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THE STEROLS OF SOME MARINE RED ALGAE

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Abstract—Cholesterol has been identified as the major sterol in several marine red algae. In two species cholesterol is accompanied by a second sterol believed to be 22-dehydrocholesterol. However in two algae, *Rhodymenia palmata* and *Porphyra purpurea*, desmosterol is the predominant sterol. It seems probable that only cholesterol or closely related C_{27} sterols occur in the Rhodophyta.

INTRODUCTION

THE first intensive study of the sterols of the algal classes was carried out by Heilbron, Phipers and Wright in 1934.[1] A doubly unsaturated sterol which they named fucosterol was isolated from the brown algae *Pelvetia caniculata* and *Fucus vesiculosus* and its structure was later determined.[2] Heilbron and co-workers also suggested that fucosterol was present in the red alga *Rhodymenia palmata* and that it occurred in addition to sitosterol in certain members of the Chlorophyta.[3] A more comprehensive study was undertaken by Carter, Heilbron and Lythgoe[4,5] who isolated the sterols of a large number of species of algae belonging to several Orders, collected mainly around Aberystwyth, Wales. Identification of the sterols was based upon the melting points of the sterols and in some cases of the steryl acetates. Of the thirteen species of Rhodophyta examined (see Table 1) it was considered that eight contained sitosterol, three contained fucosterol whilst two contained unidentified sterols.

More recently Tsuda and his associates[6–9] in a detailed survey of the red algae of the Pacific demonstrated convincingly that in most cases these algae contained cholesterol. This was one of the first instances of the isolation of cholesterol, previously regarded as belonging exclusively to the animal kingdom, from a plant source. From a total of sixteen species studied (see Table 1) twelve were found to contain cholesterol, three cholesterol and possibly chalinasterol,[6–8] and one contained 22-dehydrocholesterol.[9]

The situation in the literature[10] was thus confused, especially as in some cases the reported

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1 I. M. HEILBRON, R. F. PHIPERS and H. R. WRIGHT, *J. Chem. Soc.* 1572 (1934).
2 H. B. MACPHILLAMY, *J. Am. Chem. Soc.* **64**, 1732 (1942); D. H. HEY, J. HONEYMAN and W. J. PEEL, *J. Chem. Soc.* 2881 (1950).
3 I. M. HEILBRON, E. G. PARRY and R. F. PHIPERS, *Biochem. J.* **29**, 1376 (1935).
4 P. W. CARTER, I. M. HEILBRON and B. LYTHGOE, *Proc. Roy. Soc.* **B128**, 82 (1939).
5 I. M. HEILBRON, *J. Chem. Soc.* **79**, (1942).
6 K. TSUDA, S. AKAGI and Y. KISHIDA, *Science* **126**, 927 (1957).
7 K. TSUDA, S. AKAGI and Y. KISHIDA, *Chem. Pharm. Bull. (Tokyo)* **6**, 101 (1958).
8 K. TSUDA, S. AKAGI, Y. KISHIDA, R. HAYATSU and K. SAKAI, *Chem. Pharm. Bull. (Tokyo)* **6**, 724 (1958).
9 K. TSUDA, K. SAKAI, K. TANABE and Y. KISHIDA, *Chem. Pharm. Bull. (Tokyo)* **7**, 747 (1959); *J. Am. Chem. Soc.* **82**, 1442 (1960).
10 For a review of algal sterols see J. D. A. MILLER, In *Physiology and Biochemistry of Algae* (Edited by R. A. LEWIN), p. 357. Academic Press, New York (1962).

differences in sterol occur within the same genus. There was also apparently no correlation between taxonomic position and type of sterol in the Rhodophyta. However it must be remembered that the work of Heilbron and his co-workers[4] was carried out when relatively few phytosterols had been definitely identified and their structures conclusively elucidated. In view of the development of new analytical techniques and in particular gas–liquid chromatography, we decided to investigate the sterols of some marine red algae in an attempt to clarify the situation. The present work shows that cholesterol, or the closely related sterol, desmosterol, is found in all the red algae examined; this is in agreement with the observations of Tsuda and his colleagues.

