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Composition of the lipophilic extract from the sponge *Suberites domuncula**

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Abstract: The composition of the lipophylic extract from the sponge *Suberites domuncula* was investigated. Lipids and their fatty acids, as well as volatile compounds and sterols were identified. Stanols are the main class of steroids in the investigated sponge. A high concentration of unsaturated long chain fatty acids (C_{26}–C_{28}) was identified. The presence of branched and odd fatty acids indicates associated bacteria in the sponge.

Keywords: lipids, sterols, volatiles, GC/MS, sponge.

INTRODUCTION

Suberites domuncula Olivi is widely distributed in the Mediterranean Sea. There are only a few investigations on its secondary metabolites,[1] but there are several papers on its bioactive proteins, such as suberitine with neuorotoxic and haemolytic activity.[2,3] Furthermore *S. domuncula* was used as a model for the study of sponge cell cultures[4,5] and sponge aggregates, called primmorphs.[6]

The sterol composition of sponges is often very complex. Sterols come from the diet, which includes different planktonic species and detritus. Variations in their composition at different locations and seasons explain the variations which are sometimes found in the sterol composition of the same species from different collection sites and periods. Another reason for the complex sterol composition is the ability of a significant number of marine sponges to transform the dietary sterols in order to satisfy the specific requirements of the cell membranes. Until now the sterol composition of different species from the genus *Suberites* have been investigated: *S. vestigium*[7] contains C26–C29 mono- and di-unsaturated sterols, *S. carnosus*[8] contains ergosterol and related sterols. In *S. domuncula*[9] and *S. japonicus*[10] con-

* Dedicated to Professor Miroslav J. Gašić on the occasion of his 70th birthday.

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ventional C26–29 sterols with a saturated ring system (stanols) predominate. Stanols are rare in marine organisms and in almost all cases they are present as traces. This is an indication that the sponge *S. domuncula*, *S. japonicus*, and probably other *Suberites* sp. can transform the dietary Δ^5 sterols into stanols by a specific biological reduction of this double bond. In fact, the presence of stanols with double bonds in the side chain shows that the biological reduction in the investigated species proceeds only in the ring system.

The lipids in marine organisms, contrary to those from terrestrial organisms, often possess a very complex composition, probably connected with the specific membrane requirements of sponges in the marine environment. In the lipids from *Suberites*, a large number of fatty acids has been found, part of which contain up to 28 carbon atoms. Such acids are characteristic for different sponge species.

It is known that the volatile fractions from plants often contain compounds which are insoluble in water but have defensive functions or are attractants, repellents, antifeedants, insecticides, *etc*.[11,12] Previously, research has been almost exclusively on the volatile compounds from terrestrial plants, while there have been few investigations on marine algae,[13] and marine sponges.[14] Volatile compounds from algae can go through the food chains to filter-feeders such as sponges. In some cases they can accumulate in the invertebrates and possess defensive functions.

As part of a general project on the development of sponge cell cultures as producers of valuable compounds, our efforts were concentrated on the investigation of lipophilic compounds of this sponge in order to obtain information for the design of a medium for sponge cell cultures. It is also of interest to identify some important allelochemicals, which could be responsible for the survival and development of the sponge.

MATERIALS AND METHODS

Animal material

Specimens of *Suberites domuncula* Olivi (Suberitidae, Hadromerida) were collected by SCUBA from 20–25 m depth in the gulf of Naples (Italy) and kept in flow-through seawater tanks during transport to the laboratory.

