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A comparative study on the sterol composition of some brown algae from the Black Sea*

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Abstract: The sterol composition of the brown algae *Stilophora rhizodes* (Turner) J. Agardh, *Punctaria latifolia* Grev. and *Punctaria plantaginea* (Roth.) Grev. from the Black Sea was investigated. Fifteen sterols were identified in the sterol fractions. The main ones were cholesterol and 24-methylenecholesterol. Characteristic for brown algae, fucosterol was present in low concentrations. The results obtained were compared with recent data for the sterol composition of other Black Sea brown algae. Some conclusions concerning the evolutionary position of brown algae are made.

Keywords: Algae, *Stilophora rhizodes*, *Punctaria latifolia*, *Punctaria plantaginea*, sterols, chemoevolution.

INTRODUCTION

There are 36 species of brown algae at the Bulgarian shore of the Black Sea.[1] Till now there have been only limited investigations on their chemotaxonomy and chemoevolution. Lipids,[2] sterols[3,4] and polysaccharides[5] have been used as basis for taxonomic conclusions concerning brown algae. Recently we showed that the composition of the volatile compounds, obtained by distillation-extraction, could give some extra information concerning taxonomic and evolutionary problems in brown algae (unpublished results) but the data is still incomplete.

It is accepted that different algal classes possess characteristic sterol composition. Compared to brown algae, red algae (Rhodophyta) are considered evolutionary less advanced. Cholesterol and, in some cases, its biogenetic precursor cholesta-5,24(25)-dien-3β-ol (desmosterol) dominate in the evolutionary lower red algae.[3,6,7] Some of the evolutionary higher red algae contain 24-methyl-cholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol), which is the precursor of the sterols alkylated at C-24. The C-24 alkylated

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sterols and the sterols with a C-22 double bond are in low concentrations.[8] 5α-Chlolestanol was found in low concentrations in some red algae.

The main sterol in brown algae (Phaeophyta) is fucosterol.[6,7] It is biosynthesized through alkylation of 24-methylenecholesterol.

The alkylation of 24-methylenecholesterol in the evolutionary lower green algae (Chlorophyta) leads to the production of an isomer of fucosterol, called isofucosterol, while in the evolutionary advanced green algae and in higher plants the alkylation and further reduction of the C-24(28) double bond leads to an accumulation of sitosterol.[9,10]

In this study, for the first time, the sterol composition from three evolutionary lower species of brown algae: *Stilophora rhizodes* (Turner) J. Agardh (family Spermatochnaceae, order Chordariales), *Punctaria latifolia* Grev. and *Punctaria plantaginea* (Roth.) Grev. (family Punctariaceae, order Dictyosiphonales) from the Black Sea have been analyzed and compared to the sterol composition of prevously investigated Black Sea brown algae, which are at different evolutionary level: *Striaria attenuata* (Grev.) Grev. (family Striariaceae, order Dictyosiphonales), *Scytosiphon lomentaria* (Lyngb.) Link. and *Colpomenia peregrina* (Sauv.) Hamel (family Scytosiphonaceae, order Scytosiphonales), *Zanardinia prototypus* (Nardo) Nardo (family Cutleriaceae, order Cutleriales), *Cystoseira barbata* (Good. et Wood) Ag. and *Cystoseira crinita* (Desf.) Bory (family Cystoseiraceae, order Fucales).

According to the classification of Lobban and Wynne, the orders investigated are arranged in the following evolutionary sequence: Chordariales (evolutionary least advanced), Dictyosiphonales, Scytosiphonales, Cutleriales, Fucales.[11]

The only chemical analysis of *Stilophora rhizodes* concerns its polysaccharide composition.[12] The polysaccharide composition of *Punctaria latifolia*[13] and *Punctaria plantaginea*[5] was also studied. *Punctaria latifolia* was investigated for its amino acid content.[14]

EXPERIMENTAL

Plant material

The three investigated samples were collected from the south part of the Bulgarian coast of the Black Sea: *Stilophora rhizodes* – in the area of Sinemoretz, *Punctaria latifolia* – in the area of Varvara and *Punctaria plantaginea* – in the area of Resovo. The distance between the collection sites is less than 20 km. All the samples were collected in May 2001.

Voucher-specimens were determined by Dr. Stefka Dimitrova-Konaklieva and deposited in the herbarium of the Faculty of Pharmacy, Medical University, Sofia.

