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Phospholipid fatty acids and sterols of two *Cinachyrella* sponges from the Saudi Arabian Red Sea: comparison with *Cinachyrella* species from other origins

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Abstract

Phospholipid class compositions, fatty acids and sterols of the sponges *Cinachyrella alloclada* and *C. kükenthali* from the Saudi Arabian Red Sea were studied and compared with previous results for other *Cinachyrella* spp. collected in Senegal (East Atlantic) and New Caledonia (West Pacific). More than 50 fatty acids were identified as methyl esters and *N*-acyl pyrrolidides in each phospholipid mixture by GC/MS. Six fatty acids not hitherto found in nature were identified, namely 17-methyltetracosanoic in *C. kükenthali* and 18-methyltetracosanoic, 18-methylpentacosanoic, 18-methylhexacosanoic, 18,24-dimethyl-hexacosanoic and 6-bromo-5,9-nonacosadienoic acids in *C. alloclada*. Approximately 20 Δ5,9 unsaturated fatty acids were found, including three 6-brominated acids. The presence of bacteria was evidenced by the relatively high proportions of phosphatidylglycerol and high levels of branched short-chain fatty acids. A total of 20 free 3β-hydroxysterols were found by GC/MS, including clerosterol in relatively high amounts and gorgosterol in low amounts. The latter sterol has not been reported to date in a sponge. Comparisons with *Cinachyrella* species from other geographical areas show marked differences for both phospholipid fatty acid and sterol compositions.
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1. Introduction

Marine sponges are the most primitive multicellular animals and contain many bioactive and new metabolites, including lipids (Litchfield and Morales, 1976; Bergquist, 1978; Djerassi and Lam, 1991). Sponges have been able to adapt to their environments and this could be due to special structural features in their cell membranes, in particular unusual phospholipid fatty acids and sterols, since sterol–phospholipid interactions are assumed to play a major role in cell membranes (Morales and Litchfield, 1977; Djerassi and Lam, 1991). Several hypothetical intermediates have been further identified, thus providing a better understanding of already reported or novel metabolic pathways (Morales and Litchfield, 1977;

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Carballeira et al., 1986; Djerassi and Lam, 1991; Barnathan and Kornprobst, 1992; Barnathan et al., 1996). Furthermore, particular fatty acids appear as biomarkers for symbiotic microorganisms (Gillan et al., 1988). In contrast with other living organisms, sponge phospholipid fatty acids are to a large extent as long as 23–34 carbon atoms. However, approximately 100 unprecedented sterols have been found in marine sponges with unique bioalkylations of the side chain and variations of the sterol nucleus (Bergquist, 1978; Bergquist et al., 1991; Djerassi and Lam, 1991). Sterols show great variation in quantity and composition (Walkup et al., 1981; Bergquist et al., 1991; Djerassi and Silva, 1991; Kerr and Baker, 1991; Aiello et al., 1999), although *Plakortis halichondroides* seems to be devoid of sterols (Carballeira and Shalabi, 1990).

This work aims at extending knowledge on sponge lipids, especially for the genus *Cinachyrella*, and on their distribution based on species and geographical origin. Little is known about sponge lipids from the Red Sea. The family Tetillidae, including the *Cinachyrella* genus, has been reevaluated (Rützler, 1987). We report here the phospholipid fatty acid and sterol compositions of two *Cinachyrella* species from Saudi Arabia Red Sea and compare these with previous data for sponges of the same genus originating from Senegal (tropical East Atlantic) and New Caledonia (tropical East Pacific) (Barnathan et al., 1992a,b, 1994; Barnathan, 1993; Barnathan and Kornprobst, 2000). Several new long-chain fatty acids are described.

2. Material and methods

2.1. General experimental procedures

Phospholipid analyses were performed on HPTLC silica gel (Merck) plates using a Camag densitometer and AMD apparatus (TLC scanner II, 366 nm, CATS evaluation software) with phospholipid standards purchased from Sigma. Gas chromatography-mass spectrometry (GC/MS) coupling experiments were performed on a Hewlett Packard HP-5890 chromatograph linked to a HP-5989-A mass spectrometer (70 eV) equipped with a HP-9000-345 integrator, and using a 30-m×0.32-mm-i.d. fused silica capillary column coated with DB-1 (0.25-μm phase thickness). The carrier gas was helium. The column temperature was programmed for fatty acid methyl esters (FAMEs) and pyrrolidides from 180 to 310 °C at 3 °C min^{-1}, and for sterols and steryl acetates from 170 to 300 °C at 3 °C min^{-1}. Infrared spectra were recorded on a Perkin Elmer 257 spectrophotometer for liquid films between KBr plates.

2.2. Sponge material

All sponge specimens studied belong to the Tetillidae family (Demospongia, Tetractinomorpha, Spirophorida) and were collected in November 1997 in shallow waters (5–10 m deep) approximately 100 km north of Jeddah, Saudi Arabia. Both species were identified by comparison with authentic samples from Senegal. *Cinachyrella allo-clada* Uliczka has several large porocalices and *C. kükenthali* Uliczka is devoid of porocalices (Barnathan et al., 1992a; Barnathan, 1993). Voucher specimens were deposited in the Faculty of Marine Science, King Abdulaziz University, Jeddah.

