Sterol and Lipid Composition of Three Adriatic Sea Sponges

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The sterol and fatty acid composition of three Adriatic Sea sponges (*Geodia cydonium* and two unidentified *Tedania* sp.), collected at the same time and same place, was established. Twenty-four sterols and forty fatty acids were identified. The identical ecological conditions, including the diet, allowed us to apply the results obtained for taxonomical conclusions, based on the biodiversity of the investigated sponges. On the basis of the sterol composition they can be separated into two groups: *Tedania* and *Geodia* sponges. The sterol and fatty acid composition indicates that the two investigated *Tedania* samples might be different species or subspecies.

Key words: Fatty Acids, Adriatic Sea Sponges, Sterols

Introduction

Sponges are between the evolutionary most primitive animals in the world, being the first multicellular animals. In animals, the sterols have two important functions – serve as precursors of some hormones and are one of the main constituents of the lipid cell membrane. The terrestrial organisms possess a simple sterol composition, 3–6 sterols, one of them accounts for more than 70% from the total sterols (cholesterol in animals, sitosterol in plants and ergosterol in fungi). In marine organisms such simple composition possesses only part of the evolutionary advanced invertebrates, and in particular the marine sponges are characterized by a very complex sterol composition, largely derived from the food. Sponges are filter feeders, using plankton and detritus for food. The significant diversity of sterols in sponges can be due to specific requirements of their cell membranes, which will depend partially on the environmental factors: salinity, water temperature, pollution, etc., as well as on the diet.

Sponges are characterized by a wide diversity in their fatty acid (FA) composition. Contrary to plants and animals, which contain FAs with up to 20 carbon atoms, marine sponges often contain FAs with longer chains up to 30 carbon atoms. Sponge FAs often contain novel patterns of branching and desaturation, which could be connected with their specific cell membrane requirements.

We investigated three sponges (*Geodia cydonium*, *Tedania* 1 sp. and *Tedania* 2 sp.) collected in autumn from Adriatic Sea at the same location. This means that the effect of the environment must be identical for the three sponges investigated. In addition, the sterol and lipid composition of their diet must be very similar, so we can expect that the three sponges might have similar sterol and fatty acid composition. If there are significant differences, they must be due to some dietary preferences (*e.g.* size of plankton organisms or their chemical composition) or due to some transformations of dietary sterols and lipids in the organism of the sponges. In the both cases, the obtained differences will be connected with the biodiversity of the sponges and can be used for taxonomical conclusions, characterizing the corresponding species.

Geodia cydonium Fleming belongs to the class Demospongiae, order Astrophorida, family Geodiidae. There are very limited investigations on the sterol composition of sponges in the family Geodidae. *G. megastrella* from Labrador was investigated for its sterol composition (Kingston *et al.*, 1979); the main component was 24-methyl-cholesta-5,24(28)-dien-3β-ol (72% from the total sterols), while cholesterol was present in unusually low content (9%). Geodisterol, a trihydroxy sterol

with an aromatic A ring was isolated from *Geodia* sp. (Wang and Crews, 1996). Steroidal ketones were found in *G. cydonium* (Migliuolo *et al.*, 1990). Sterols and some rare fatty acid were identified in *Geodinella robusta* (Makarieva *et al.*, 2002), a related genus of *Geodia.* There are no studies on the lipid composition of the sponge *G. cydonium.*

Tedania sp. belongs to the class Demospongiae, order Poecilosclerida, family Tedaniidae. There are only two reports on the sterol and lipid composition of sponges of genus *Tedania.* Thirteen sterols were identified from the sponge *T. excavata* (Seldes *et al.*, 1988). Among them, Δ^5-sterols were the principal components (cholesterol about 60% from the total sterols) with traces of stanols. *T. ignis* was investigated for its sterol and phospholipid fatty acid composition (Carballeira and Maldonado, 1987); the major sterols were cholesterol (70%) and sitosterol (11%), while palmitic (16:0), behenic (22:0) and 5,9-hexacosadienoic (26:2) acid were the main fatty acids.

