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ECHINODERM STEROLS

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Abstract—The sterols of a representative of each of the five classes of echinoderms have been examined. The sterols are mixtures of from three to six sterols, ranging from 27 to 30 carbons. Sterols of asteroids (sea stars) and holothurians (sea cucumbers) are Δ^7-sterols, while sterols of echinoids (sea urchins), ophiuoids (brittlestars), and crinoids (sea lilies) are Δ^5-sterols.

A RECENT paper from this laboratory[1] dealt with the distribution of quinone pigments in the five classes of the phylum Echinodermata. We showed that structure patterns among echinoderm pigments suggest a close phylogenetic relationship of echinoids (sea urchins) and ophiuroids (brittlestars) on one hand, and of asteroids (sea stars) and holothurians (sea cucumbers) on the other. The position of the fifth class, the crinoids (sea lilies), could not be ascertained on the basis of available data. We further pointed out that Hashimoto's[2] recent study of the distribution of steroidal sapogenins provides additional support for a close relationship between asteroids and holothurians. Earlier work on the distribution of sterols in echinoderms, which has been reviewed by the late Werner Bergmann,[3] had already foreshadowed an identical phylogenetic relationship.

Our work on quinone pigments from the echinoderms[1] afforded us an opportunity to examine the sterols of the animals which we had collected primarily for the pigment work. Since most of the previous work on sterols had been carried out prior to the introduction of efficient separation methods and of mass spectrometric techniques, we felt that a careful study of the sterol mixtures would be of interest. In fact, existing data on marine sterols—m.ps, rotations, and occasionally combustion analyses—are in most cases almost certainly data for unresolved sterol mixtures rather than for individual sterols. This situation was clearly recognized by Bergmann.[3]

In contrast to our knowledge of echinoderm pigments where until recently[1] virtually all research had been carried out with echinoids, most of the published information on echinoderm sterols pertains to sterols from asteroids.[3] Asteroid sterols are typically mixtures of mono- and diunsaturated "stellasterols", both bearing a characteristic Δ^7-double bond.[4] Sterols with a Δ^7-double bond have also been recognized in the holothurians, although this class of animals has been inadequately studied, in part at least because of its low fat content. Our knowledge of the sterols of the remaining three classes of echinoderms is even sketchier. A common feature appears to be the Δ^5-double bond. A possible anomaly is reported by Bergmann[5] who mentions the isolation of a new sterol from the slate pencil sea urchin (binomial not provided) and cites as evidence for the Δ^7-unsaturation its reactivity in the Liebermann–Burchard test. Other work seems to indicate that cholesterol is the predominant sterol of echinoids. The ophiuroids have been studied

inadequately and Bergmann in his review[3] expressed doubt that a single pure sterol had been isolated from this class. Sterols from crinoids are known to be levorotatory, but few additional data have been reported. Bolker in a recent communication[6] assigned to "crinosterol" the structure of 24-methyl-22-dehydrocholesterol, i.e. the 24-epimer of brassicasterol. Absence of VPC and/or mass spectral data in Bolker's communication coupled with our own experience cast an element of doubt on the homogeneity of his crinosterol.

In the course of our work we have examined the sterol mixture of one representative of each of the five echinoderm classes: the sea urchin *Echinothrix diadema* Linn., the brittlestar *Ophiocoma insularia* Lyman, the sea star *Acanthaster planci* Linn., the sea cucumber *Holothuria atra* Jager, and the sea lily *Antedon* sp. Fresh or air-dried animals were extracted with acetone and the ensuing aqueous residue was extracted with benzene, leading to a crude oil containing the sterol fraction. The oil was saponified and the unsaponifiable residue was chromatographed on Decalso, which furnished a crystalline sterol mixture. This mixture was converted to the trimethylsilyl ethers (TMSE) and separated by preparative GLC. Relative retention times and mass spectral data are summarized in Table 1. The identity of individual sterols, except where noted, was established by comparison with retention times and mass spectra of authentic samples. In most cases, it was not possible to ascertain the stereochemistry of the side chain because of the small amount of sterol available.