2.3. Extraction, isolation and analysis of phospholipids

The sponges were washed in seawater, carefully cleaned, cut in small pieces and steeped twice in chloroform/methanol (1:1 v/v) for 24 h at room temperature. The combined extracts yielded the crude total lipids. Phospholipids were separated from other lipids by column chromatography on silica gel (70–230 mesh) with hexane, dichloromethane (neutral lipids), acetone (glycolipids) and methanol (phospholipids) as successive eluents. The general methods used for isolation and analysis of lipid mixtures have been previously described (Barnathan et al., 1992a,b).

2.4. Preparation of fatty acid methyl esters and N-acyl pyrrolidides

The fatty acids obtained were converted into methyl esters by reaction (30 min under reflux) with methanolic hydrogen chloride. *N*-Acyl pyrrolidides were prepared by direct treatment of methyl esters with pyrrolidine/acetic acid (10:1 v/v) for 2 h under reflux and purified by TLC on 0.5-mm silica gel layers using hexane/diethyl ether (1:2 v/v).

Table 1
Lipid composition of the *Cinachyrella* sponges from different locations

Species	Lipid composition (%)				
	Neutral lipids		Glycolipids Acetone	Phospholipids Methanol	Sterols
	Hexane	DCM			
C. alloclada Red Sea	40.0	43.6	8.4	8.0	19.9
C. kükenthali Red Sea	37.9	36.5	16.9	8.7	26.9
C. alloclada Senegal[a]	40.8–41.6	39.8–45.0	8.9–9.6	5.1–9.6	17.3–23.4
C. kükenthali Senegal[a]	35.3–46.8	44.1–56.7	4.7–6.5	1.6–3.3	18.3–26.8
C. aff. *schulzei* New Caledonia	39.7	52.6	4.2	3.5	17.1

Free sterols were isolated as a part of neutral lipids eluted with dichloromethane.
[a] Values for three different sponge collections; see Section 2.

2.5. Isolation, purification and acetylation of sterols

Free sterol mixtures were readily isolated from all the sterol-containing chromatographic fractions (dichloromethane) obtained during the lipid class separation, by washing the dried product with methanol and purifying by column chromatography and/or preparative TLC. In some cases pure sterol samples were prepared by recrystallisation in methanol to obtain better mass spectra. The total sterols were acetylated with acetic anhydride/pyridine (1:1 v/v) for 20 h at room temperature. Sterols were analyzed in free form and as acetates by GC/MS.

GC retention times (RT) and mass spectral data (MS, 70 eV) of the new fatty acids are as follows:

17-Methyltetracosanoic acid pyrrolidide: RT, 28.95 min; MS, m/z (% relative intensity): 435 (M^+, 0.8), 420 (0.3), 406 (0.3), 392 (0.5), 378 (0.4), 364 (0.5), 350 (0.8), 336 (0.8), 322 (0.2), 308 (1.1), 294 (0.6), 280 (0.5), 266 (0.7), 252 (0.6), 238 (0.5), 224 (0.7), 210 (1.7), 196 (1.8), 182 (1.9), 168 (2.4), 154 (1.8), 140 (2.5), 126 (17.2), 113 (100), 98 (12.8).
18-Methyltetracosanoic acid pyrrolidide: RT, 28.52 min; MS, m/z: 435 (M^+, 0.8), 420 (0.3), 406 (0.3), 392 (0.5), 378 (0.4), 364 (0.8), 350 (0.7), 336 (0.2), 322 (1.1), 308 (0.5), 294 (0.5), 280 (0.6), 266 (0.5), 252 (0.8), 238 (0.7), 224 (1.0), 210 (2.0), 196 (1.1), 182 (2.0), 168 (2.3), 154 (1.8), 140 (2.6), 126 (16.4), 113 (100), 98 (11.8).
18-Methylpentacosanoic acid pyrrolidide: RT, 30.42 min; MS, m/z: 449 (M^+, 1.2), 434 (1.0), 420 (0.9), 406 (0.6), 392 (0.5), 378 (0.5), 364 (1.3), 350 (1.7), 336 (0.4), 322 (1.5), 308 (1.2), 294 (1.1), 280 (1.2), 266 (1.3), 252 (1.1), 238 (1.4), 224 (1.1), 210 (1.2), 196 (1.5), 182 (1.8), 168 (1.9), 154 (2.1), 140 (1.9), 126 (23.1), 113 (100), 98 (18.4).
18-Methylhexacosanoic acid pyrrolidide: RT, 32.40 min; MS, m/z: 463 (M^+, 2.0), 448 (0.8), 434 (1.0), 420 (1.0), 406 (0.8), 392 (0.9), 378 (1.0), 364 (2.2), 350 (2.1), 336 (0.1), 322 (2.6), 308 (0.8), 294 (1.1), 280 (1.2), 266 (1.0), 252 (1.4), 238 (1.9), 224 (1.6), 210 (1.0), 196 (2.4), 182 (2.7), 168 (2.5), 154 (2.3), 140 (2.5), 126 (15.3), 113 (100), 98 (7.8).
18,24-Dimethylhexacosanoic acid pyrrolidide: RT, 32.73 min; MS, m/z: 477 (M^+, 1.6), 462 (1.8), 448 (1.6), 434 (0.1), 420 (1.3), 406 (1.4), 392 (1.8), 378 (2.6), 364 (3.3), 350 (2.6), 336 (0.1), 322 (2.8), 308 (1.8), 294 (1.8), 280 (1.7), 266 (1.6), 252 (2.1), 238 (2.1), 224 (2.8), 210 (3.1), 196 (2.6), 182 (3.4), 168 (3.8), 154 (3.6), 140 (3.4), 126 (19.4), 113 (100), 98 (12.9).
6-Bromo-5,9-nonacosadienoic acid pyrrolidide: RT, 40.34 min; MS, m/z: 486 ($[M\text{-}Br]^+$, 8.1), 260 (9.7), 258 (9.5), 224 (2.5), 180 (12.9), 178 (12.4), 154 (3.7), 140 (2.1), 126 (16.1), 113 (100), 98 (18.9).