The present paper describes studies on sterol and fatty acids composition of three sponges from the Adriatic Sea.

Materials and Methods

Collection of sponges

Geodia cydonium, Tedania 1 sp. and *Tedania* 2 sp. were collected by dredging (at a depth of 20 m) near Rovinj, Croatia, in October 2001, and frozen at – 20 °C until extracted. *Geodia cydonium* Fleming (Geodiidae, Astrophorida, Demospongiae) shows a globular form with a light gray color (voucher No. S3R/01). *Tedania* 1 sp. shows a slippery surface due to mucus and a dark blue color (voucher No. S5R/01). *Tedania* 2 sp. shows a smooth surface and a brown color (voucher No. S6R/01). Both *Tedania* samples are lobulate with large oscula, soft and fragile. Professor R. Pronzato of the DIP.TE.RIS dell'Università Di Genova, Italy identified all specimens. Voucher specimens are maintained in the ICB-CNR collection.

Extraction and isolation of sterols and fatty acids

The three sponges (140, 70 and 50 g dry weight after extraction, from *G. cydonium, Tedania* 1 sp. and *Tedania* 2 sp., respectively) were consecutively extracted with methanol, methanol/chloroform (1:1 v/v) and chloroform. The extracts were combined, 300 ml water were added and the chloroform layers were removed. The water/methanol layers were re-extracted with 350 ml *n*-butanol (yields: 880, 800 and 580 mg dry chloroform extracts, and 220, 990 and 750 mg dry butanol extracts from *G. cydonium, Tedania* 1 sp. and *Tedania* 2 sp., respectively).

The chloroform extracts were evaporated under reduced pressure at 40 °C. Portions from the dry residues (400 mg each) were subjected to column chromatography on silica gel (1:75). Light petroleum ether followed by light petroleum ether/acetone mixtures, chloroform and chloroform/methanol mixtures in ascending polarity were used as eluents. The fractions containing sterols [those eluted with petroleum ether/acetone (10:1 v/v) and chloroform] were combined and purified by preparative thin layer chromatography (TLC) on silica gel G with light petroleum ether/acetone (10:1 v/v). The sterol fractions (21, 30 and 64 mg from *G. cydonium*, *Tedania* 1 sp. and *Tedania* 2 sp., respectively) were investigated by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

Quantitative analyses were performed on a Pye Unicam 304 gas chromatograph equipped with a FID and a 30 m × 0.32 mm i.d., 0.25 μm film thickness fused silica capillary column SPB-1 at 230 °C, programmed to 300 °C at 4 °C/min with a 10 min hold. Injector and detectors temperatures were at 300 °C.

GC/MS analyses were performed on a Hewlett Packard 5973 mass spectrometer coupled with a 6890 series II plus gas chromatograph, equipped with a capillary column SPB-50 (30 m × 0.32 mm i.d., 0.25 μm film thickness). The MS source was at 250 °C and the ionization voltage at 70 eV. The GC oven temperature was at 270 °C, programmed to 290 °C at 4 °C/min with a 20 min hold.

Part of the chloroform extracts (100 mg each) was esterified with 5 ml of 15% acetyl chloride in dry methanol at 55 °C for 3 h. The fatty acid methyl esters (FAMEs) were purified by TLC on silica gel G with petroleum ether/acetone 95:5 (v/v) (yields: 27, 14 and 35 mg from *G. cydonium*, *Tedania* 1 sp. and *Tedania* 2 sp., respectively) and analyzed by GC and GC/MS.

Quantitative analyses were performed by GC with a capillary column SP–2340 (30 m × 0.25 mm i.d.) at 150 °C, programmed to 210 °C at 4 °C/min with a 10 min hold. Injector and detectors temperatures were at 250 °C.