The sea urchin *Echinothrix diadema* elaborates a three-component sterol mixture of which the major constituent (Ed-1) has the identical GLC retention time as cholesterol. The minor peaks corresponded in relative retention times to campesterol (Ed-2) and β-sitosterol (Ed-3). The absolute stereochemistry of Ed-2 at C-24 is not known as it has not been possible to resolve campesterol from its C-24 epimer Δ^5-ergostenol by GLC.[7] Similarly, GLC did not permit us to draw conclusions about the configuration of the side chain of Ed-3; it appears reasonable to assume that our ethylcholesterol is identical with β-sitosterol, a substance of widespread occurrence in nature. The mass spectrum of the sterol mixture showed molecular ion peaks at *m/e* 386 (cholesterol), 400 (campesterol), and 414 (β-sitosterol). This fully confirms previous reports that cholesterol is the principal sterol of sea urchins.[3, 8, 9]

The brittlestar *Ophiocoma insularia* was found to contain six sterols as indicated by GLC. The IR, NMR, and mass spectra and the GLC retention time of the major sterol (Oi-1), m.p. 147–148°, $[\alpha]_D$ −36°, were identical with those found for cholesterol. The component next in abundance, Oi-5, m.p. 121–122°, $[\alpha]_D$ −37°, had prominent bands in its IR spectrum at 3400 (OH), 840, 800 (Δ^5), and at 823 cm^{-1} (a second trisubstituted double bond).[10] The NMR spectrum in deuterochloroform showed the C-3 proton at 3·55 δ, the C-6 olefinic proton at 5·35 δ, and a methyl doublet of an ethylidene group at 1·56 δ (J = 7 c/s). The comparable values for fucosterol are 1·57 δ (J = 6·7 c/s).[11] An identical doublet was seen in the NMR spectrum of sargasterol, which we isolated for comparison from the alga *Sargassum ringgoldianum* Harvey.* The mass spectrum of Oi-5 had two molecular ion peaks at *m/e* 412 and 414, which showed that some monounsaturated 29-carbon sterol accompanied the diunsaturated one. The base peak at *m/e* 314 caused by loss of C_7H_{14} (98) from the 412 molecular ion was fully analogous to the mass spectral behavior of sargasterol[12] and has also been observed for fucosterol[13] and isofucosterol.[14] A 6-membered

* We are indebted to Dr. Tamao Yoshida for collecting this material in Chiba Prefecture, Japan.

cyclic rearrangement has been proposed for this fragmentation.[13] We have observed similar fragmentation in 24-methylenecholesterol,[12] as have other workers in 24-methylenelophenol,[15] and citostadienol.[13,15] The influence of side chain unsaturation of sterols on mass spectral fragmentation has recently been studied in detail by Wyllie and Djerassi.[16] The spectral evidence and physical constants (m.p. and optical rotation) indicate that Oi-5 is fucosterol rather than isofucosterol,[10,14] which is reported to have an IR band for its second trisubstituted double bond at low frequency (813 cm^{-1})[10] or sargasterol for which a m.p. of 132–133° and a rotation of −47·5° have been reported.[17] It is associated with a small amount of β-sitosterol. The remaining minor components were shown to be brassicasterol (Oi-2), campesterol (Oi-3), and stigmasterol (Oi-4). These results confirm the previous supposition that ophiuroid sterols are complex mixtures of Δ^5-sterols. Interestingly enough, the major constituent is cholesterol, thereby again hinting at a close relationship of ophiuroids and echinoids.