Extraction

Each sample: 32.3 g (dry weight) from *Stilophora rhizodes*, 36 g (dry weight) from *Punctaria latifolia* and 29.5 g (dry weight) from *Punctaria plantaginea* was homogenised in methanol and consecutively extracted with 500 ml methanol, 500 ml methanol–chloroform (1:1) and 500 ml chloroform. The extracts were combined and 250 ml water was added to each sample. The chloroform extracts were removed. Yields of the chloroform extracts: *Stilophora rhizodes* 0.82 g, *Punctaria latifolia* 1.1 g, *Punctaria plantaginea* 0.8 g.

Isolation and analysis of the sterols

The chloroform extract of each alga was evaporated under reduced pressure at 40 ºC. Part of the dry residues (185 mg from *Stilophora rhizodes*, 275 mg from *Punctaria latifolia* and 180 mg from *Punctaria*

plantaginea) were subjected to silica gel column chromatography (1:40). The column was eluted with 100 ml petroleum ether, followed by 100 ml petroleum ether – acetone (15:1), 100 ml petroleum ether : acetone (10:1), 200 ml chloroform, 100 ml chloroform : methanol (99:1), 100 ml chloroform : methanol (97:3), 100 ml chloroform : methanol (4:1), 100 ml chloroform : methanol (3:1), 100 ml chloroform : methanol (1:1), 200 ml methanol.

The fractions containing sterols (eluted with petroleum ether : acetone (10:1), chloroform and chloroform : methanol (99:1)) were identified by thin layer chromatography (TLC) on silica gel G. Further purification by preparative TLC on silica gel G (petroleum ether : acetone (10:1) as the solvent system) was performed. The total sterol mixture was investigated by gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS).

GC: A Pye Unicam 304 (Scientific Instrument Company of Philips, Cambridge, England) equipped with a flame ionization detector (FID) and a capillary column SPB-1 (30 m × 0.32 mm, 0.25 μm film thickness, Supelco Park, Bellefonte, PA, USA) was used. The temperature programme was 230 ºC – 300 ºC at a rate of 4 ºC min^{-1} and a 10-min hold at 300 ºC. The injector temperature was 300 ºC and the detector temperature 320 ºC. The carrier gas was N_2.

GC/MS: A Hewlett Packard 6890 + MS 5973 (Hewlett Packard, Palo Alto, California, USA) with a capillary column SPB-50 (30 × 0.32 mm, 0.25 μm film thickness, Supelco Park, Bellefonte, PA, USA) was used. The carrier gas was helium and a temperature programme of 270 ºC – 290 ºC at a rate of 4 ºC min^{-1} and a 20-min-hold at 290 ºC were used. The ion source was set at 250 ºC and the ionisation voltage was 70 eV.

RESULTS AND DISCUSSION

The results obtained from the analysis of the sterol fractions are summarized in Table I. The sterol composition from the three species investigated has not been analyzed until now. Data concerning the sterol composition of Black Sea brown algae previously investigated are also included in Table I in order to compare their sterol composition with those investigated in this work.

The main sterol in *Stilophora rhizodes* is 24-methylenecholesterol, which is a biogenetic precursor of the at C-24 alkylated sterols. As was mentioned earlier, this sterol plays an important role in the biogenesis of plant sterols and is characteristic for some of the evolutionary advanced red algae.[3] The concentration of cholesterol, which is a sterol typical for red algae,[3,6,7] is also significant (11.1 %). The for brown algae characteristic fucosterol[6,7] is only at a low concentration (4.6 %). The other C_{29}-sterols are also only present at low concentrations. This shows that the process of alkylation of 24-methylenecholesterol, leading to the production of fucosterol, is suppressed in *Stilophora rhizodes*. Such an inhibition of the alkylation is characteristic only for red algae. This result could be explained by the lower evolutionary position of *Stilophora rhizodes*. The presence in this alga of 24-methyl-cholesta-4,24(28)-dien-3-one, 24-methyl-cholest-22-en-3-one and cholest-4-en-3-one is also of interest, because steroidal ketones are rarely found in algae.

There is a similarity in the sterol composition in the two *Punctaria* species investigated. Analogous to *Stilophora rhizodes*, the sterol composition appears to be much closer to that of red algae than to that of brown ones. The main sterol is cholesterol (45 % in *Punctaria plantaginea* and 76 % in *Punctaria latifolia*). The biogenetic precursor of C-24 alkylated sterols, 24-methylenecholesterol, is in high concentrations in *Punctaria plantaginea* (28.6 %), but not in *Punctaria latifolia*. Sterols with a saturated ring system (5α-stanols) are found in low concentrations in red algae. These stanols are not characteris-

TABLE I. Sterol composition (% of the total sterol mixture).

