestanyl (**2**), and 24-ethylcholestanyl (**3**) acetates by GLC-Mass spectrometry. The presence of the high molecular ion peaks at m/e 430, 444, and 458, together with the ion at m/e 257 (M^+–R–AcOH, R=side chain) confirmed that **1**, **2**, and **3** were fully saturated stanyl acetates with C_8, C_9, and C_{10} side chains, respectively. The secondary less polar band contained cholesteryl (**4**), 24-methylcholesteryl (**5**), and 24-ethylcholesteryl (**6**) acetates. The mass spectra of **4**, **5**, and **6** gave no molecular ion but afforded the intense ions (M^+–AcOH) at m/e 368, 382, and 396 and the prominent ions (M^+–AcOH–108) at m/e 260, 274, and 288, and the ions (M^+–AcOH–121) which are characteristic of Δ^5-steryl acetates[10]. Thus, **4**, **5**, and **6** were identified as Δ^5-steryl acetates with C_8, C_9, and C_{10} side chains. The four bands with the lesser polarity in $AgNO_3$-TLC were corresponded to 24-norcholesta-5, 22-dienyl (**7**), cholesta-5, 22E-dienyl (**8**), 24-methylcholesta-5, 22-dienyl (**9**), and 24-ethylcholesta-5, 22-dienyl (**10**) acetates. The mass spectra of **7**, **8**, **9**, and **10** gave no molecular ion peak but intense ions (M^+–AcOH) at m/e 352, 366, 380, and 394, the ions at m/e 282 by the cleavage at C(20)–C(22) with one hydrogen transfer and the loss of acetic acid[10,11], and the pair of ions at m/e 255 (M^+–R–AcOH) and 253 (M^+–R–2H–AcOH)[11]. In addition, the mass spectra of **9** and **10** afforded the ions (M^+–AcOH–43)[10] at m/e 337 and 351, respectively. Thus, **7**, **8**, **9**, and **10** were identified as $\Delta^{5,22}$-steryl acetates with C_7, C_8, C_9, and C_{10} side chains.

By the similar manner to that of the horseshoe crab sterols, the sterols of the sea caterpillar and warf monkey were characterized. The steryl acetates of the sea caterpillar and warf monkey were also composed of **1**, **2**, **3**, **4**, **5**, **6**, **8**, **9**, and **10** (Table 2). In addition, the sea caterpillar contained two 24-ethylidenecholesteryl acetates, 24-E-ethylidenecholesteryl (**11**)[12] and 24-Z-ethylidenecholesteryl (**12**)[13] acetates. The mass spectra of **11** and **12** showed the prominent ions at m/e 394

Table 2. Sterol composition of the horseshoe crab, sea caterpillar, and warf monkey.

Sterol		RRT*1		Composition (%)		
		OV-17	OV-1	Horseshoe crab	Sea caterpillar	Warf monkey
Saturated	Cholestanol	1.01	1.01	1.5	1.0	1.1
	24-Methylcholestanol	1.29	1.28	0.6	1.2	0.4
	24-Ethylcholestanol	1.61	1.60	0.8	0.5	1.9
Δ^5	Cholesterol	1.00	1.00	78.0	75.3	81.5
	24-Methylcholesterol	1.28	1.27	4.2	4.0	4.0
	24-Ethylcholesterol	1.59	1.58	6.2	0.1	1.9
$\Delta^{5,22}$	24-Norcholesta-5, 22-dienol	0.66	0.66	3.9	—	—
	Cholesta-5, 22E-dienol	0.93	0.92	2.9	7.0	1.0
	24-Methylcholesta-5, 22-dienol	1.12	1.11	2.9	5.3	2.6
	24-Ethylcholesta-5, 22-dienol	1.42	1.49	2.3	0.5	3.3
$\Delta^{5,24}$	Cholesta-5, 24-dienol	1.18	1.19	—	—	1.0
$\Delta^{5,24(28)}$	24-E-Ethylidenecholesterol	1.68	1.64	—	0.1	—
	24-Z-Ethylidenecholesterol	1.78	1.69	—	0.1	—

*1 Relative retention times of steryl acetates to cholesteryl acetate

(M^+–AcOH), 379 (M^+–AcOH–CH_3), 296 (M^+–C–23 to C–29–1H–AcOH), and 281 (m/e 296–CH_3) which were indicative of C_{29}-steryl acetates with ethylidene groups. Also, the warf monkey contained small amounts of cholesta-5, 24-dienyl acetate (**13**) which gave the mass spectral ions at m/e 366 (M^+–AcOH), 351 (M^+M^+–AcOH–CH_3), 342 (M^+–C–22 to C–27–1H), 255, 253, 228, 213, and 69.

Discussion

In earlier studies, cholesterol was reported to be the major sterols in the horseshoe crabs, *Limulus polyphemus*[14] and *Trachypleus tridentus* (*Limulus longispina*)[15]. TOYAMA and TAKAGI[15] have shown the presence of the unidentified diunsaturated Δ^5-sterol in *T. tridentus*. In the present study, the horseshoe crab, *T. tridentus*, was found to contain cholesterol as the major sterols (78.0% of total sterols) as observed in more evolved species of arthropods. Also, *T. tridentus* contained stanols (2.9%) such as cholestanol, 24-methylcholestanol, and 24-ethylcholestanol, and $\Delta^{5,22}$-sterols (12.0%) such as 24-norcholesta-5, 22-dienol, cholesta-5, 22E-dienol, 24-methylcholesta-5, 22-dienol, and 24-ethylcholesta-5, 22-dienol besides Δ^5-sterols (88.4%) such as cholesterol, 24-methylcholesterol, and 24-ethylcholesterol.

In earlier investigations on the order Isopoda (Arthropoda), only cholesterol was identified in the several species, *Ligia exotica*[16] *Cirolana harfordi*[17], *Ligia occidentalis*[17], and *Armadillidium vulgare*[17]. However, the results of the present study revealed that the warf monkey, *L. exotica*, involved cholesta-5, 22E-dienol (7.0%), 24-methyl-

cholesterol (4.0%), 24-methylcholesta-5, 22-dienol (5.3%), and other minor sterols besides large amounts of cholesterol (75.3%).

Regarding the annelids, cholesterol has been regarded to be a sole sterol occurring in this phylum of animals such as *Lumbricus terrestris*[18,19], *Nereis pelagia*[20], *Amphitrite ornata*[20], *Arenicola marina*[22], etc. until the work of KOBAYASHI *et al.*[20] and VOOGT[21]. Paying careful attention to the minor components, KOBAYASHI and coworkers[21,22] have studied the sterols of the marine annelid, *Pseudopotamilla occelata,* by using modern techniques. As a result, they have revealed that *P. occelata* contained C_{27}-sterols (74.0%) such as cholesterol (50.1%), cholesta-5, 22E-dienol, and desmosterol, C_{26}-sterols (4.9%) such as 24-norcholesta-5, 22-dienol and occelasterol, and 24-alkyl Δ^5 and $\Delta^{5,22}$-sterols (21.1%). The sterols of the sea caterpillar, *C. flava,* were slightly different with those of *P. occelata*[21]: that is, the former contained small amounts of stanols (2.7%) such as cholestanol, 24-methylcholestanol, and 24-ethylcholestanol, and 24-ethylidenesterols (0.2%) but not 24-norcholesta-5, 22-dienol and desmosterol. Also, *C. flava* contained more abundance of cholesterol than *P. occelata.*

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