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Sterols of Marine Algae

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Abstract □ The free and bound sterols of the marine algae *Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*, were isolated and identified by using a combination of TLC, GLC, and mass spectroscopic techniques. Preparative TLC enabled the fast, efficient separation of each sterol from the mixture for positive identification by GLC and mass spectroscopy. The chromatographic techniques employed permitted the detection and isolation of the more polar sterols in the presence of the more common 3β-monohydroxy sterols. To the authors' knowledge, this is the first recorded detailed sterol analysis of *L. saccharina* and the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. The mass spectra for these two sterols are presented.

Keyphrases □ Sterols, free and bound—isolated and identified from marine algae (*Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*) □ Algae, marine—isolation and identification of free and bound sterols □ Marine algae—isolation and identification of free and bound sterols □ *Laminaria saccharina*—isolation and identification of free and bound sterols

Marine algae have been the subject of numerous investigations in the fields of pharmacognosy and natural product chemistry for many years (1). Sterols found in algae (2, 3) and marine invertebrates (4) have been reported to exhibit antihypercholesterolemic activity in test animals (5–7), as has β-sitosterol in humans (8).

In most studies on the sterol content of marine and other organisms, the identification has been made on the basis of GLC retention times (9), occasionally accompanied by mass spectrometry of the mixture as a whole or of the individual GLC peaks when a GLC–mass spectrometer combined unit was available (10, 11). Column chromatography has also been employed extensively to separate simple mixtures of sterols (12), and preparative GLC can be used to complete purification that has been partially achieved by column chromatography (13).

Occasionally, new compounds have appeared when the natural sterol mixtures were analyzed by GLC. It is then necessary to isolate the individual component to characterize it fully (14, 15). Many sterols can be readily isolated from mixtures by preparative TLC in sufficient quantities to obtain NMR spectra and melting points as well as mass spectra (16). These physical data are necessary to give positive identification of unknown as well as known compounds (14, 15).

The preparative TLC methods used are simple techniques that can be employed along with GLC in analytical screening of algae as well as other biological material to determine sterol, triterpenoid, or other natural product content. These or other similar TLC methods have been in use for some time by workers studying sterol metabolism (17).

In the present work, the dried algae were extracted initially with a chloroform–methanol mixture to obtain the free sterols or their esters and subsequently with aqueous methanolic potassium hydroxide to obtain the water-soluble or bound sterols (18). In the two brown algae examined, the bound and unbound sterols were similar in composition and in approximately equal quantities, while the red algae showed the bound fraction to be present in approximately one-third the amount of free sterols. Isolation and separation were achieved completely by preparative

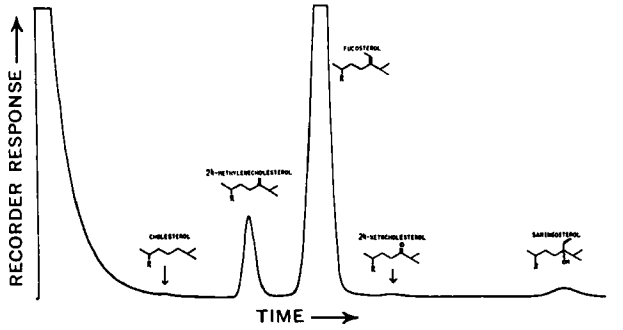


Figure 1—*GLC separation of* L. saccharina *sterols on 1.5% OV-17.*

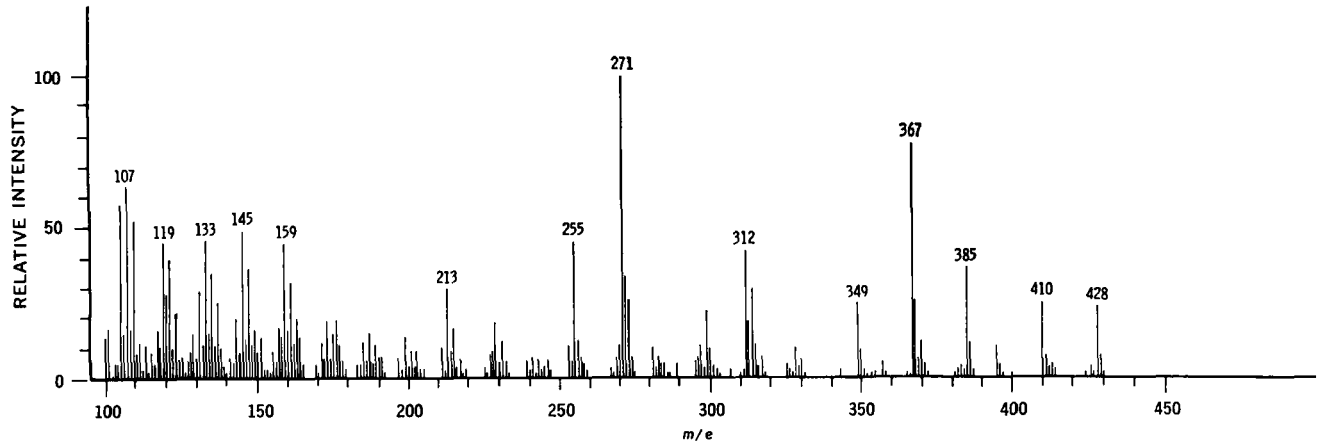


Figure 2—*Mass spectrum of saringosterol isolated from* L. saccharina.