GC/MS analyses of FAMEs mixture were carried out with a fused silica capillary column CP-

Wax 52 CB (30 m × 0.25 mm i.d. × 0.20 μm film thickness) at 165 °C, programmed to 240 °C at 4 °C/min with a 30 min hold.

Identification of compounds by GC/MS analyses

The identification was accomplished using computer searches on a NIST98 MS Data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass spectral fragmentation. When possible reference compounds were co-chromatographed to confirm GC retention times especially when isomeric compounds have similar spectra. Only the unambiguously identified compounds were reported in the tables.

Results and Discussion

The investigations were performed mainly by GC/MS, which is a very suitable method for investigation of complex mixtures of organic compounds. The problem is that the ion currents generated depend on the characteristics of the investigated compounds and are not a true quantification. For this reason, the GC/MS analyses do not give exactly quantitative data. The data obtained from GC/MS only can be used for the characterization of the biodiversity and for comparisons between the same groups of compounds in different organisms. The results obtained can characterize the investigated organisms, indicate the presence of known biologically active compounds and allow some conclusions for the chemotaxonomy and chemoevolution of the organisms. Additionally we performed a quantitative analysis of sterols and fatty acid methyl esters, using gas chromatography.

Sterol composition

The sterols were investigated by GC/MS (qualitative analysis) and by GC (qualitative and quantitative analyses). The data are reported in Table I. It is evident that the investigated sponges, together with the investigated earlier *Geodia* and *Geodinella* species, can be separated into two groups due to their sterol composition. In the first one we include the two *Geodia* samples (*Geodia cydonium* and *Geodia megastrella*) together with *Geodinella robusta*, while in the second group are included the two *Tedania* samples. The sponges from both groups contain the usual complex mixtures of sterols for marine invertebrates, all of them identified earlier in different sponges.

The sterol composition of *Geodia* is characterized by unusually low contents of C_{27} sterols, while in *Tedania* sp. the same sterols are more than 50% of the total sterols. In *Geodia* dominated C_{28} sterols, which are in much lower contents in *Tedania* sponges. In all cases, the contents of C_{29} sterols are the lowest and are similar in all sponges.

The main sterol in *G. cydonium* is 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylene cholesterol), while in the second group cholesterol is the main sterol.

Only in rare cases the sponge biosynthesized small amounts of sterols *de novo.* Almost all sterols come from the diet and they can be incorporated in the cell membranes unchanged or they can be transformed by the sponges or by associated bacteria or unicellular algae, in order to satisfy the specific cell membrane requirements of the sponge.

Since the sponges were collected at the same time and place, the environmental conditions are identical (water temperature, salinity, diet, pollution, etc.), so there must be no specific requirements of the cell membranes, leading to changes in the sterol composition. This is in agreement with the typical sterol composition for most of the marine invertebrates, which we obtained.

Most of the identified sterols are present in both investigated groups of sponges, but their contents varied. Some sterols are characteristic for the phytoplankton (mainly alkylated sterols), while cholesterol and similar sterols are characteristic for zooplankton and sometimes for some unicellular algae (Dinoflagellates, Diatomea) (Elyakov and Stonik, 1988; Goad, 1978). The differences obtained are characteristic for the both investigated groups of sponges and can be used for chemotaxonomic conclusions.

Contrary to many sponges, the two *Tedania* sp. do not transform the dietary sterols. To some extent, the differences in the sterol composition obtained might be due to some dietary preferences, zooplankton for *Tedania* sp. and phytoplankton for *Geodia.* The unusually high contents of 24-methyl-cholesta-5,24(28)-dien-3β-ol in *Geodia* may be due to the significant portion of phytoplankton in their diet (there are some phytoplankton species in which this sterol is the main one) (Elyakov and Stonik, 1988; Goad, 1978), but we can not exclude the role of 24-methyl-cholesta-5,24(28)-dien-3β-ol as a precursor of C_{29} ster-

Table I. Main sterols from three Adriatic Sea sponges (% from the total sterols).