Steroids	*S. rhizodes*	*P. latifolia*	*P. plantaginea*	*S. attenuata*	*S. lomentaria*	*C. peregrina*	*Z. prototypus*	*C. barbata*	*C. crinita*
24-nor-Chol-5-en-3β-ol	–	–	–	–	–	–	< 0.1	–	–
24-nor-Cholesta-5,22-dien-3β-ol	–	–	–	< 0.1	–	–	–	–	–
24-nor-Cholest-22-en-3β-ol	–	0.7	–	–	–	–	–	–	–
27-nor-24-Methyl-cholesta-5,22-dien-3β-ol or (22*Z*)-cholesta-5,22-dien-3β-ol	–	–	3.9	–	–	–	–	–	–
(22*E*)-Cholesta-5,22-dien-3β-ol	< 0.1	< 0.1	2.6	–	–	–	< 0.1	–	–
(22*E*)-Cholest-22-en-3β-ol	–	0.7	–	–	–	–	–	–	–
Cholest-5,24(25)-dien-3β-ol	–	–	–	–	–	–	< 0.1	–	–
Cholesterol	11.1	76.0	45.0	22.0	2.0	26.0	6.0	2.2	2.4
5α-Cholestan-3β-ol	< 0.1	< 0.1	< 0.1	–	–	–	–	–	–
24-Methyl-cholesta-5,22-dien-3β-ol	–	1.2	13.0	2.0	< 0.1	1.0	< 0.1	< 0.1	2.2
5α-Cholest-7-en-3β-ol	–	2.7	–	–	–	–	–	–	–
24-Methyl-cholest-5-en-3β-ol	–	–	1.3	< 0.1	–	4.0	< 0.1	–	–
24-Methylcholest-22-en-3-one	< 0.1	–	–	–	–	–	–	–	–
24-Methyl-cholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol)	73.2	6.1	28.6	18.0	15.0	45.0	5.0	2.4*	3.7*
24-Methyl-cholest-24(28)-en-3β-ol	–	3.5	–	–	–	–	–	–	–
Cholest-4-en-3-one	< 0.1	–	–	–	–	–	–	–	–
24-Ethyl-cholesta-5,24(28)*E*-dien-3β-ol (fucosterol)	4.6	–	–	16.0	76.0	15.0	85.0	95.4	61.2
24-Ethyl-cholesta-5,24(28)*Z*-dien-3β-ol (isofucosterol)	–	–	–	6.0	–	–	1.0	–	–
24-Ethyl-cholesta-5,22-dien-3β-ol	–	–	–	11.0	3.0	–	2.0	< 0.1	25.4
24-Ethyl-cholest-5-en-3β-ol	4.6	2.4	–	16.0	–	10.0	–	< 0.1	< 0.1
24-Methyl-cholesta-4,24(28)-dien-3-one	6.4	–	–	–	–	–	–	–	–

*a sum in % concentration of 24-methyl-cholesta-5,24(28)-dien-3β-ol and 24-methyl-cholest-5-en-3β-ol

tic for brown and green algae but were detected in traces in the three species investigated. Higher concentrations of sterols with a saturated ring system were found in *Punctaria latifolia*. Such sterols are of practical value, because they possess anticancer activity.[15] Some of the stanols possess a double bond in the side chain. Almost every sterol with a double bond at C-5 has a corresponding stanol possibly produced through reduction in the organism. The possiblity for such a reduction is supported by the identification of the rare sterol 24-nor-cholest-22-en-3β-ol in *Punctaria latifolia*. This sterol could be obtained through a supposed biological reduction of 24-nor-cholesta-5,22-dien-3β-ol, a sterol characteristic for the phytoplankton but not for the macroalgae. Maybe the latter sterol has been absorbed by the alga and subjected to a biological reduction leading to the production of the corresponding 5α-stanol (24-nor-cholest-22-en-3β-ol). Such a process has not been observed in brown algae, so its mechanism is of interest. Certain information is given by the identification in *Punctaria latifolia* of an isomer of cholesterol with a double bond at C-7 instead of C-5. During transformation of the double bond from C-5 to C-7 stanols are produced as intermediates[16,17] and this could explain the identification of a reasonable number of stanols in that alga.