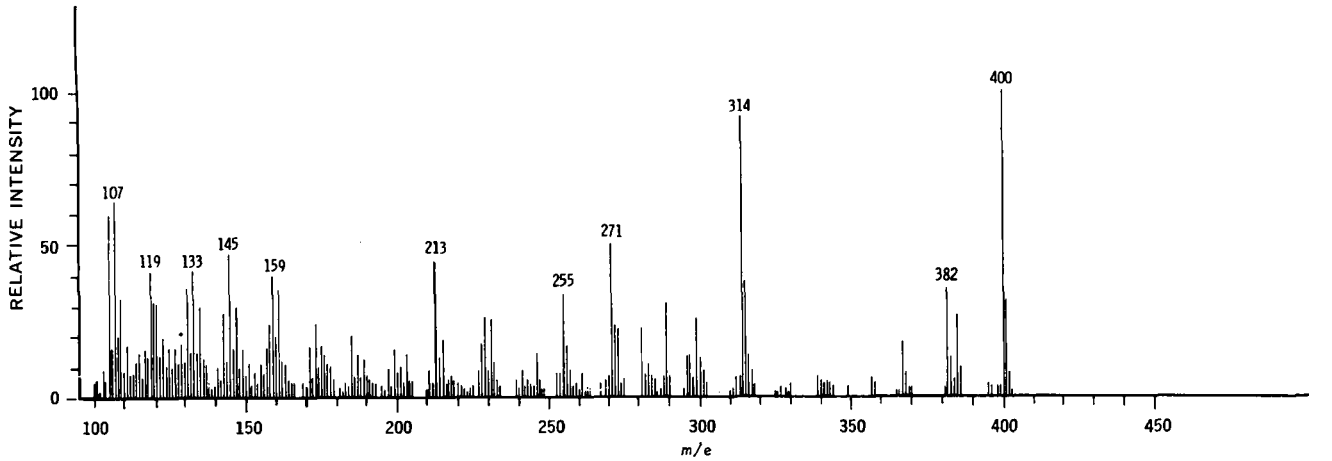


Figure 3—*Mass spectrum of 24-ketocholesterol isolated from* L. saccharina.

TLC using GLC to monitor the separation and to identify the separated sterols tentatively.

EXPERIMENTAL[1]

Chromatographic Analysis—The extraction procedure was intended to determine both free and bound sterols and included two stages with methods similar to those used in a study of *Euglena gracilis* (19). For each species studied, 100-g samples of dried, ground algae were thoroughly extracted with 400 ml of methanol–chloroform (2:1) under reflux for 1 hr. This extract was concentrated and saponified using standard procedures (20). The plant cell debris was subsequently boiled with 400 ml of potassium hydroxide (10%) in methanol-water (9:1) for 1 hr; the extract was diluted with an equal volume of water and extracted with ether.

The two extracts thus obtained were purified, following drying and evaporation of the ether, by preparative TLC utilizing 20 × 20-cm plates coated with silica gel $HF_{254\&366}$ (10 g/plate); not more than 0.25 g of extract was applied per plate. After development in hexane–ether–ethanol (80:20:4), visualization was accomplished by spraying a narrow band with concentrated sulfuric acid and warming at 60° until color appeared. The band(s) with R_f in the sterol region that turned red or violet was (were) marked by following the band(s) across the plate under longwave UV light, and the entire section (except for that sprayed) was scraped into a sintered-glass funnel and extracted with ether.

GLC analyses were then carried out on the ether solutions using cholestane as an internal standard. The chromatograph[2] was equipped with glass columns (2 m × 3 mm) and hydrogen flame detectors. It was operated isothermally at either 240° with column packing of 1.5% OV-17 on Gas Chrom Q (100–120 mesh) or 215° with a mixture of 3% XE-60 and 3% NGS on the same support. The samples were injected into the OV-17 column as free sterols or their acetates and onto the mixed phase column as the trimethylsilyl ether derivatives.

With each species analyzed, the sterol composition of the free and bound sterols was not significantly different. Therefore, the two extracts were combined before a separation of the individual components was begun.

Isolation of Individual Sterols—After GLC analysis of the total sterols, the mixture was acetylated (20 parts pyridine and 20 parts acetic anhydride) at room temperature overnight. The remaining acetic anhydride was destroyed with excess methanol, and the mixture was poured onto ice and extracted with ether. The acetates were subjected to the exact preparative TLC conditions described for obtaining the sterols from the crude lipid, except that not more than 0.025 g of the mixture was applied per plate. The steryl acetates separated into three bands. The least polar top band (R_f 0.90–0.97) contained the major portion with two minor bands below (R_f 0.75–0.79 and R_f 0.67–0.72). After scraping and extracting the adsorbent, the composition of the bands was monitored by GLC.