Sterols	*G. cydonium*	*Tedania* 1 sp.	*Tedania* 2 sp.
24-Norcholest-5-en-3β-ol	–	–	0.2
24-Norcholesta-5,22-dien-3β-ol	0.4	0.1	2.6
24-Norcholest-22-en-3β-ol	0.1	–	–
Cholesta-5,22-(*Z*)-dien-3β-ol (ocellasterol)	2.0	3.6	4.2
Cholesta-5,22-(*E*)-dien-3β-ol	3.5	5.3	7.3
Cholesta-22-(*E*)-en-3β-ol	0.2	–	–
Cholest-5-en-3β-ol	13.5	48.1	33.7
5α-Cholestan-3β-ol	2.0	–	–
Cholest-4-en-3-one	–	–	0.9
22,23-Cyclopropyl-cholest-5-en-3β-ol	–	–	2.0
24-Methyl-cholesta-5,22-dien-3β-ol	9.2,	12.6	13.8
24-Methyl-cholesta-5,24(28)-dien-3β-ol	42.0	7.7	0.9
24-Methyl-cholest-22-en-3β-ol	0.5	–	–
24-Methyl-cholest-24(28)-en-3β-ol	3.0	–	–
23-Methyl-cholest-5-en-3β-ol	–	–	8.8
24-Ethyl-cholesta-5,22-dien-3β-ol	4.0	2.2	0.3
24-Ethyl-cholesta-5,23-dien-3β-ol	–	1.5	–
24-Ethyl-5α-cholest-22-en-3β-ol	0.2	–	–
24-Ethyl-cholest-5-en-3β-ol	13.9	10.7	13.8
24-Ethyl-5α-cholestan-3β-ol	2.0	–	–
24-Ethyl-cholesta-5,24(28)-(*E*)-dien-3β-ol	0.1	–	0.1
24-Ethyl-cholesta-5,24(28)-(*Z*)-dien-3β-ol	1.0	2.5	2.1
24-Ethyl-cholesta-7,25-dien-3β-ol	1.5	–	–
24-Ethyl-5α-cholestan-24(28)-(*E*)-en-3β-ol	0.1	–	–
C_{26} sterols	0.5	0.1	2.8
C_{27} sterols	21.2	57.0	46.1
C_{28} sterols	54.7	20.3	25.5
C_{29} sterols	22.8	16.9	16.3

Values are given as the mean of three measurements. The standard deviations (related to peak proportion on the chromatograms) are ± 0.1 for compounds with a percentage > 10 and ± 0.05 for the others.

ols, which were identified in the investigated sponges. The transformation of 24-methyl-cholesta-5,24(28)-dien-3β-ol into C_{29} sterols could proceed with different intensity in the two groups of sponges investigated and this will lead to accumulation of this sterol in the *Geodia* sp. A significant amount of 24-ethyl-cholest-5-en-3β-ol in both groups of sponges is an indication of the presence of green macro algae detritus (Elyakov and Stonik, 1988; Goad, 1978).

Further differences observed in the two groups of sponges are the low relative content of stanols in *G. cydonium*, and the complete absence of these sterols in the investigated *Tedania* samples. Because of the complete absence of stanols in most of the marine plankton organisms (Elyakov and Stonik, 1988; Goad, 1978) we might assume that the reduction of the double bond at C-5 was performed in the organism of *G. cydonium*.

While the differences in the sterol composition of the two investigated groups of sponges are clear and we can distinguish between them, the differences between the two investigated samples of *Tedania* are much smaller. The biologists accept the two samples as identical organisms, in spite of the differences of surface and color. These differences are an indication that there are differences in the metabolism of these sponges. The composition of the main sterols is quantitatively and qualitatively similar in both *Tedania* samples, but there are differences in some minor sterols. The introduction in the molecule of a C-23 methyl group and a 22,23-cyclopropane ring, the oxidation of 3-OH to a keto group and the relatively higher content of 24-nor-sterols are characteristic only for the *Tedania* 2 sp. For this reason, we propose that the two *Tedania* sp. belong to different chemoraces or even to different subgenera or genera. Based on these results we suggest that the sterol analysis of sponges from the genera *Tedania* and *Geodia* could be used for chemotaxonomic conclusions.

