STEROLS FROM SOME BLACK SEA ULVACEAE

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Abstract—The free and esterified sterol composition of Ulva rigida and Enteromorpha linza (Ulvaceae) from the Black Sea was investigated. The main sterol component of Ulva rigida was identified as fucosterol. The influence of the season and water pollution on the sterol composition was investigated. The origin of the C_{26}- and C_{25}-sterols and of saringasterol in these seaweeds is discussed.

INTRODUCTION

The representatives of the family Ulvaceae are among the most wide-spread species of green algae (Chlorophyta) in the Black Sea. They are represented by four genera. Ulva consists of one species, Ulva rigida, while Enteromorpha has ten species.

Ulva rigida Ag. and Enteromorpha linza (L) J. Ag. produce a substantial part of the total biomass of the benthos along the Bulgarian Black Sea coast. For this reason, they are of great interest as far as their utilization is concerned and also as an important link of the food chains in the Black Sea.

Here we report the results of our investigations on the sterol composition of U. rigida and E. linza. The sterol content of these seaweeds in other seas has been repeatedly studied and it has been found that 28-isofucosterol (1) is the main sterol in Ulvaceae, including U. rigida and E. linza. Small quantities of cholesterol (2), 22-dehydrocholesterol (3), 24-methylcholesta-5,22-dien-3-ol (4), 24-methylenecholesterol (5) and 24-ethylcholesterol (6) have also been found in Ulvaceae [1-7]. The data for the sterols found in Mediterranean U. rigida and E. linza are given in Table 1, and they are of special interest for the present study as this sea is connected with the Black Sea but differs significantly in its ecological conditions.

RESULTS AND DISCUSSION

The free sterols and sterol esters were separated by column chromatography, followed by preparative TLC on silica gel–silver nitrate. Isolated individual sterols or simple sterol fractions were investigated by GLC, GC-MS and ¹H NMR. The identified sterols and their concentrations are given in Table 1. It is evident that the percentage of the component sterols is similar to those in Ulva and Enteromorpha species from the Mediterranean [1, 2], the 24-ethylidene cholesterol being the main sterol in both algae. In all Ulvales studied up to now [1-7], as well as in other green algae, only the Z-28-isomer, i.e. isofucosterol (1) was found, while the E-28-isomer, fucosterol (7), was found exclusively in the evolutionary lower

brown algae (Phaeophyta), where it comprises more than 90% of the total sterol mixtures.

A GLC analysis of the total sterols from both algae showed that 28-isofucosterol in E. linza was the main sterol (Table 1). One of the secondary ones had the R_t of fucosterol (7). However, the main peak in the GLC trace of Ulva rigida sterols had an R_t of fucosterol (7) instead of isofucosterol (1). The mass spectra of the two isomers are identical and their chromatographic behaviour on GLC and TLC on silica gel–silver nitrate was very close. The ¹H NMR spectra of these two isomers, which is the best method for distinguishing between them revealed the characteristic difference in H-25 signals. In the fucosterol spectrum it appears at δ2.2, while in isofucosterol spectrum it is at 2.8 [8]. The ¹H NMR spectrum of 24-ethylidencholesterol isolated from E. linza indicated a 90% content of isofucosterol (1) and 10% of fucosterol (7), while that isolated from U. rigida consists on average of 80% fucosterol and only 20% isofucosterol. As the latter figures contradict those for Ulva collected in other seas we carried out an investigation on five U. rigida samples, collected at different locations and seasons. In all cases the main sterol was fucosterol (75-85% from the total 24-ethylidencholesterol). This is an important discovery, since until now isofucosterol was accepted as a taxonomic marker for Ulvales and fucosterol was regarded as evolutionary lower than isofucosterol. In some Ulva species the presense of isofucosterol was confirmed by ¹H NMR spectra [1, 2] while in other cases the C-28 stereochemistry was not absolutely proved. From our data it is reasonable to conclude that fucosterol is not a reliable marker for the evolutionary position of plant species. A recent investigation of some Indian Cassia species [9] is in support of this conclusion. Thus, Cassia fistula was found to contain 17% isofucosterol, which is quite natural for a higher plant. Cassia siamea does not contain any 24-ethylidencholesterol, and Cassia sofora contains 8.7% fucosterol and no isofucosterol.