The top band was subjected to a further preparative TLC separation using a system in which the silica gel $HF_{254\&366}$ was impregnated with silver nitrate (50 g silica gel–10 g silver nitrate). The elution solvent mixture was hexane–benzene (5:3). The bands on these silver nitrate plates were easily visualized under longwave UV light so spraying with sulfuric acid was not re-

[1] The samples extracted were obtained from the Gulf of St. Lawrence through the courtesy of Mr. J. Claude Wallace, Marine Plants Experimental Station, Miminegash, Prince Edward Island. The plants were dried and sorted from foreign material prior to grinding and extraction.

[2] F&M 700.

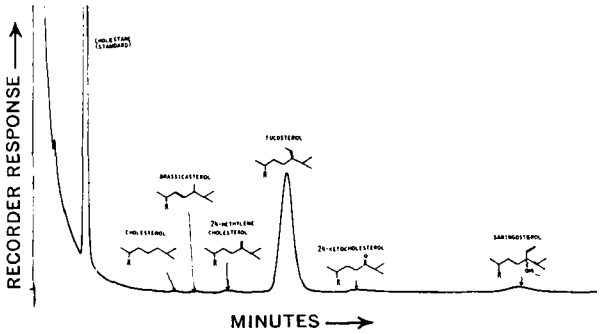


Figure 4—*GLC separation of* A. nodosum *sterols on 1.5% OV-17.*

quired. The bands were treated as already described, and GLC indicated complete separation.

Mass spectra were then recorded[3]. Melting points and IR spectra were consistent with those reported for the individual compounds.

RESULTS AND DISCUSSION

In the figures, the structure of R is as shown in Structure I, with attachment of the side chain at position 17.

Phaeophyta—*Laminaria saccharina* was found to contain 0.20% sterols. GLC showed the relative sterol composition to be: fucosterol, 87%; 24-methylenecholesterol, 11%; cholesterol, 0.05%; 24-ketocholesterol, 0.05%; and saringosterol, 1.8% (Fig. 1). Following isolation by preparative TLC, mass spectral analyses were obtained for the various sterols. The mass spectrum of the isolated saringosterol (Fig. 2) compared favorably with the previously reported fragments (11, 21). The spectrum of 24-ketocholesterol (Fig. 3) was identical to that of the authentic compound synthesized from fucosterol in this laboratory[4].

To the knowledge of the authors, this is the first detailed sterol analysis of *L. saccharina*, although fucosterol was reported previously (22). This is also the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. These results are consistent with the sterol pattern found in other brown algae (10) and thus support the phylogenetic classification.

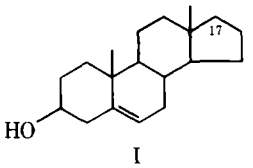
Ascophyllum nodosum was found to contain 0.10% sterols. The mixture was composed of cholesterol (0.05%), brassicasterol (<1%), 24-methylenecholesterol (2%), fucosterol (90%), 24-ketocholesterol (<1%), and saringosterol (6%) (Fig. 4). This is in good agreement with previous documentation (11).

Knights (11) suggested, upon finding 24-ketocholesterol and saringosterol in dried *A. nodosum* but not in fresh material, that these sterols might be due to aerial oxidation of fucosterol. However, 24-ketocholesterol was reported in another phaeophyte, *Pelvetia canaliculata* (23), and saringosterol was identified in several brown algae (2).

Rhodophyta—*Furcellaria fastigiata* was found to contain approximately 0.02% sterols. GLC showed cholesterol to be the major component (>99%), with minor traces of 22-*trans*-dehydrocholesterol, 24-methylenecholesterol, β-sitosterol, fucosterol, isofucosterol, and desmosterol. No further purification of the sterols was attempted because of the small amounts present.

Gibbons *et al.* (3) reported cholesterol as the only sterol present while Idler and Wiseman found 0.004% sterols, 62.7% as cholesterol and 23.2% as 24-methylenecholesterol (24). These differences may be due to the influence of seasonal collection. Significant seasonal variation in sterol content has been shown in the red alga *Rhodymenia palmata*, with varying ratios of cholesterol–desmosterol (24), and *Halosaccian*, changing from almost pure demosterol in spring to 100% cholesterol in late fall[5].

The GLC analyses of the complete sterol mixtures of the *L. saccharina* and *A. nodosum* are shown in Figs. 1 and 4. The TLC techniques employed to isolate the sterols and the GLC conditions used enabled the detection of the more polar sterols, 24-ketocholesterol and saringosterol, without the aid of column chromatography. As can be seen, the gas–liquid chromatogram readily shows the relative quantities of these rare compounds in the presence of the more common 3β-monohydroxy sterols. Preparative TLC also enabled the fast, efficient separation of each of these sterols for positive identification and possible future use.



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[3] DuPont CEC 21-110B double-focusing high-resolution mass spectrometer.

[4] Unpublished data.

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