3. Results and discussion

3.1. Lipid and phospholipid classes

Total lipids accounted for 1.6–3.2% of dried sponge residues after extraction in all the *Cinachyrella* specimens studied, as for other specimens from various origins previously investigated (Barnathan et al., 1992a, 1994; Barnathan, 1993). Table 1 allows a comparison between several *Cinachyrella* sponges from different locations regarding the lipid composition that appears very similar.

Table 2
Phospholipid composition of the *Cinachyrella* sponges from the Red Sea

Phospholipid	Composition (wt.%)	
	C. alloclada	*C. kükenthali*
lyso-Phosphatidylcholine	2.6	–
Phosphatidylcholine	48.0	44.5
Phosphatidylethanolamine	6.7	14.9
Phosphatidylinositol	17.0	19.1
Phosphatidylglycerol	12.1	13.5
Phosphatidylserine	8.5	8.0
Diphosphatidylglycerol	5.1	–

Neutral lipids were always predominant, whereas phospholipids are the minor lipid class.

As determined by quantitative thin layer chromatography (Table 2), the major phospholipid classes were phosphatidylcholine (44–48%) and phosphatidylinositol to a lesser extent. Phosphatidylglycerol occurred at high levels, suggesting that bacteria were present, thus contributing to the chemicals further analyzed. The demospongic acids ($\geq C_{23}$) amounted to 17.8% in *C. kükenthali* and 35.2% in *C. alloclada*. As previously reported, they were generally mostly found as aminophospholipids, namely phosphatidylethanolamine and phosphatidylserine, whereas the short-chain acids usually predominate in the common animal phosphatidylcholine (Djerassi and Lam, 1991).

3.2. Phospholipid fatty acid composition

More than 50 fatty acids were identified in each sponge, ranging from 10 to 30 carbon atoms (Table 3). Each fatty acid was identified, as the methyl ester or pyrrolidide, from its mass spectrum and its gas chromatography (GC) mobility (equivalent chain length, ECL values). Mass spectra of all fatty acid methyl esters analyzed showed molecular ion peaks, which allowed determination of the ECL values. *N*-Acyl pyrrolidides are known as useful derivatives for fatty acid analysis by gas chromatography/mass spectrometry (GC/MS) since they have a more pronounced tendency to retain the positive charge under electron impact and give homologous fragments with an interval of 14 or 12 amu. A reduced or missing peak for a given fragment flanked by elevated expected peaks indicates a methyl branch at this position (Anderson, 1978). In addition, we have recently reported that ECL values determined for methyl esters and *N*-acyl pyrrolidides are found very similar for both derivatives with the exception of highly branched fatty acids (Mirallès et al., 1995). The fatty acids identified in Red Sea sponges are presented in Table 3.

3.3. Branched saturated fatty acids

As observed for species collected in other locations, both Red Sea sponges also contained a large range of iso and anteiso fatty acids typical for the occurrence of bacteria. Other branched fatty acids identified were 10-methylhexadecanoic (3.9%), 13-methylhexadecanoic (10.3%), 10-methyloctadecanoic and 12-methyleicosanoic (1.1%) acids, likely originating from bacteria (Vacelet, 1975; Bobbie and White, 1980; Walkup et al., 1981; Gillan et al., 1988; Carballeira et al., 1998). The rare 13-methylhexadecanoic acid was identified from Gorgonians (Mirallès et al., 1995). 11-Methyloctadecanoic acid was also found in some sponges (Walkup et al., 1981; Dasgupta et al., 1984). The main MS fragmentations are given in Fig. 1 for the new fatty acids described in this paper.

In the phospholipids of *C. alloclada* from the Red Sea, three new homologous 18-methyl branched long-chain acids accounted for 13.4% of the total acids. These new 18-methyl branched acids, with 25 (ECL=24.38), 26 (ECL=25.35) and 27 (ECL= 26.38) carbon atoms, respectively, were readily identified from the mass spectra of their pyrrolidide derivatives (see Fig. 1 and Section 2). The corresponding molecular ions were present (M^+ at m/z 435, 449 and 463, respectively). In addition, the fragment ion at m/z 336 was diminished and flanked by elevated peaks at m/z 322 and m/z 350, which clearly pointed towards the methyl group at C-18. Mass spectra for FAMEs depicted for all three acid McLafferty peaks at m/z 74 and the corresponding molecular ion peak. It is important to note that these 18-methyl fatty acids were only identified in *C. alloclada* from the Red Sea.