The GLC of the sterols from the sea star *Acanthaster planci* showed that it also contained a mixture of six sterols. Separation by preparative GLC, followed by crystallization from methanol-ether furnished colorless crystals of constituent Ap-2, m.p. 124–126°. It has a higher retention time than cholesterol by a factor of 1·13, which is in good agreement with that of Δ^7-cholestenol. The mass spectrum of this compound has a molecular ion peak at *m/e* 386 and the cracking pattern was found to be similar to that described for Δ^7-cholestenol by Knights.[18] The principal constituents Ap-4, m.p. 145–146°, showed a molecular ion peak at *m/e* 400 in its mass spectrum, which is 14 mass units higher than Δ^7-cholestenol (Ap-2). The fragmentation pattern was similar to that of Ap-2 provided a correction is made for an extra C-24 Me group. The presence of weak mass peaks at *m/e* 382 (M-18) and 367[M-(15 + 18)] coupled with the absence of characteristic intense mass peaks of Δ^5-monounsaturated C-28 sterol (campesterol)[12,17] at *m/e* 289[M-(H_2O + C_7H_9)], and at 315[M-(H_2O + C_5H_7)] in the mass spectrum[12] of Ap-4, further confirmed the identity of the major component as Δ^7-ergostenol. Of the remaining four minor components, Ap-3 has a molecular ion peak at *m/e* 398 and a higher retention time than its Δ^5-analogue, brassicasterol. The cracking pattern of Ap-3 differs from brassicasterol by having an intense mass peak at *m/e* 271 [M-(side chain + 2H)], a significant peak at 246,[19,20] which seems to be characteristic of a Δ^7-sterol and which we have observed in the mass spectra of all sea star sterols, and a weak peak at *m/e* 380[M-18]. On the basis of the above evidence Ap-3 is therefore $\Delta^{7,22}$-ergostadienol. The component Ap-1 was not completely separated from Ap-3 and was a very minor component. The mass spectrum has molecular ion peaks at *m/e* 384/386 and similar cracking patterns as observed for synthetic $\Delta^{7,22}$-cholestadienol.[12] The other minor component, Ap-5, has a higher retention time than β-sitosterol and showed molecular ion peaks at *m/e* 414/412. Thus it seems to be a mixture of Δ^7-stigmastenol associated with its diunsaturated analogue. The GLC retention time of the remaining sterol Ap-6 did not correspond to any of the available reference sterols. It has a molecular weight of 426 (mass spectrum). We have designated it acanthasterol and it appears to be the Δ^7-analogue of gorgosterol.[21] Structural elucidation of gorgosterol, which we isolated from a coelenterate, is in progress in our laboratory. These results fall well within the boundaries of Bergmann's prediction on the basis of earlier incomplete work, that sea star sterols are mixtures of Δ^7-mono- and diunsaturated sterols.[3]

The sterol mixture which we have isolated from the holothurian *Holothuria atra* showed considerable resemblance to the sea star sterol mixture on the basis of a preliminary positive selenium dioxide test and prominence of the 830 cm^{-1} IR band, which is characteristic of a Δ^7-double bond.[22] The GLC of the sterol showed five components, which were represented in the mass spectrum by molecular ion peaks at *m/e* 384 (Ha-1), 386 (Ha-2), 398 (Ha-3), 400 (Ha-4) and 412/414 (Ha-5). The major constituent is Ha-2, Δ^7-cholestenol. Except for the absence of acanthasterol (Ap-6) the mixture proved to have a composition identical with that of the sea star sterol mixture. The proportions of individual sterols are different but not their identity. These results again emphasize the parallelism in sterol composition of asteroids and holothurians. No previous attempts had been made to separate and identify individual sterols in a sea cucumber, although the presence of Δ^7-sterols had been recognized by color tests.[3]

The GLC of the sterol mixture isolated from the crinoid *Antendon sp.* from Eniwetok showed six components. The major component, An-3, corresponded to brassicasterol. This observation was further confirmed by the presence of an IR band at 970 cm^{-1} (Δ^{22}-*trans*)[23] and a strong molecular ion peak at *m/e* 398 in the mass spectrum of the sterol. The other components were identified as 22-dehydrocholesterol (An-1), cholesterol (An-2), campesterol (An-4), stigmasterol (An-5), and β-sitosterol associated with some diunsaturated material (An-6). The identification was based on relative retention times as compared with authentic samples and on the corresponding molecular ion peaks in the mass spectrum. Crinoid sterols therefore are Δ^5-sterols and the make-up of the mixture resembles most closely that of the ophiuroid.

The echinoderm sterols which we have described are known to occur naturally except for $\Delta^{7,22}$-cholestadienol and acanthasterol. $\Delta^{7,22}$-Cholestadienol has been synthesized.[23] However, some of these sterols have not previously been isolated from marine invertebrates. The most interesting of these is fucosterol (Oi-5). It and its C-20 epimer, sargasterol, are the common sterols of some marine algae.[17,24] Isofucosterol which was synthesized by Dusza[10] has been recently isolated from oat seeds.[14] Fucosterol is of biogenetic interest, since it has a 24-ethylidine group which is a suggested intermediate in the biosynthesis of 24-ethylsteroids.[25]

It is evident from this account that the picture of echinoderm sterols is more complex than has been described in the literature. It is not known whether echinoderms synthesize these sterols *de novo* or whether they modify ingested sterols. In our own experience the sterol composition is characteristic of a particular genus or species and is independent of the environment. For instance, the sea star *Acanthaster planci* from two distant locations, Oahu and Eniwetok, elaborates closely parallel sterol mixtures. Similarly, the brittlestar *Ophiucoma insularia* from two different locations on Oahu afforded parallel sterol mixtures.