The observed results could be explained with the lower evolutionary position of the three investigated algae. This is in accordance with the evolutionary scheme of Lobban and Wynne.[13] The three investigated algae might be considered close to red algae in terms of their high content of cholesterol and its derivatives and to brown algae in terms of their content (especially in *Stilophora rhizodes* and *Punctaria plantaginea*) of methylated at C-24 sterols. The higher concentration of cholesterol, the lower content of alkylated sterols and the presence of a whole series of 5α-stanols in *Punctaria latifolia* compared to *Punctaria plantaginea* are an indication that of the two *Punctaria* species, *P. latifolia* is at a lower evolutionary level. An earlier analysis of the volatile components of these three species (unpublished results) also supports this observation.

In order to create a more detailed picture for the application of the sterol composition in chemoevolution of brown algae, the data for the sterol composition of some earlier investigated Black Sea brown algae were also used for comparisons. The earlier investigated *Striaria attenuata*[4] belongs to the same order, Dictyosiphonales, as the *Punctaria* species. The concentration of cholesterol (22.0 %) and 24-methylenecholesterol (18.0 %) in *Striaria attenuata* is lower compared to the *Punctaria* species. Reasonable concentrations of at C-24 alkylated sterols, such as fucosterol (18.7 %), stigmasterol (13.0 %) and sitosterol (18.7 %), were found but compared to the evolutionary higher algae these concentrations appeared to be relatively low.

The proposed method for investigating algal evolution through their sterol composition is confirmed by the data of the sterol composition of *Colpomenia peregrina* and *Scytosiphon lomentaria*.[18] They are accepted to be evolutionary more advanced than *Stilophora rhiz*odes, *Striaria attenuata* and the *Punctaria* species.[11] This is in accordance on the one hand with the higher concentrations of at C-24 alkylated sterols (24-methylenecholesterol, fucosterol and sitosterol) and on the other hand with the lower content of cho-

lesterol, found in *Colpomenia peregrina*.[18] The sterol composition of *Scytosiphon lomentaria* seems to be characteristic for a more advanced alga than the ones investigated. The content of fucosterol is 76 %. Only the relatively high concentration of 24-methylenecholesterol (15 %) is an indication that this alga is not that evolutionary advanced.[18]

The evolutionary higher brown algae *Zanardinia prototypus*,[4] *Cystoseira barbata* and *Cystoseira crinita*[19] contain more than 80 % of C_{29}-sterols from the total sterol mixture. Fucosterol is the main sterol in all three species and very low concentrations of sterols containing less than 29 carbon atoms were detected.

It is logical to expect that sterols, as important cell mumbrane constituents, would change their composition at different environmental conditions in order to fulfil their functions. In order to clarify this problem, the sterol composition of *Cystoseira crinita* from two different seas: the Black Sea and the Mediterranean Sea, which differ strongly in salinity and water temperature, were compared.[20] While the fucosterol concentrations in the two samples were very close, in the Mediterranean sample there was no stigmasterol. Instead of this sterol the concentrations of cholesterol and 24-methylenecholesterol were significantly increased. The increase of cholesterol reduces the cell membrane permeability and this is probably connected with the elevated salinity in the Mediterranean Sea, but more investigations must be performed to support this hypothesis.

It can be concluded that the sterol composition of brown algae could be used to reach some evolutionary conclusions, but it is necessary to support the results obtained by other analyses, such as the analysis of the volatiles and the analysis of the *n*-butanol fraction, in order to confirm the observations made.

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ИЗВОД

УПОРЕДНА СТУДИЈА МРКИХ АЛГИ ИЗ ЦРНОГ МОРА НА САСТАВ СТЕРОЛА

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У овом раду проучаван је састав стеролских фракција изолованих из мрких алги *Stilophora rhizodes* (Turner) J. Agardh, *Punctaria latifolia* Grev. и *Punctaria plantaginea* (Roth.) Grev. сакупљених из Црног мора. Изоловано је петнаест стерола, од којих су главни чиниоци холестерол и 24-метиленхолестерол, док је стероид карактеристичан за мрке алге, фукостерол, изолован у малој концентрацији. Подаци добијени у овом раду упоређени су са скорим резултатима проучавања састава стеролских фракција из других мрких алги Црног мора. Изнета су нека запажања у вези са еволуционим положајем мрких алги.

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