Fatty acid composition

In order to analyze the FA composition of *G. cydonium*, *Tedania* 1 sp. and *Tedania* 2 sp., the total FAs were transformed to methyl esters and analyzed by GC and GC/MS. The results obtained are summarized in Table II. It is evident that there are significant differences in the FA composition between the sponges from genus *Geodia* and those from genus *Tedania.*

In G. cydonium there is a relatively lower content of the long chain fatty acids (more than 22 carbon atoms), compared to the same group of acids in the investigated *Tedania* samples. FAs with longest chain (27–29 carbon atoms) were present only in *G. cydonium.* Furthermore, in both *Tedania* sp. the long chain fatty acids contain mainly an even number of carbon atoms, while in *G. cydonium* predominate those with odd number of carbon atoms. It is interesting to underline the unusually low contents of polyunsaturated fatty acids (PUFAs) in the investigated sponges. These acids are typical for the sponges from the class Demospongiae (Bergquist *et al.*, 1984). Both *Tedania* samples contain higher contents of PUFAs and lower contents of saturated and iso-fatty acids than *G. cydonium.* The last may be explained with the lower contents of bacteria in *Tedania* samples.

Evidently, the sponges from the genera *Geodia* and *Tedania* strongly differ in their FA content. The ecological factors (season, water temperature, salinity, pollution and diet) are identical, so the differences obtained must be due to the biological differences in the investigated sponges.

Only quantitative differences were observed in the FA compositions of both *Tedania* samples. The most important differences appeared in the PUFAs and in the relative contents of fatty acids with 24 and 26 carbon atoms.

These differences afforded an additional proof for the suggestion that the investigated two samples of *Tedania* belong to two different subgenera or genera.

Table II. Main fatty acids from three Adriatic Sea sponges (% from the total FAs).

Fatty acids	*G. cydonium*	*Tedania* 1 sp.	*Tedania* 2 sp.
12:0	0.1	–	–
14:0	5.5	1.6	0.8
i-14:0	1.8	0.2	0.3
i-15:0	2.7	0.2	0.3
15:0	2.0	0.2	0.3
16:0	12.1	8.1	4.6
16:1	3.9	2.8	2.2
16:2	1.0	1.6	1.8
16:3	4.6	0.6	0.7
16:4	1.7	–	–
17:0	0.5	0.3	1.2
18:0	9.4	4.2	3.7
18:1	11.2	8.1	5.7
18:2	0.2	–	0.2
18:3	1.5	0.8	0.3
19:0	0.2	0.6	0.7
20:0	0.5	2.7	3.0
20:1	0.8	–	–
20:3	0.2	–	–
20:4	0.9	2.4	1.9
20:5	–	11.2	3.5
21:0	–	–	–
22:0	0.8	0.6	0.5
22:1	0.6	2.5	4.2
23:0	0.8	0.6	0.6
24:0	0.2	3.9	1.3
24:1	1.0	5.6	12.6
25:0	0.6	0.1	0.5
25:1	4.5	–	–
25:2	0.3	3.5	2.3
26:0	2.2	–	–
26:1	1.2	–	–
26:2	3.2	25.0	32.2
26:3	–	10.1	12.4
27:2	10.3	–	–
27:3	0.8	–	–
28:2	2.8	–	–
28:3	4.7	–	–
29:2	0.7	–	–
29:3	1.4	–	–

Values are given as the mean of three measurements. The standard deviations (related to peak proportion on the chromatograms) are ± 0.1 for compounds with a percentage > 10 and ± 0.05 for the others.

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