Isolation and analysis of sterols and fatty acids

Fresh sponges were extracted with a mixture of chloroform and methanol (2:1 v/v), followed by filtration and addition of an equal volume of water. The lower layer, containing the total lipids, was evaporated under reduced pressure. The chloroform-methanol extract was evaporated under reduced pressure at a temperature of 40 ºC. Part of the dry residue (200 mg) was subjected to silica gel column chromatography. Fractions containing sterols were combined and investigated by gas chromatography (GC: Pye Unicam 304 equipped with a flame ionisation detector) with a capillary column SPB-1 (30 m × 0.32 mm, 0.25 µm film thickness), the injector was at 300 ºC, the detector at 320 ºC, and the oven initially at 230 ºC and then to 300 ºC at 4 ºC/min with a 10 min hold, and by gas chromatography/mass spectroscopy (GC/MS: Hewlett Packard 5989B mass spectrometer coupled with a 5890 series II plus gas chromatograph) with a capillary column SPB-50 (30 m × 0.32 mm, 0.25 µm film thickness), the oven initially at 270 ºC and then to 290 ºC at 4 ºC/min with a 20 min hold. The source was at 250 ºC.

Part of chloroform-methanol extract (200 mg) was hydrolysed with methanolic 2 M KOH at reflux for 1 h. After neutralization, the reaction mixture was extracted with Et_2O. The ethereal extract was purified on a

silica gel column to obtain a mixture of fatty acids, which was methylated with CH_2N_2. The FAMEs mixture was analysed by GC/MS with a capillary column HP5 MS (cross linked 5 % PH ME siloxane); 30 m × 0.25 mm; 0.25 μm film thickness. The source was at 250 ºC, quadrupole at 100 ºC, the oven held for three minutes at 100 ºC and then heated to 300 ºC at 10 ºC/min. The injector and detector were at 280 ºC.

Isolation and analysis of volatile compounds

Part of chloroform-methanol extract (240 mg) was subjected to a four-hour distillation-extraction in a Lickens-Nickerson apparatus.[15] The volatile compounds were extracted from the distillate with Et_2O (yield: 3 mg, 1.25 % of the extract). They were investigated by GC/MS with a capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film thickness). The temperature was programmed from 40 ºC to 280 ºC at a rate of 6 ºC min^{-1}.

Analysis of polar compounds

The water-methanol layer (10 mg dry residue) was subjected to silylation. It was dissolved in 50 μl pyridine and 75 μl of bis-(trimethylsilyl)-trifluoracetamide was added. The mixture was heated at 80 ºC for 30 min and analysed by GC/MS, with a capillary column HP5 (23 m × 0.25 mm, 0.5 μm film thickness). The source was at 250 ºC, quadrupole at 100 ºC, the oven at 100 ºC and then to 310 ºC at 5 ºC/min with a 10 min hold. The injector and detector were at 290 ºC.

RESULTS AND DISCUSSION

Sterols

The results of the sterol analyses are summarized in Table I. Analogous to the earlier investigated *S. domuncula* and *S. japonicus*, stanols are the main steroids. 5α-Cholestanol appeared to be the main sterol. All other sterols were present in lower concentrations. The sterol mixture is characteristic for a diet containing mainly zooplankton, accompanied by some amounts of phytoplankton (characterized by 24-methyl-cholest-5,22-dien-3β-ol, which is transformed to 24-methyl-5α-cholest-22-en-3β-ol) and of detritus from macro algae, characterized by the presence of C_{29} sterols. The absence of 4-methyl sterols is an indication that dinoflagellates do not have a significant participation in the investigated sponge. There is only one unusual sterol: 3-ethoxy-5α-cholestane. This sterol has not been found in nature and it could be expected that it was an artefact, but no ethanol was used in the extraction and purification procedures. It is interesting that while *S. domuncula* and *S. japonica* contain almost exclusively stanols, in *S. vestigium*, and *S. carnosus* Δ^5 sterols predominate. It could be assumed that there might be two subgenera of *Suberites*, characterised by the ability to saturate the C-5 double bond.