TABLE 1. PUBLISHED DATA ON THE STEROLS OF RED ALGAE

	After Heilbron *et al.*[3–5]		After Tsuda *et al.*[6–9]	
Order	Genus and species	Sterol	Genus and species	Sterol
Bangiales	*Porphyra umbilicalis*	Fucosterol	—	—
Nemalionales	*Lemanea mamillosa*	Sitosterol	—	—
Gelidiales	*Gelidium corneum*	Sitosterol	*Acanthopeltis japonica*	Cholesterol, Chalinasterol
			Gelidium amansii	Cholesterol, Chalinasterol
			G. japonicum	Cholesterol
			G. subcostatum	Cholesterol
			Pterocladia tenuis	Cholesterol, Chalinasterol
Cryptonemiales	*Corallina officinalis*	Unidentified	*Cyrtymenia sparsa*	Cholesterol
			Gloidopeltis furcata	Cholesterol
			Grateloupia elliptica	Cholesterol
			Tichocarpus crinitus	Cholesterol
Gigartinales	*Ahnfeltia plicata*	Unidentified	*Chondrus giganteus*	Cholesterol
	Chondrus crispus	Sitosterol	*C. occellatus*	Cholesterol
	Gigartina stellata	Sitosterol	*Iridophycus cornucopiae*	Cholesterol
	Phyllophora membranifolia	Sitosterol	*Rhodoglossum pulcherum*	Cholesterol
	Plocamium coccineum	Sitosterol	*Hypnea japonica*	22-Dehydro-cholesterol
Rhodymeniales	*Rhodymenia palmata*	Fucosterol	*Coeloseira pacifica*	Cholesterol
Ceramiales	*Polysiphonia fastigiata*	Sitosterol	*Rhodomela larix*	Cholesterol
	P. nigrescens	Fucosterol		
	Ceramium rubrum	Sitosterol		

RESULTS

Thirteen species of the Class Rhodophyta were chosen to include members of as many families as possible within the ecological limits of the collecting area on the Cardiganshire coast (Table 2). Each species was carefully freed from animal and foreign plant material. However, the treatment employed did not exclude the possibility of the presence of microscopic flora and fauna but the quantities of such contaminants compared with the bulk of the algae were negligible and considered unlikely to interfere with the sterol analysis.

Extraction of the non-saponifiable lipid gave in all cases a dark-brown viscous oil (0·075–0·28% of the fresh weight of the algae) which was chromatographed on alumina (Brockmann, grade III). The sterol fraction was further purified by preparative thin-layer chromatography

on silica gel. All the algae contained sterol which gave a blue–green colour (λ_{max} 635 nm) with the Liebermann–Burchard test and which reached maximum intensity at 35 min. This is typical of sterols containing a Δ^5 bond.[11]

TABLE 2. SPECIES OF RHODOPHYTA EXAMINED FOR STEROL

Order	Family	Genus and Species*	Habitat
Gigartinales	Gigartinaceae	*Chondrus crispus*	Rock pools, mid tide
	Gigartinaceae	*Gigartina stellata*	Rock pools, mid tide
	Furcellariaceae	*Furcellaria fastigiata*	Rock pools, low tide
	Phyllophoraceae	*Ahnfeltia plicata*	Rock pools, high tide
	Plocamiaceae	*Plocamium vulgare [coccineum]*	Very low tide
Ceramiales	Rhodomelaceae	*Laurencia pinnatifida*	Rock pools, mid tide
	Rhodomelaceae	*Polysiphonia lanosa [fastigiata]*	Epiphytic on *Ascophylum nodosum*
	Rhodomelaceae	*Polysiphonia nigrescens*	Rock pools, mid tide
Cryptonemiales	Corallinaceae	*Corallina officinalis*	Rock pools, mid tide
	Dumontiaceae	*Dilsea carnosa [edulis]*	Rock pools, mid tide
	Polyideaceae	*Polyides caprinus*	Very low tide
Rhodymeniales	Rhodymeniaceae	*Rhodymenia palmata*	Very low tide
Bangiales	Bangiaceae	*Porphyra purpurea*	Rock pools, mid tide

* Names are according to M. PARKE and P. S. DIXON, *J. Marine Biol. Assoc. U.K.* **44**, 499 (1964).