We have now for the first time an accurate picture of the sterol patterns in representatives of the five classes of echinoderms. The major feature, the occurrence of Δ^7-sterols in asteroids and holothurians and of Δ^5-sterols in echinoids, ophiuroids, and crinoids, which had been suggested by the early fragmentary work, was fully confirmed. We further see that the sterol constituents of the asteroids and the holuthurians are virtually indentical and we now have a clear indication that crinoid sterols resemble ophiuroid sterols more closely than those of any other class. The pertinent data of all sterols encountered in this work are summarized in Table 1.

Sterol code	Source	Appropriate abundance in ‰ based on GLC chromatograms	Assigned structure	Retention time relative to* cholesterol		Mass spectral data	
				This work	Ref sample	Mol wt,	No of carbons
An-1	crinoid	4·5	$\Delta^{5,22}$-cholestadien-3β-ol-(22-dehydrocholesterol)	0·88		384	27
Ed-1	echinoid	97	cholesterol	1·00	1·00	386	27
Oi-1	ophiuroid	66·5	cholesterol	1·00			
An-2	crinoid	20·5	cholesterol	0·99			
Ap-1	asteroid	1·0	$\Delta^{7,22}$-cholestadien-3β-ol	1·00		384	27
Ha-1	holothurian	4·5		1·00			
Oi-2	ophiuroid	1·5	24-methyl-$\Delta^{5,22}$-cholestadien-3β-ol (brassicasterol)	1·11	1·13	398	28
An-3	crinoid	47·0		1·10			
Ap-2	asteroid	7·5	Δ^{7}-cholesten-3β-ol	1·13		386	27
Ha-2	holothurian	43·5		1·13			
Ed-2	echinoid	1·5	24-methyl-Δ^{5}-cholesten-3β-ol	1·23	1·30	400	28
Oi-3	ophiuroid	2·5	(campesterol or 22,23-dihydro-	1·25			
An-4	crinoid	9·0	brassicasterol)	1·24			
Ap-3	asteroid	16·5	24-methyl-$\Delta^{7,22}$-cholestadien-	1·24		398	28
Ha-3	holothurian	24·0	3β-ol	1·25			
Oi-4	ophiuroid	1·5	24-methyl-$\Delta^{5,22}$-cholesten-3β-	1·35	1·37	412	29
An-5	crinoid	5·0	ol (stigmasterol)	1·36			
Ap-4	asteroid	71·0	24-methyl-Δ^{7}-cholesten-3β-ol	1·55		400	28
Ha-4	holothurian	19·0	(Δ^{7}-ergostenol)	1·57			
Oi-5	ophiuroid	28·0	mixture of 24-ethyl and 24-	1·60	1·60/1·60	414/412	29
An-6	crinoid	14·0	ethylidine-Δ^{5}-cholesten-3β-ol	1·56			
Ap-5	asteroid	3·0	mixture of 24-ethyl and 24-	1·80		414/412	29
Ha-5	holothurian	9·0	ethylidine-Δ^{7}-cholesten-3β-ol	1·81			
Ap-6	asteroid	1·0	acanthasterol	2·45		426	30

* Retention time of cholesterol 7·5 min.

In a recent monograph on the physiology of echinoderms[26] the authors briefly cite the reported occurrence of some chemical constituents in echinoderms and express the view that "to base a classification on these data would lead to absurd results." The results may be "absurd", but all available evidence is certainly consistent. Our spinochrome work,[1] Hashimoto's steroidal saponin research,[2] and the sterol distribution which is described here, all point to a close phylogenetic relationship of asteroids and holothurians and another relationship of echinoids, ophiuroids, and crinoids.

EXPERIMENTAL

M.ps determined on a Fisher–Johns apparatus and uncorrected. Rotations determined with an ETL-NPL Type 143A photoelectric polarimeter. IR spectra measured with a Beckman IR-5 instrument. NMR spectra recorded with a Varian A-60 spectrometer. Chemical shifts are expressed in ppm and referred to TMS $\delta = 0$. Mass spectra were measured on a Hitachi—Perkin-Elmer RMU-6D spectrometer, at 70 eV with a direct inlet system. GLC was carried out in an Aerograph A-705 instrument with hydrogen flame detector and chromatograms were recorded on a Varian G-10 recorder.