The new 17-methyltetracosanoic acid was identified in *C. kükenthali* (4.3%) from the mass spectrum of its pyrrolidide (ECL=24.33) (Fig. 1) since the molecular ion was at m/z 435 and the corresponding fragmentation at m/z 322 was diminished and flanked by elevated peaks at m/z 308 and m/z 336, which pointed towards the methyl group at C-17. Some 19-methyl branched long-chain fatty acids were identified from sponge

Table 3
Phospholipid fatty acids identified from the Red Sea *Cinachyrella alloclada* and *C. kükenthali*

Fatty acid	Composition (wt.)	
	C. alloclada	*C. kükenthali*
Octanoic (8:0)	1.3	1.4
Decanoic (10:0)	1.5	2.3
Tetradecanoic (14:0)	1.4	2.1
4,8,12-Trimethyltridecanoic (br-16:0)	8.5	7.1
13-Methyltetradecanoic (i-15:0)	0.9	6.9
12-Methyltetradecanoic (a-15:0)	–	3.1
Pentadecanoic (15:0)	0.4	1.3
10,13-Dimethyltetradecanoic (br-16:0)	–	1.5
14-Methylpentadecanoic (i-16:0)	1.3	2.5
9-Hexadecenoic (16:1)	1.8	3.4
Hexadecanoic (16:0)	8.5	8.1
10-Methylhexadecanoic (br-17:0)	3.9	–
13-Methylhexadecanoic (br-17:0)	–	10.5
15-methylhexadecanoic (i-17:0)	1.7	3.7
14-Methylhexadecanoic (a-17:0)	1.8	1.2
9-Heptadecenoic (9-17:1)	–	1.0
Heptadecanoic (17:0)	0.9	1.3
9,12-Octadecadienoic (18:2)	0.2	0.6
9-Octadecenoic (18:1)	3.0	1.7
11-Octadecenoic (18:1)	3.0	2.5
Octadecanoic (18:0)	12.5	7.3
10-Methyloctadecanoic (br-19:0)	0.7	–
11-Methyloctadecanoic (br-19:0)	2.8	0.2
17-Methyloctadecanoic (i-19:0)	1.0	1.5
6-Nonadecenoic (19:1)	0.3	0.3
9-Nonadecenoic (19:1)	–	1.7
11-Nonadecenoic (19:1)	1.8	–
Nonadecanoic 19:0	1.4	0.3
14-(or 15)-Eicosenoic (20:1)[a]	0.4	0.1
12-Methyleicosanoic (br-21:0)	1.1	–
Docosanoic (22:0)	–	0.4
21-Methyldocosanoic (i-23:0)	0.7	–
17-Tetracosenoic (24:1)	0.7	0.6
Tetracosanoic (24:0)	0.2	0.3
17-Methyltetracosanoic (br-25:0)[b] #1	–	4.3
18-Methyltetracosanoic (br-25:0)[b] #2	4.5	–
Pentacosenoic (25:1)	0.3	0.5
18-Methylpentacosanoic (br-26:0)[b] #3	0.6	–
19-Hexacosenoic (26:1)	3.7	4.1
18-Methylhexacosanoic (br-27:0)[b] #4	7.4	–
25-Methyl-19 (or 20)-hexacosenoic (i-27:1)	1.3	–
18,24-Dimethylhexacosanoic (br-28:0)[b] #5	0.9	–
20-Heptacosenoic (27:1)	3.0	–
Nonacosanoic (29:0)	2.1	–
Δ5,9-Demospongic acids		
5,9-Octadecadienoic (18:2)	0.4	4.2
5,9,x-Hexacosatrienoicc	0.3	–
24-Methyl-5,9-pentacosadienoic (i-26:2)	–	0.4
23-Methyl-5,9-pentacosadienoic (a-26:2)	–	0.2
5,9-Hexacosadienoic (26:2)	0.2	0.4
25-Methyl-5,9-hexacosadienoic (i-27:2)	–	0.5
24-Methyl-5,9-hexacosadienoic (a-27:2)	–	2.0
5,9-Heptacosadienoic (27:2)	0.6	0.5
25-Methyl-5,9-heptacosadienoic (a-28:2)	–	3.0
5,9-Octacosadienoic (28:2)	4.0	0.8
Methyl-5,9-octacosadienoic (br-29:2)[a]	0.3	–