TABLE 3. MELTING POINTS AND RELATIVE RETENTION DATA OF STEROLS

	M.p.*		Relative retention times [a]		
	Sterol	Steryl acetate	QF-1[b]	SE-30[c]	Hi-EFF 8B[d]
Cholesterol	149·5–150	114–115	2·95 (4·80)	2·30 (3·21)	7·07 (5·86)
Desmosterol	120–121	92–93	3·24 (5·05)	2·47 (3·63)	9·30 (7·67)
7-Dehydrocholesterol	150	130	—	—	—
22-Dehydrocholesterol	135	126	— (4·30)	— (2·83)	— (5·77)
Campesterol	158	138	4·06	3·26	9·80
Brassicasterol	148	157	—	2·63	7·59
Ergosterol	168	181	—	2·89	10·52
Chalinasterol	145–146	135–136	—	—	—
β-Sitosterol	137	127	4·83	4·05	11·89
Clionasterol	148	144	—	—	—
Stigmasterol	170	144	4·06	3·58	9·83
Poriferasterol	156	147	4·06	3·58	9·83
α-Spinasterol	174	187	4·83	—	11·20
Chondrillasterol	169	175	—	—	—
Fucosterol	124	118	4·83	4·05	13·27

* See Ref. 12.

[a] Relative to cholestane. Values in parenthesis are for the steryl acetates.

[b] Column 180 × 0·3 cm, 1% QF-1 on 80–100 mesh HMDS Chromosorb W. Column 225°; Injector 245°; Detector 250°; Nitrogen 40 ml/min.

[c] Column 180 × 0·3 cm, 1% SE-30 on 80–100 mesh HMDS Chromosorb W. Column 226°; Injector 245°; Detector 250°; Nitrogen 40 ml/min.

[d] Column 120 × 0·3 cm, 0·7% Hi-EFF 8B on 80–100 mesh HMDS Chromosorb W. Column 216°; Injector 240°; Detector 240°; Nitrogen 40 ml/min.

[11] P. R. MOORE and C. A. BAUMANN, *J. Biol. Chem.* **195**, 615 (1952).

TABLE 4. ANALYSIS OF STEROLS OF RED ALGAE

	Relative retention times*			M.P.		Identity of sterol(s) and approximate percentage composition
	QF-1	SE-30	Hi-EFF 8B	Sterol	Steryl acetate	
1. *Chondrus crispus*	3·00	2·36	7·10	144	114	Cholesterol, 100
2. *Gigartina stellata*	3·00	2·31	7·14; 9·16	140	—	Cholesterol, 99; desmosterol, 1
3. *Furcellaria fastigiata*	2·95 (4·73)	— (3·15)	6·98	142	—	Cholesterol, 100
4. *Ahnfeltia plicata*	3·00	2·32	6·89	144–145	114	Cholesterol, 100
5. *Plocamium vulgare*	3·00	2·33	—	143–144	—	Cholesterol, 100
6. *Laurencia pinnatifida*	3·00	2·30	6·98; 9·02	144–145	—	Cholesterol, 98; desmosterol, 2
7. *Polysiphonia lanosa*	3·00	—	6·89	144–145	113	Cholesterol, 100
8. *Polysiphonia nigrescens*	— (4·58; 5·10)	— (3·09; 3·51)	7·00; 9·30	—	—	Cholesterol, 97; desmosterol, 3
9. *Corallina officinalis*	3·00	2·30	6·80	144	—	Cholesterol, 100
10. *Dilsea carnosa*	3·00; 2·61 (4·90; 4·30)	(3·18; 2·90)	6·98; 6·25	134	—	Cholesterol, 85; 22-dehydrocholesterol, 15
11. *Polyides caprinus*	3·00; 2·60 (4·85; 4·25)	2·36; 2·24 (3·21; 2·96)	7·10; 6·25	140	—	Cholesterol, 90; 22-dehydrocholesterol, 10
12. *Rhodymenia palmata*	3·25	2·47	9·46; 6·80	118	94–95	Desmosterol, 99; cholesterol, 1
13. *Porphyra purpurea*	3·29; 3·00	2·42; 2·30	9·46; 6·80	118	—	Desmosterol, 89; cholesterol, 11

* Conditions for gas–liquid chromatography are as given in Table 3. Where two values are given the first is for the major component. Values in parenthesis are the relative retention times of the steryl acetates.

Gas–liquid chromatography with the three stationary phases listed in Tables 3 and 4 revealed that the algae 1–9 contained one major sterol, which in all cases had retention data corresponding to cholesterol. In no case was a sterol peak observed which corresponded to β-sitosterol, fucosterol, or chalinasterol. However with algae 2, 6 and 8 a minor component was present as indicated and which corresponded to desmosterol. The i.r. spectra of the sterols from algae 1 to 9 were identical to that of cholesterol, with peaks at 805 and 845 cm^{-1} (Δ^5). Additional evidence for the identity of cholesterol was provided by the melting points of the isolated sterols and, in some cases, of the acetates, as shown in Table 4. A sample of the sterol from *Gigartina stellata* was analysed by mass spectrometry and showed a parent ion at m/e 386. Other peaks were observed at m/e 371 [M-CH_3]; 368 [M-H_2O]; 353 [M-(CH_3+H_2O)]; 301; 275; 273 [M-side chain]; 255 [M-(side chain+H_2O)]; 247; 231 [M-(side chain and part of ring D+H)] and 213. This provides further confirmation for the presence of cholesterol in this alga.