TABLE I. Sterols composition (% of the total sterols) of *Suberites domuncula*

Sterols	
5α-24-nor-Cholest-22-en-3β-ol	1.0
5α-(22*Z*) Cholest-22-en-3β-ol (occelasterol)	3.6
5α-(22*E*) Cholest-22-en-3β-ol	5.0
Cholest-5-en-3β-ol (cholesterol)	0.5
5α-Cholestan-3β-ol	56.0

TABLE I. Continued

Sterols	
5α-Cholest-24(25)-en-3β-ol	1.6
24-Methyl-5α-cholest-22-en-3β-ol	10.8
24-Methyl-5α-cholest-24(28)-3β-ol	3.7
24-Methyl-5α-cholestan-3β-ol	2.9
24-Ethyl-5α-cholest-22-en-3β-ol	1.6
24-Ethyl-5α-cholestan-3β-ol	10.2
24-Ethyl-5α-cholest-24(28)-en-3β-ol	1.2
24-Propyl/isopropyl-5α-cholest-24(28)-en-3β-ol	tr[a]
3-Ethoxy-5α-cholestan	tr

[a]Tr trace < 0.1 %

Fatty acids

The total FAMEs obtained by saponification followed by methylation of the total lipids were analysed by GC/MS, and the data are reported in Table II. Analogous to other sponges,[16] high concentrations of unsaturated long chain fatty acids were identified in the investigated sponge (C_{26}–C_{28}). In *S. compacta* from the Japanese Sea,[17] the main fatty acids were C_{26}, followed by C_{22}, C_{28} and C_{18}. This may be explained by differences in the environment. Of interest is the absence of 18:2, 18:3, 20:4 and 20:5 acids in the by us investigated sample. Similar to *S. massa*,[18] in *S. domuncula* a few branched fatty acids were identified, mainly with a methyl group at C-12, which together with the presence of a low concentration of odd FA is an indication for the presence of associated bacteria.

Volatile compounds

The volatile compounds were obtained by steam distillation in a Lickens-Nickerson apparatus and analysed by GC/MS. The results obtained are summarized in Table III. Analogous to other investigated sponges,[14] there is a limited number of volatile compounds in *S. domuncula*, and *n*-hydrocarbons predominated (about 30 % from the total volatile compounds). The main hydrocarbons contain 17–26 carbon atoms. The hydrocarbons with an odd number of carbon atoms predominate. Besides *n*-hydrocarbons, two unsaturated hydrocarbons with a double bond at C-1 and two aromatic hydrocarbons were identified.

Analogous to other investigated marine organisms, three free fatty acids were found in the volatile compounds. Such compounds are often accepted as resulting from hydrolysis during the isolation procedure. Distillation-extraction is a relatively mild process and degradation of the lipids was not expected, so the identified fatty acids might exist in the free state in the sponges. These compounds possess some biological activities (antibacte-

TABLE II. Fatty acids composition of *Suberites domuncula*

Fatty acids	% of total FA	Fatty acids	% of total FA
13:0	tr[a]	18:2	0.4
13:0-i	0.4	18:0 (12,15-dimethyl)	2.0
14:0	0.5	19:0	tr
14:0-i	1.0	19:0-a	tr
14:0-a	0.7	19:0 (12,16-dimethyl)	tr
15:1	tr	20:0	1.3
15:0	0.6	20:0 (12,17-dimethyl)	0.4
15:0-i	3.6	21:0	1.2
15:0-a	1.2	21:0 (12,18-dimethyl)	0.4
16:1	1.4	21:0 (12,17-dimethyl)	tr
16:0	2.1	22:0	0.5
16:0-i	tr	22:0 (12,19-dimetyl)	tr
16:0-a	0.4	23:0	0.4
16:0 (4,8,11-trimethyl)	tr	24:1	0.6
16:0 (12,13-dimethyl)	tr	24:0	tr
17:1	2.3	25:1	tr
17:0	0.7	25:2	1.0
17:0-i	tr	26:1	14.8
17:0-a	tr	26:2	14.1
17:0 (11,14-dimethyl)	tr	27:2	1.4
17:0 (4,13-dimethyl)	tr	28:1	4.5
17:0 (12,14-dimethyl)	0.5	28:2	1.0
18:1	3.8	28:3	23.2