Table 3 *(Continued)*

Fatty acid	Composition (wt.)	
	C. alloclada	*C. kükenthali*
27-Methyl-5,9-octacosadienoic (i-29:2)	0.6	–
26-Methyl-5,9-octacosadienoic (a-29:2)	0.4	–
5,9-Nonacosadienoic (29:2)	1.0	Trace
6-Bromo-5,9-heptacosadienoic (Br-27:2)	0.2	Trace
5,9,23-Triacontatrienoic (30:3)	1.0	0.2
6-Bromo-5,9-octacosadienoic (Br-28:2)	1.0	Trace
6-Bromo-5,9-nonacosadienoic (Br-29:2)[b] #6	0.2	–
$\Sigma\Delta5,9$	10.2	12.2
ΣLong-chain fatty acids ($\geq C_{23}$)	35.2	17.8

br, branched; i, iso; ai, anteiso; trace, <0.1%. Other minor fatty acids (< 0.2%) in *C. alloclada*: 9-17:1, i-20:0, 20:0, br-21:0, n-23:0, br-29:0, 29:1, 30:1, 31:2; and in *C. kükenthali*: n-12:0, br-14:0, 16:1, i-9-17:1, 9-17:1, 18:3, 20:0, 26:2, 30:2.

[a] The position of the methyl branch and/or double bond is still ambiguous.

[b] Unprecedented as natural compounds numbered as in Fig. 1.

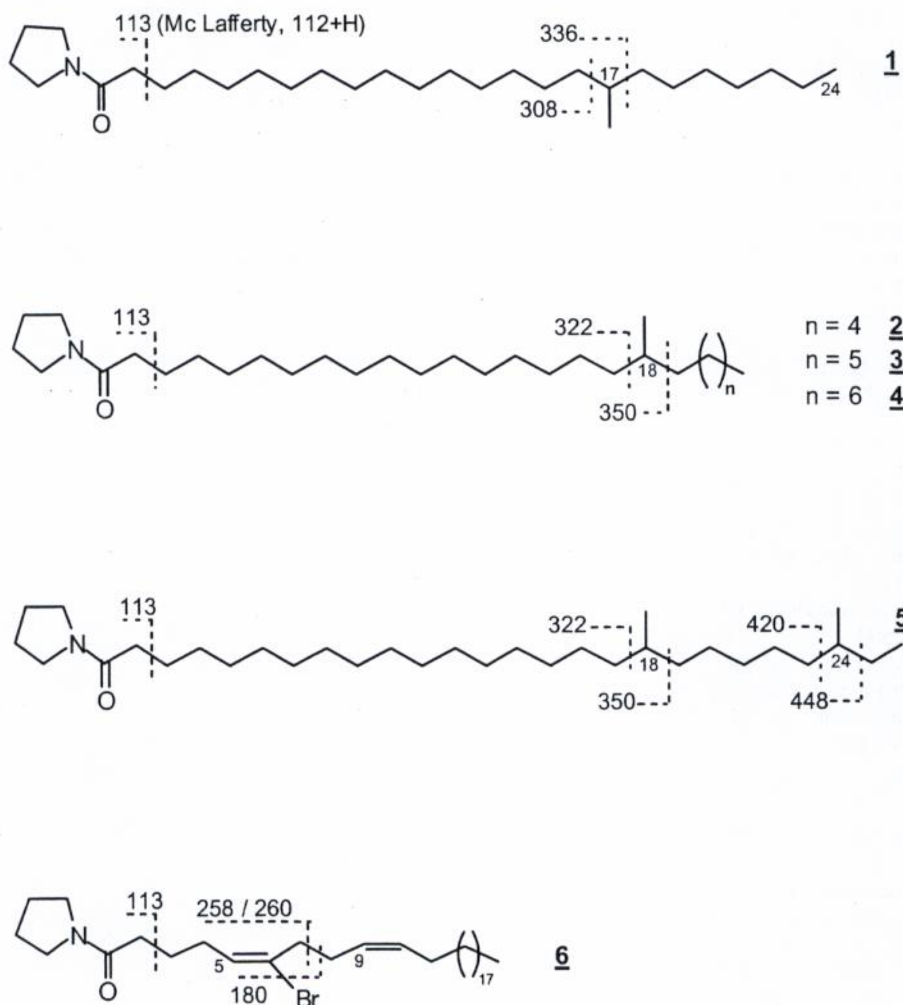
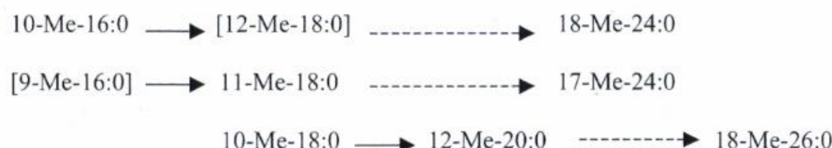


Fig. 1. Main mass spectral fragmentation (m/z) of the new fatty acids as pyrrolidide derivatives. Compounds are numbered as indicated in Table 3.



Scheme 1. Hypothetical but still unknown.

phospholipids (Dasgupta et al., 1984). The occurrence of bacteria in the sponges is supported by the huge levels of phosphatidylglycerol and phosphatidylinositol that are characteristic of bacterial membranes (Kates, 1964; Walkup et al., 1981; Gillan et al., 1988; Djerassi and Lam, 1991), and by the identification of several branched, short-chain acids typically of bacterial origin, such as iso- and anteiso-pentadecanoic, iso- and anteiso-heptadecanoic, 10-methylhexadecanoic, 13-methylhexadecanoic, 10-methyloctadecanoic, 16-methyl-9-hexadecenoic and 11-methyl-octadecanoic acids. Thus, the new, branched, long-chain acids probably arise from shorter precursors of exogenic origin through a homologation process, such as in Scheme 1.