The algae 10 and 11 were shown by gas–liquid chromatography to contain two sterols, the major sterol in each case was cholesterol whilst the minor sterol is believed on the basis of retention data to be 22-dehydrocholesterol. This view is strengthened by the observation of a small peak at 975 cm^{-1} in the i.r. spectrum of the sterol from *Dilsea carnosa* which is indicative of a *trans* Δ^{22} bond. However, insufficient material was obtained to permit a separation and further study of this minor compound.

Gas–liquid chromatography of the sterol from *Rhodymenia palmata* (12) revealed one major component with a retention time identical to that of demosterol and also small amounts of cholesterol. With the Liebermann–Burchard reagent the sterol gave a green colour (λ_{max} 635 nm) maximal after 35 min but the intensity was less than observed with an equal amount of cholesterol and is in accordance with the observation of Avigan *et al.*[13] who showed a lower colour response for desmosterol. The i.r. spectrum was the same as that of cholesterol except that it exhibited an additional peak at 830 cm^{-1} which indicated a second trisubstituted double bond as found in desmosterol.[13,14] The melting points of both the sterol and its acetate were in good agreement with the reported values for desmosterol. The identity of desmosterol was substantiated by the mass spectra of the sterol. This had a mass peak at m/e 384 with other peaks at m/e 369 [M-CH_3]; 366 [M-H_2O]; 351 [M-CH_3+H_2O]; 300; 271 [M-side chain+2H] and 253 [M-(side chain+2H+H_2O)].

Gas–liquid chromatography of the sterol from *Porphyra purpurea* (13) showed that desmosterol also predominated in this algae but a significant amount of cholesterol was also present. This appears to be the first report of desmosterol in algae.

DISCUSSION

The identification of cholesterol as the predominant sterol in the majority of British red algae examined reconciles the British and Japanese work in this field. There is no difference in the sterol type, i.e. (C_{27}) in the algae found in British or Japanese waters, and hence the need to explain the previous apparent divergence on taxonomic or geographical grounds is eliminated. Our own demonstration of cholesterol in *Chondrus crispus* collected near Aberystwyth also confirms the recent demonstration of cholesterol in this species collected

[12] W. Bergmann, In *Comparative Biochemistry* (Edited by M. Florkin and H. S. Mason), Vol. 3, p. 103. Academic Press, New York (1962).
[13] J. Avigan, D. Steinberg, H. E. Vooman, M. J. Thompson and E. Mosettig, *J. Biol. Chem.* **235**, 3123 (1960).
[14] W. M. Stokes, F. C. Hickey and W. A. Fish, *J. Biol. Chem.* **232**, 347 (1958).

on the coast of Nova Scotia.[15] The finding of desmosterol in *Rhodymenia palmata* and *Porphyra purpurea* is interesting. Desmosterol is a comparatively recent discovery[16] and its melting point is similar to that of fucosterol (Table 3). It is therefore understandable that in their earlier investigation the Heilbron group misinterpreted the melting point which they obtained for the sterol from *R. palmata*.

Cholesterol has been reported in small amounts in various higher plants[17] where it accompanies sterols containing an extra alkyl group at C_{24}. However in the majority of the Rhodophyta, cholesterol or closely related C_{27} sterols occur unaccompanied by any sterols containing a C_{-24} alkyl group (see Table 1 for three possible exceptions). This may be of biogenetic interest. Possibly the members of this phytological division have not developed the enzyme(s) necessary for the transmethylation reaction from *S*-adenosylmethionine.

Desmosterol is considered to be a possible intermediate in cholesterol biosynthesis in animals[18] and the presence of small amounts of desmosterol in some red algae can be satisfactorily explained on this basis. However the reason for the accumulation of large amounts of desmosterol in *R. palmata* and *P. purpurea* is obscure. A failure of these algae to elaborate a Δ^{24} hydrogenating enzyme appears untenable because of the presence of small amounts of cholesterol. Possibly desmosterol fulfils some specific physiological function in these organisms more efficiently than does cholesterol. However both this point and also the significance of the presence of 22-dehydrocholesterol in some red algae must await further investigations.