The reference compounds cholesterol and stigmasterol were obtained commercially and were recrystallized to constant m.p. Synthetic samples of brassicasterol, $\Delta^{7,22}$-cholestadienol and 22-dehydrocholesterol were supplied by Professor K. Tsuda and authentic samples of campesterol and β-sitosterol were obtained from Dr. M. J. Thompson.

Isolation of crude sterols. Freshly collected or dried animals were extracted with acetone until a colorless extract was obtained. The resultant extract was concentrated to an aqueous concentrate or a dry residue, which was in turn extracted with benzene. Concentration of the benzene extract afforded an orange-brown oil.

The oil was saponified with 10% KOH in MeOH for ca. 2 hr under N_2. The unsaponifiable matter was extracted with ether and benzene. The combined extracts were washed with water until neutral to litmus, dried over anh Na_2SO_4, and evaporated to dryness.

The crude unsaponifiable residue was taken up in isooctane and chromatographed on a column of Decalso (Matheson, Coleman & Bell). Elution with isooctane, 5%, 10%, and finally 25% ether in isooctane furnished the sterols in the last fraction. One or two crystallizations from MeOH–ether yielding crystalline sterol mixtures.

Separation of sterols. Analytical GLC was carried out using a 2 m × 4 mm i.d. glass column packed with 1% GE-XE-60 on 110/120 mesh Gas-Chrom Q (Applied Science Labs, Inc.).

Preparative GLC was performed on 3 m × 9 mm i.d. glass columns packed with 3% GE-XE-60 on Gas-Chrom Q. The column exit was connected to the detector end, provided with a capillary splitter, which was adjusted to give a split ratio of about 1:10 between the hydrogen flame detector and manually operated fraction collector.

The carrier gas was N_2 at 20 psi. The flow rate at the detector as well as collector ends was determined with a 10 ml soap bubble flow meter and injections were made with a 10 μl (analytical) and 50 μl (preparative) Hamilton syringe. Injector temperature was 280°, detector 270°, column 220° (analytical) or 240° (preparative). The liquid phase, GE-XE-60 (1 and 3%) was applied to Gas-Chrom Q by a filtration and evaporation technique, as described by Horning *et al.*[27]

TMSE were prepared by the method of Eneroth *et al.*[28] and ca. 30 μl of 3–5% soln of the TMSE reaction mixture was directly injected for preparative GLC. Fractions were collected manually in glass tubes having aerosol scrubbers at the top and glass wool at both ends. Generally, only the center of the peak was collected. After collection each bottle was rinsed several times with ether to remove all sterol TMSE and the solvent was removed under reduced pressure. The free sterols were then recovered by refluxing the TMSE in methanol for 2–3 hr followed by 1–2 recrystallizations from MeOH–ether.

Ophiuroid sterols. Preparative GLC of TMSE of the sterol mixture (150 mg) of the brittlestar *Ophiucoma insularia* afforded, Oi-1 (42 mg), Oi-2 (6 mg), Oi-3 (3 mg), Oi-4 (2·5 mg) and Oi-5 (20 mg). The free sterols were regenerated and recrystallized to furnish crystalline Oi-1, m.p. 147–148°, $[\alpha]_D$ −36° and Oi-5, m.p. 121–122°, $[\alpha]_D$ −37°. The other 3 fractions did not crystallize and were further characterized and compared by mass spectrometry and GLC retention time only.

Asteroid sterols. A typical batch of 50 mg of the sterol mixture isolated from the sea star *Acanthaster planci* yielded, 1 mg Ap-1, 1·5 mg Ap-2, 5·7 mg Ap-3, 10·7 mg Ap-4, 1·5 mg Ap-5, and 1·8 mg Ap-6. In

order to isolate enough of the interesting sterols Ap-2 and Ap-6, more sterol mixture was separated by preparative GLC. Work-up of the fractions left Ap-2, m.p. 124–126°; Ap-3, m.p. 154–156°; Ap-4, m.p. 145–146°. Fractions, Ap-1, Ap-5, and Ap-6 did not crystallize. All fractions were characterized by mass spectral and GLC retention time comparisons.

Echinoid, holothurian, and crinoid sterols. The sterol mixtures of the sea urchin *Echinothrix diadema*, the sea cucumber *Holothuria atra* and the sea lily *Antedon sp.* were not separated preparatively. Retention times of the analytical GLC were compared with those of authentic samples run under identical conditions. Mol wts were determined mass spectrometrically.

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