10,13-Dimethyltetradecanoic acid has only been observed in a Carribean sponge (Carballeira and Maldonado, 1989). The mass spectrum of its pyrrolidide (ECL = 15.07) exhibited a molecular ion peak at m/z 309 (16:0 acid structure), and two homologous fragment ions at m/z 224 and m/z 280 were diminished (methyl branches at C-10 and C-13, respectively). A branched octacosanoic acid was identified as pyrrolidide derivative by GC/MS (ECL = 27.23) (Fig. 1). The spectrum exhibited the usual base peak at m/z 113, a molecular ion at m/z 477 (28:0 fatty acid structure) and two very weak fragment ions at m/z 336 and m/z 434 (methyl branches at C-18 and C-24, respectively). The spectrum of the corresponding methyl ester exhibited the usual base peak at m/z 74, the molecular ion peak at m/z 438 and an enhanced fragment ion at m/z 395 ($C_{25:0}$ fragment). Thus, we identified the new 18,24-dimethylhexacosanoic acid.

3.4. Isoprenoid fatty acids

Both Red Sea sponges contain 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD) at significant levels (8.5 and 7.1% for *C. alloclada* and *C. kükenthali*, respectively) as shown in Table 4.

In all the other *Cinachyrella* sponges we have studied, isoprenoid acids were largely predominant. In contrast with all *Cinachyrella* previously studied, the Red Sea specimens did not contain the rare 5,9,13-trimethyltetradecanoic acid (5,9,13-TMTD) that seemed to be a specific pattern for this sponge genus (Barnathan et al., 1993; Kornprobst and Barnathan, 1998). No trace of the latter acid was detected in any *Cinachyrella* from the Red Sea, in ion monitoring GC/MS analyses of the methyl ester mixture (searching of molecular ion corresponding to a 17:0 acid structure in the area between pentadecanoic acid methyl ester and 14-methylpentadecanoic acid methyl ester). Here too, the comparison between *Cinachyrella* species collected in different locations shows interesting differences. The average content of 4,8,12-TMTD is the same for species from the Red Sea and New Caledonia, but 5,9,13-TMTD is lacking in Red

Table 4
Isoprenoid phospholipid fatty acids in the *Cinachyrella* genus

Species	Composition (%)	
	4,8,12-TM-13:0	5,9,13-TM-14:0
C. alloclada Red Sea	8.5	–
C. kükenthali Red Sea	7.1	–
C. alloclada Senegal[a]	14.5–36.7	0.4–1.5
C. kükenthali Senegal[a]	19.6–24.4	0.3–0.6
C. aff. *schulzei* New Caledonia	8.8	0.5

See Section 2; TM, trimethyl.

[a] Values for three different sponge collections. See Section 2.

Table 5
Fatty acid distribution in some *Cinachyrella* sponges from different locations

Cinachyrella species	Distribution (%)				
	Saturated		Monoenoic	Δ-5,9-dienoic	Δ-5,9,23–30:3
	Linear	Branched			
C. alloclada Red Sea	31	38	20	10	1
C. kükenthali Red Sea	26	44	18	12	Trace
C. alloclada Senegal	26–31	28–36	18–22	11–12	3–11
C. kükenthali Senegal	11–21	31–37	17–20	11–21	2–11
C. aff. *schulzei* New Caledonia	30	15	24	24	7

Trace, <0.1%.

Sea sponges. On the contrary, *Cinachyrella* species from Senegal contain much more 4,8,12-TMTD than the former, but the content of 5,9,13-TMTD is the same as for the New Caledonia species. In addition, it should be noted that phytanic acid was not detected in both sponges.

3.5. Unsaturated fatty acids

It appears from Table 3 that the phospholipid fatty acid compositions of both *Cinachyrella* from the Red Sea are quite similar, even for Δ5,9 fatty acids: 13 acids (12.2%) in *C. kükenthali* and 13 acids (10.2%) in *C. alloclada*. The infrared spectrum of the phospholipid FAMEs displayed no prominent absorption at approximately 960–980 cm^{-1}, indicating the absence of (*E*)-unsaturation. It should be noted that high amounts of the short-chain 5,9-octadecadienoic acid (4.2%) were found in *C. kükenthali.* However, brominated Δ5,9 fatty acids found in *C. alloclada* only occur at trace levels in *C. kükenthali*, and this could be related to the amount of very long-chain fatty acids, which is greater in *C. alloclada* (35.2%) than in *C. kükenthali* (17.8%) (Wijekoon et al., 1984; Lam et al., 1989; Carballeira and Emiliano, 1993; Carballeira and Reyes, 1995; Barnathan et al., 1994).