EXPERIMENTAL

Preparation of Algae for Extraction

In general, amounts of alga varying between 100 and 300 g fresh weight were collected and stored in the deep freeze for periods up to 48 hr. Following this treatment any visible animals present were washed out with running water and the species under investigation was separated by hand from other plant material present.

Extraction of Non-saponifiable Lipids

The alga was homogenized in ethanol (4 vol. ethanol per unit fresh weight of alga) with an Ultra-Turrax homogenizer. KOH solution was added to give a final concentration of 15% KOH in 85% ethanol and the solution refluxed for 90 min. The residue was removed by filtration through a pad of glass wool and washed first with a small volume of ethanol and then with diethyl ether. Two volumes of water were added to the filtrate which was then exhaustively extracted with diethyl ether. The ethereal extract was washed with several volumes of water, dried (Na_2SO_4) and the major portion of the diethyl ether was removed by distillation and the last few millilitres under a stream of nitrogen.

Isolation of Sterols

The non-saponifiable lipid was chromatographed on alumina, Brockmann, grade III, (10 g of alumina/100 mg of lipid) and eluted successively with petroleum ether (40–60°), 2%

[15] A. SAITO and D. R. IDLER, *Can. J. Biochem.* **44**, 1195 (1966).

[16] W. M. STOKES, W. A. FISH and F. C. HICKEY, *J. Biol. Chem.* **220**, 415 (1956); U. H. M. FAGERLUND and D. R. IDLER, *J. Am. Chem. Soc.* **79**, 6473 (1957); W. BERGMANN and J. P. DUSZA, *J. Org. Chem.* **23**, 459 (1958).

[17] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Science* **140**, 198 (1963); C. DJERASSI, J. C. KNIGHT and H. BROCKMANN, *Chem. Ber.* **97**, 3118 (1964); R. D. BENNETT, S. T. KO. and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).

[18] For a review of sterol biosynthesis see R. B. CLAYTON, *Quart. Rev.* **19**, 168, 201 (1965).

diethyl ether in petroleum ether (E/P), 6% E/P, 9% E/P, 20% E/P and diethyl ether.[19] The major 4-desmethyl sterols were eluted in the 20% E/P fraction.[19]

Thin-layer Chromatography

The sterols were purified further by preparative thin-layer chromatography on layers of silica gel impregnated with Rhodamine 6G.[20] developed with chloroform or ethyl acetate–benzene (1:5 v/v), cholesterol or β-sitosterol was chromatographed as marker, and the sterol bands detected under u.v. light. The sterol zones were then scraped off and eluted with dry diethyl ether.

Gas–Liquid Chromatography

A Varian-Aerograph 1522B was used, fitted with flame ionization detectors and using nitrogen as carrier gas. On-column injections were employed. Column packings were prepared from 80–100 mesh HMDS-treated Chromosorb W by the filtration technique.[21] The stationary phases used were: 1% QF-1; 1% SE-30 and 0·7% HiEFF 8B[22] (cyclohexanedimethanol succinate) all obtained from Applied Science Laboratories Inc., Pa. The approximate percentage composition of sterol mixtures was determined from peak areas on the assumption that the detector response was the same for all sterols.

Purification of Sterols for Melting Points

The sterols and steryl acetates were recrystallized to constant melting point from chloroform–methanol.

Preparation of Steryl Acetates

The sterols were acetylated with pyridine–acetic anhydride in the usual manner.

Liebermann–Burchard Test

A suitable quantity of sterol was dissolved in 1 ml of glacial acetic acid and 2 ml of the Liebermann–Burchard reagent (19 ml of acetic anhydride + 1 ml conc. H_2SO_4) added.

Infra-red Spectra

These were determined as Nujol mulls on NaCl discs on the Perkin Elmer Infracord.

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[19] L. J. Goad and T. W. Goodwin, *Biochem. J.* **99**, 735 (1966).
[20] J. Avigan, D. S. Goodman and D. Steinberg, *J. Lipid Res.* **4**, 100 (1963).
[21] E. C. Horning, W. J. A. Vanden Heuvel and B. G. Creech, *Methods Biochem. Analy.* **11**, 69, (1963).
[22] B. A. Knights, *J. Gas Chromatog.* **338** (1964).