[a]Tr trace < 0.4 %

rial, insecticidal) that could improve the resistance of the sponge towards pathogens and predators. Our recent research showed that algae from the Black Sea contain a larger number of free fatty acids than the algae from the Mediterranean Sea. This is in accordance with our results for *S. domuncula*. The common diterpene hydrocarbon 2,6,10,14-tetramethyl hexadecane, and a sesquiterpene ketone (M^+ = 218; fragmentation different from aristolone) were detected. Few other compounds have also been identified. Of interest is the presence of aromatic compounds with a *tert*-butyl group.

TABLE III. Volatile compounds from *Suberites domuncula*[a]

Compound	% of total volatile compounds	Compound	% of total volatile compounds
Hydrocarbons		*Acids*	
Tridecane	0.1	Hexanoic acid, 2-ethyl	0.2
Tetradecane	0.2	Nonanoic acid	0.1
Pentadecane	0.4	Octadecanoic acid	10.0
Hexadecane	0.6	*Terpenes*	
Heptadecane	1.4	Hexadecane-2,6,10,14-tetra-methyl	1.4
Octadecane	1.6	Sesquiterpene ketone ($M^+ = 218$)	0.2
Nonadecane	1.6	*Others*	
Icosane	2.0	7,9-Di-*tert*-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	6.0
Henicosane	4.0	*tert*-Butylated hydroxy tolu-ene	1.0
Pentacosane	8.0	Formamide, *N*,*N*-dibutyl	0.2
Hexacosane	6.0	Phenethyl alcohol	0.1
1-Tetradecene	0.4		
1-Hexadecene	0.6		
Biphenyl	0.2		
Phenathrene	1.8		

[a]Only interesting compounds are reported.

TABLE IV. Polar compounds from *Suberites domuncula*[a]

Compound	% of total silylated compounds	Compound	% of total silylated compounds
N-containing compounds		Glutamine	0.4
Glycine	32	3-Pyridine carboxylic acid	tr
Alanine	1.3	Creatinine	0.2
Valine	0.6	*Fatty acids*	
Leucine	1.0	Palmitic acid	0.7
Isoleucine	0.6	Oleic acid	tr[b]
Proline	1.1	Oleic acid isomer	tr
Threonine	0.7	Stearic acid	0.5
Aspartic acid	0.6	*Others*	
Lysine	0.5	2-Chloro-1,1-biphenyl	tr
Ornithine	0.4	Glycerol	0.2
Aminomalonic acid	2.7	Phosphatidic acid	0.5
α-Aminoadipic acid	0.2		

[a]Only interesting compounds are reported. [b]Tr trace < 0.1 %

Polar compounds

The water-methanol layer contains the polar compounds, which were silylated and analysed by GC/MS, and the data are reported in Table IV. Mainly, free amino acids and their derivatives were identified. An unusually high concentration of glycine was detected, which has never been found in intact marine organisms. Free fatty acids were obtained in low concentrations; similar to those in the intact sponges *Ircinia muscarum* and *Dysidea avara* from the same region.[4]

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ИЗВОД

ИСПИТИВАЊЕ САСТАВА ЛИПОФИЛНОГ ЕКСТРАКТА СУНЂЕРА *Suberites domuncula*

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У овом раду испитиван је састав липофилног екстракта сунђера *Suberites domuncula*. Идентификовани су липиди и одговарајуће масне киселине, заједно са испарљивим супстанцама и стеролима. Станоли представљају главни састојак стероидне компоненте испитиваног сунђера, а идентификована је и висока концентрација незасићених масних киселина дугог низа (C_{26}–C_{28}). Присуство разгранатих масних киселина непарног броја угљеникових атома указује на присуство бактерија у испитиваном сунђеру.

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