The new 6-bromo-5,9-nonacosadienoic acid was characterized as the pyrrolidide derivative (Fig. 1), since the mass spectrum showed the usual base peak at m/z 113, brominated ions at m/z 258 and 260 of equal intensity due to the double allylic cleavage between C-7 and C-8 positions, an enhanced ion at m/z 180 due to the same cleavage after bromine loss, and the ion at m/z 486 after bromine loss from the molecular ion (Wijekoon et al., 1984; Barnathan et al., 1994). In addition, a plot of the retention time vs. carbon number of the already known brominated 6-bromo-Δ5,9-27:2 (ECL=28.38) and 6-bromo-5,9-28:2 acids (ECL=29.36) (Barnathan et al., 1994), and the new brominated Δ5,9-29:2 acid (ECL=30.23), showed a quasi-linear relationship.

Comparison of the fatty acid distribution in *Cinachyrella* species collected in different oceanic areas shows marked differences that should be mentioned (Table 5).

First of all, the presence of the Δ5,9,23-30:3 acid was noted, which occurred in important amounts in *Cinachyrella* from Senegal (Barnathan et al., 1992a), with 11% in both species studied, but in low amounts (up to 1%) in Red Sea species. An intermediate value of 7% was observed for a species collected in New Caledonia (Barnathan et al., 1994). This implies that the total amount of Δ5,9 fatty acids mainly encountered in sponges is quite low for Red Sea sponges (10 and 12%) compared to Senegal (23 and 25%) and New Caledonia (30%). This variation is correlated with the respective content of saturated fatty acids, approximately 70% for Red Sea species, 56% for Senegalese species and 46% for the New Caledonian species.

3.6. Sterol composition

Sponge sterols are known to occur essentially in free form. Free sterols were readily isolated from the dichloromethane chromatographic fractions on crystallization from methanol. Sterols were analyzed by GC/MS in free form and as acetates. As shown in Table 6, the sterol compositions of both Red Sea sponges appear very similar, despite the fact that *C. alloclada* contains Δ7 sterols lacking in *C. kükenthali* and Δ22 sterols that occur only in low amounts in *C. kükenthali*.

The major components were brassicasterol, cho-

Table 6
Sterol compositions of *Cinachyrella* sponges from different locations

Sterol	Unsaturation pattern	M_w	Composition (%)				
			C. alloclada		*C. kükenthali*		*C.* aff. *schulzei* New Caledonia
			Red Sea	Senegal	Red Sea	Senegal	
24-Norcholestadienol	5,22	370	–	0.3/0.5	–	Trace/0.3	0.4
22(*Z*)-Dehydrocholesterol	5,22	384	2.3	0.4/0.8	1.4	0.2/0.6	1.5
22(*E*)-Dehydrocholesterol	5,22	384	5.3	8.4/7.0	4.8	3.2/4.7	7.2
22-Dehydrocholestanol	0	386	–	–/–	1.2	–/–	–
Cholesterol	5	386	14.5	22.4/22.6	11.3	19.3/19.6	18.9
Lathosterol	7	386	–	0.9/1.3	–	0.7/1.8	–
Cholestanol	0	388	3.1	2.1/3.2	5.4	3.3/10.6	0.6
Brassicasterol/crinosterol	5,22	398	24.3	15.8/13.8	18.8	14.9/8.7	18.5
24-Methylenecholesterol	5,24 (28)	398	0.3	Trace/0.3	0.4	Trace/1.5	tr.
Campesterol/dihydrobrassicasterol	5	400	11.0	8.1/9.3	14.2	6.2/6.6	4.4
Campesten-7-ol	7	400	–	–/–	2.1	–/–	–
22-Dehydrocampestanol	22	400	–	–/–	3.4	–/–	–
Campestanol	0	402	1.1	–/–	2.8	–/–	–
Poriferasterol/stigmasterol	5,22	412	0.8	4.5/4.4	1.0	5.0/2.1	7.1
23,24-Dimethylcholestadien-5,22-ol	5,22	412	–	2.2/–	–	3.1/–	–
Clerosterol	5,25	412	11.8	–/–	10.7	–/–	–
Fucosterol	5,24 (28)	412	–	1.8/2.6	–	3.2/9.5	2.0
Isofucosterol	5,24 (28)	412	–	–/–	–	0.3/0.4	2.4
Poriferasten-7-ol	7	412	–	–/–	0.7	–/–	–
Stigmasten-7-ol	7	412	–	–/–	1.0	–/–	–
Clionasterol	5	414	18.4	33.0/30.0	13.7	40.6/33.6	37.0
Sitosterol	5	414	0.7	–/–	0.9	–/–	–
22-Dehydrostigmastanol	22	414	1.6	–/–	2.0	–/–	–
Stigmastanol	0	416	4.2	–/–	3.6	–/–	–
Unidentified C_{30}[a]	5, X	426	0.3	–/–	0.5	–/–	–
Gorgosterol	5,22:23-CH_2	426	0.3	–/–	0.1	–/–	–

Data for Senegal samples show first value for shallow water sponges/second value for sponges at depth of 15–25 m.
[a] Base peak at $m/z=296$ and $(M\text{-}AcOH)^+$ at $m/z=408$.