The origin of the sterols with unusually short side chains is an obscure problem in the chemistry of marine sterols [10]. It was supposed that the C_{26}-sterol, 24-norcholesta-5,22-dien-3β-ol (8) is produced by phytoplankton [11] and it was actually found in some microalgae [11]. There are two reports for the isolation of this sterol

R =

1   5   9

2   6   10

3   7   11

4   8
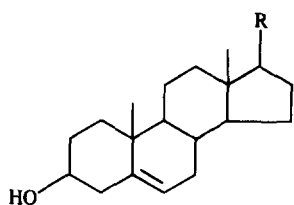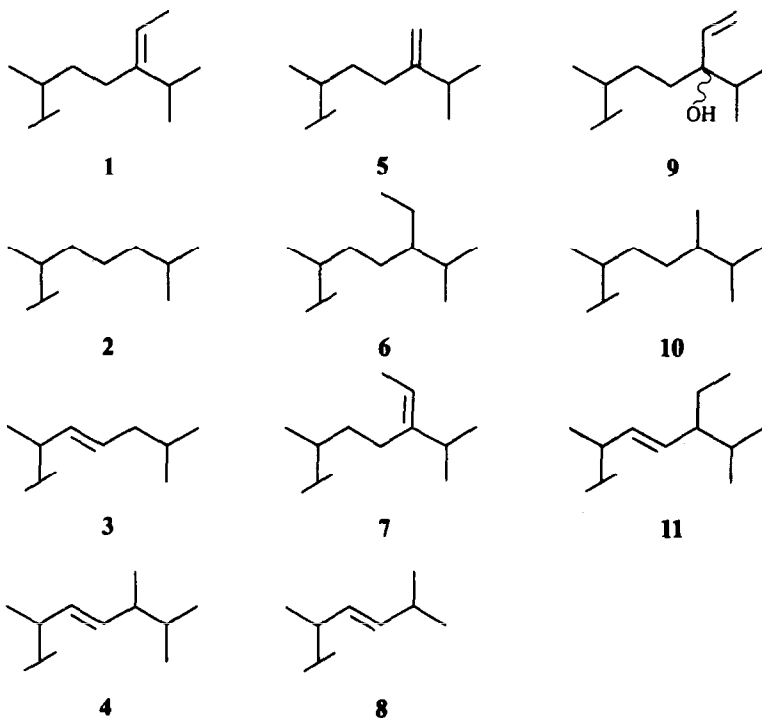
from red algae [12, 13]. In one of them labelled precursors were incorporated into all sterols with the exception of 8 [12]. Probably the season was unsuitable for the biosynthesis of this sterol or it was produced from microorganisms which are always present in the algae.

In our previous investigation on *U. rigida* sterols, we found some GLC evidence for the presence of sterol 8 and its lower homologues. The first of them was identified by GC-MS and comparison with an authentic sample. Its lower homologue (M_r 356) was a C_{25}-sterol with two double bonds, one of them at C-5 and the other in the side chain, probably at C-22. Other lower homologues were also present in the sterol mixture. The amounts of these sterols were insufficient for elucidation of their full structures.

This was the first isolation of sterols with short side chains from green algae and their total quantity was 30% from the total free sterols in these algae. Since the algae were collected in waters containing large amounts of phytoplankton, we could assume that the latter may be the source of these sterols. In this connection, we analysed three other samples of *U. rigida*, collected at different

locations, one of them from waters with a high, and the others with a very low phytoplankton content. In the last two samples phytoplankton was carefully washed from their surface. Again, the polluted sample contained about 30% sterols with short side chains, the quantity of the C_{26}-sterol being greater than in the previous sample. In the 'cleaned' samples, the quantity of the short chain sterols was very low (less than 1% from the total sterols) and even this amount could be due to some residual phytoplankton. These data showed that *U. rigida* does not contain short side chain sterols. The presence of C_{26}-sterol 8 in some red algae could be due to the same reason.

A significant influence of antropogenic pollution on the *U. rigida* sterol composition was also established. The fucosterol content in algae, collected in regions polluted with waste waters, was considerably higher (Table 1). Morphological transformations in this algae, i.e. an increase of the size and some changes in the thallus consistency, were also observed.

A compound more polar than the free sterols was also isolted from *E. linza*. Its mass spectrum showed a molecular weight of 428 and a fragmentation pattern

Table 1. Sterol compositions of *Ulva rigida* and *Enteromorpha linza* (% from the total sterol mixture)

		1	2	4	5	6	7	8	10	11	12	13
ULVA RIGIDA												
Mediterranean	FS*	80	20	tr.	—	—	—	—	tr.	tr.	—	—
Black Sea												
Summer, clear water	FS	15	17	tr.	tr.	5	63	—	tr.	tr.	—	—
	SE	18	3	tr.	tr.	4	73	—	tr.	1	—	—
	SG	1	4	14	14	—	2	—	—	—	—	—
Summer, phytoplankton polluted waters	FS	10	16	—	—	—	40	30	tr.	tr.	—	—
Summer, waste water	FS	26	1	1	tr.	—	72	—	tr.	tr.	—	—
Spring	FS	13	27	tr.	tr.	8	52	—	tr.	tr.	—	—
Spring, unusually warm waters	FS	20	1	tr.	tr.	—	79	—	tr.	tr.	—	—
	SE	12	18	tr.	tr.	tr.	46	20	tr.	tr.	—	—
	SG	2	2	—	tr.	tr.	7	—	—	—	6	84
ENTEROMORPHA LINZA												
Black Sea												
Summer, clear waters	FS	62	11	tr.	tr.	5	16	—	—	2	—	—
	SE	48	36	—	tr.	tr.	12	—	—	4	—	—
Spring	FS	56	14	—	12	tr.	15	—	—	tr.	—	—
Spring, unusually warm waters	FS	70	9	—	3	—	18	—	—	—	—	—