Table 7
Comparison of sterol content according to unsaturation pattern and location

Unsaturation pattern	Sterol composition (%)				
	C. alloclada		*C. kükenthali*		*C.* aff. *schulzei* New Caledonia
	Red Sea	Senegal	Red Sea	Senegal	
Δ0	8.4	2.1/3.2	13.0	3.3/10.6	0.6
Δ5	44.4	63.6/61.9	40.1	66.1/59.8	60.4
Δ7	–	0.9/1.3	3.8	0.7/1.8	–
Δ22	1.6	–/–	5.4	–	–
Δ5,22	32.7	31.6/26.5	26.0	26.4/16.4	35.1
Δ5,24 (28)	0.3	1.8/2.9	0.3	3.5/11.4	3.9
Δ5,25	11.8	–/–	10.7	–/–	–

The unidentified C_{30} sterol and gorgosterol are not taken into account. Data for Senegal samples show first value for shallow water sponges/second value for sponges at a depth of 15–25 m.

lesterol, campesterol and clionasterol, which account for more than 10% in each sponge, but the major sterol is not the same according to the geographic sponge location. For both Senegalese species and for *C.* aff. *schulzei* from New Caledonia, the major sterol is clionasterol, at 33, 40 and 37%, respectively (Barnathan et al., 1992b). For both Saudi Red Sea species, the major sterol

is brassicasterol, at 19 and 24%, respectively. Comparison of the sterol composition according to unsaturation pattern and geographical locations gives interesting information.

It is clearly evident from Table 7 that important differences should be noted according to the geographical location. Δ22 and Δ5,25 sterols have only been identified in *Cinachyrella* species from the Red Sea. Clerosterol accounted for approximately 11% in both species. Conversely, Saudi Red Sea species are also poor in 24-methylene cholesterol. Stanols are quite lacking in the New Caledonia species, but are present in Senegal and Red Sea species, especially in the latter.

The as yet unidentified C_{30} sterol X (Table 6) is likely a sterol of 30 carbon atoms with a Δ24 (24′) unsaturated side chain, since the base peak is at m/z 296 and the ion due to the loss of acetic acid from the molecular ion at m/z 408 is less than 10% (Goad and Akihisa, 1997). Unfortunately, the low concentration of this sterol prevented further analysis. Another C_{30} sterol at low levels (0.1 and 0.3% in the species from the Red Sea) has been characterized from its mass spectrum as a Δ5 sterol with an extra double bond on the side chain. As a Δ5 sterol, its acetate exhibited intense ions at m/z 408 ($[M\text{-}AcOH]^+$) and m/z 255 (loss of the side chain from the latter ion) (Goad and Akihisa, 1997). The base peak was at m/z 296, due to the cleavage of cyclopropane, and the relative intensity of the ion at m/z 408 was 87 and 94% in two successive GC/MS experiments. Other primary cleavages were at m/z 337 (between C-23 and C-24) and m/z 213 (cleavage of the D ring). Moreover, comparison with an authentic sample isolated from a tunicate of the Indian Ocean and purified by HPLC confirmed that this sterol is gorgosterol (Viracaoundin, 2001). Thus, the most striking result of this study is the first report of gorgosterol in a marine sponge.

Gorgosterol was first isolated from gorgonians (Ciereszko et al., 1968). It has been shown that gorgosterol is associated with Zooxanthellae, the symbiotic unicellular algae (Withers et al., 1982). Diatoms and dinoflagellates are known to be important components of phytoplankton, and thus form the foundation of marine food chains. Gorgosterol is widespread in attached invertebrates such as gorgonians, but the association between marine sponges and Zooxanthellae appears to be less frequent. The partially verified biosynthesis starts from dinosterol supplied by dinoflagellates and requires successive transmethylation for side chain elaboration (Goad, 1978; Goad and Akihisa, 1997). The methyl group introduced arises from a transmethylation reaction requiring *S*-adenosylmethionine as a methyl group donor. The transfer of the electrophilic *S*-methyl group produces a cation, which is stabilized by proton loss. Of interest was the identification in Senegalese *Cinachyrella* sponges of the rare 23,24-dimethylcholesta-5,22-dien-3Δ-ol, an intermediate in the biosynthesis of gorgosterol, likely of symbiotic origin (Barnathan et al., 1992a).

To summarize, these *Cinachyrella* sponges are of particular interest since they can synthesize new methyl-branched long-chain fatty acids from short precursors, likely of bacterial origin. A new brominated Δ5,9 acid was characterized. In addition, the sterol composition of all the *Cinachyrella* species, regardless of geographic origin, appears similar, although clerosterol was found at high levels in the Red Sea sponges. Moreover, the latter sponges contain gorgosterol found for the first time in a sponge, whereas its biosynthetic intermediate, namely 23,24-dimethylcholesterol, was found in the Senegalese sponge specimens. The Central Pacific (New Caledonia), East Atlantic (Senegal) and Central Red Sea have very different ecological conditions, especially water temperature (small variations from 24 and 30 °C throughout the year for New Caledonia and Saudi Arabia, but larger variations from 14 to 28 °C along Senegalese coasts), but also for turbidity (Senegalese waters are rarely clear, whereas seawater in New Caledonia and the Central Red Sea are rarely turbid) and perhaps salinity. It is likely that at least some of these factors are implied in the differences observed in lipid compositions.

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