*FS, Free sterols; SE, sterol esters; SG, sterol glycosides.

typical for dihydroxysterols. On the basis of this fragmentation and of the ^{1}H NMR spectrum we supposed that this compound was saringasterol (9). This was confirmed by the comparison of its mass spectrum with that of an authentic sample [14]. Until now, saringasterol was found mainly in brown algae [14–16], but there were some doubts that saringasterol was an artefact, produced from fucosterol during the drying of the biomass. A few years ago, saringasterol was isolated from three *Ulva* species [7], but it was not specified whether dry or fresh algae was investigated. In our work we used fresh algae and this showed that in *E. linza* saringasterol is not an artefact. In fresh *U. rigida* we found only traces of saringasterol.

In some algae, including *Ulva gigantea* [17] a number of sterol glycosides have been isolated. In *U. rigida* we found traces of compounds with chromatographic behaviour typical of sterol glycosides. After acid hydrolysis, cholesterol and fucosterol were identified in amounts insufficient for quantitative analysis. After acid hydrolysis of more polar fractions we noted the presence of sterols in the isolated aglycones, together with compounds with a longer R_t on GLC. From the GLC data (Table 1), it was evident that there was an increase in cholesterol concentration as compared with that in the free sterol fraction. The same results were also obtained in earlier investigations [17].

There were some investigations on the seasonal variations of sterols in *U. rigida* [3, 4]. The quantity of isofucosterol attained its maximum level in winter and minimum level in spring, while cholesterol content was at its maximum during the summer. In Table 1, we show the seasonal variations in the sterol content of Black Sea *U. rigida*. In spring, the sterol composition was simpler with an increase of cholesterol. Unusually warm water in the spring of 1983 (7–8° higher than that in 1982) led to considerable changes in the sterol composition (Table 1). These differences show the big influence of sea temperature on the biosynthesis and storage of sterols in marine algae and can explain the apparently contradictory results in some earlier publications.

EXPERIMENTAL

The gas chromatograms were run on a JEOL 20K instrument with an FID and 2 m packed glass column (3 mm i.d.) with 2.8 % OV-17 on chromosorb Q. The GC-MS were run on a JEOL JMS D-300 double focusing mass spectrometer with a JEOL JMA data aquisition and processing system. A JEOL 20K gas chromatograph was coupled via an all glass jet separator and a 4 m packed glass column (3 mm i.d.) with 2.8% OV-17 on chromosorb Q was used. The EI conditions were: 70 eV energy, 300 A ionizing current, ionization chamber temperature 200°. ^{1}H NMR spectra were measured on TESLA 60 MHz, internal standard TMS.

Ulva rigida and *Enteromorpha linza* were preserved in the herbarium of the Pharmaceutical Faculty, Academy of Medicine, and identified by S. Dimitrova-Konaklieva from the same faculty. Algae were collected in August 1981, 1982 and 1983 near the cape of Kaliakra and near the Kitten village; *U. rigida* samples polluted with phytoplankton were collected in 1978 and 1980 near Sozopol, and those polluted with antropogenic waters in 1981 near Nessebar. The spring samples were collected in April 1982 and 1983, near the cape of Kaliakra and near Kitten village.

The fresh algae were cut up and extracted twice with MeOH and finally once with $CHCl_3$. The combined extracts were concd and lipids were extracted twice with $CHCl_3$. They were chromatographed on a silica gel column and sterol esters and free sterols were eluted with mixtures of petrol-Et_2O with a gradual increase of the latter. The sterol esters were hydrolysed by refluxing with 2 N KOH in EtOH for 2 hr, and free sterols were

isolated by preparative TLC on silica gel G (petrol–Et$_2$O, 1:1). The free sterols were subjected to preparative TLC on silica gel G/16% AgNO$_3$ with a 48 hr continuous development with petrol–CHCl$_3$ (93:7). The sterols obtained were analysed by GLC. The results given in Table 1 are average values.

When lipids from *E. linza* were chromatographed on a silica gel column, there was one main component in the fractions eluted after the free sterols. This compound was isolated in a pure state by preparative TLC (petrol–Et$_2$O–Me$_2$CO, 8:4:1) and was 0.3% of the total lipids. It was identified as saringasterol. MS *m/z* (rel. int.): 428 [M]$^+$ (10), 410 (21), 395 (10), 385 (19), 367 (71), 349 (36), 311 (19), 270 (60), 254 (36), 212 (24), 100 (100). $[\alpha]_D^{20} = -19,10°$ (CHCl$_3$); lit. data $[\alpha]_D^{20} = -31°$ [14].

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