Lipids (2012) 47:345–353
DOI 10.1007/s11745-011-3631-4

ORIGINAL ARTICLE

New 2-Methyl-13-Icosenoic Acid from the Temperate Calcisponge *Leuconia johnstoni*

Elodie Quévrain · Gilles Barnathan · Tarik Meziane · Isabelle Domart-Coulon · Vony Rabesaotra · Marie-Lise Bourguet-Kondracki

Received: 16 May 2011 / Accepted: 3 November 2011 / Published online: 27 November 2011

Abstract The fatty acid composition of the temperate calcareous marine sponge *Leuconia johnstoni* Carter 1871 (Calcaronea, Calcarea) was characterized for the first time in specimens collected off the Brittany coast of France over four years from October 2005 to September 2008. Forty-one fatty acids (FA) with chain lengths ranging from C_{14} to C_{22} were identified as fatty methyl esters (FAME) and *N*-acyl pyrrolidide (NAP) derivatives by gas chromatography–mass spectrometry (GC–MS). Twenty-two saturated fatty acids (SFA) were identified accounting for 52.1–59.0% of the total FA and dimethylacetals (DMA). In addition, among the SFA, we noticed the presence of numerous methyl-branched *iso* and *anteiso* FA, suggesting a large number of associated bacteria within *L. johnstoni*. Thirteen monounsaturated fatty acids (MUFA, 28.0–36.0% of total FA + DMA) were also identified as well as six polyunsaturated fatty acids (PUFA, 4.0–8.2%). A noticeable DMA was detected at a high level, particularly in September 2008 (11.8%), indicating the presence of plasmalogens in this sponge species. This calcareous sponge lacked the non-methylene-interrupted FA (NMI FA) with a Δ5,9 system typical of siliceous Demosponges and Hexactinellids. The occurrence of the unusual 8,13-octadecadienoic acid was reported for the first time as a minor PUFA in a calcareous sponge. The major FA, representing 20–25% of this sponge FA, was identified as the new 2-methyl-13-icosenoic acid from mass spectra of its methyl ester and its corresponding *N*-acyl pyrrolidide derivatives as well as a dimethyl disulfide adduct.

Keywords Calcareous sponge · *Leuconia johnstoni* · Fatty acids · 2-Methyl-13-icosenoic acid · 8,13-Octadecadienoic acid · Dimethylacetal

Abbreviations

amu	Atomic mass unit
DMA	Dimethylacetal(s)
DMDS	Dimethyl disulfide
DUFA	Diunsaturated fatty acid(s)
ECL	Equivalent chain length
FA	Fatty acid(s)
FAME	Fatty acid methyl ester(s)
GC–MS	Gas chromatography–mass spectrometry
MS	Mass spectrum (spectra)
MUFA	Monounsaturated fatty acid(s)
NAP	*N*-Acyl pyrrolidide(s)
NMI FA	Non-methylene-interrupted fatty acid(s)
PUFA	Polyunsaturated fatty acid(s)
SFA	Saturated fatty acid(s)
TLC	Thin layer chromatography

Introduction

Sponges are very ancient metazoans, with fossils dating back to the Early Cambrian period about 530 million years

E. Quévrain · M.-L. Bourguet-Kondracki (✉)
Molécules de Communication et Adaptation des Micro-organismes UMR 7245 MNHN-CNRS, Muséum National d'Histoire Naturelle, 57 rue Cuvier, C.P. 54, 75005 Paris, France
e-mail: bourguet@mnhn.fr

G. Barnathan · V. Rabesaotra
Groupe Mer Molécules Santé EA 2160, Equipe Lipides marins à activité biologique, Faculté de Pharmacie, Université de Nantes, BP 53508, 44035 Nantes Cedex 1, France

T. Meziane · I. Domart-Coulon
Biologie des Organismes et des Ecosystèmes Aquatiques, UMR 7208 MNHN-CNRS-UPMC, Muséum National d'Histoire Naturelle, 57 rue Cuvier, C.P. 26, 75005 Paris, France

ago. Interestingly these sessile marine invertebrates have the ability to biosynthesize a wide variety of structurally unique secondary metabolites, with no equivalent in terrestrial natural products. Because many of these metabolites display biological and pharmacological activities with potential applications in human health and biotechnological industry, they have become excellent candidates for the discovery of new bioactive natural products [1, 2]. While the siliceous Demospongia and Hexactinellida sponge classes, which represent about 90% of the species in the Porifera phylum, have been extensively studied, the chemistry of the Calcarea sponges was barely investigated. Chemical investigations on Calcispongiae have mostly concerned sponges of the Calcinea subclass and very few studies included representatives of the Calcaronea subclass [3–5].

Marine sponges have also proved to be a major source of unusual fatty acids (FA) including very long-chain (up to 34) non-methylene-interrupted fatty acids (NMI FA) with the particular unsaturation pattern Δ5,9 [6]. Although numerous usual and original FA have been identified from marine Demosponges [7] and some Hexactinellids [5], to the best of our knowledge, only five studies report on the phospholipid FA composition of a few calcareous sponge species [8–12], two of them revealing antimicrobial activity or protein kinase C inhibition activity [11, 12].

The widespread association of marine sponges with a great variety of microorganisms has generated much recent interest, as in numerous chemical studies associated microorganisms proved to be the true producers of sponge metabolites. Our investigations on the role of sponge associated bacterial communities led us to detect bacterial markers in their calcareous sponge hosts through cellular localization, chemical and microbial approaches [13, 14]. The temperate calcareous sponge *Leuconia johnstoni* Carter 1871 (class Calcispongiae, subclass Calcaronea, order Baerida, family Baeriidae) was selected for investigation of its FA composition which can be linked to the abundance of its resident bacteria. Indeed, previous studies have reported that significant levels of *i/ai* SFA can be assigned to bacterial sponge symbionts [15]. However, due to the low available biomass of this small encrusting calcareous species and the observation by thin layer chromatography (TLC) that phospholipids constituted the major class of this sponge lipids, the study was focused on the characterization of its FA composition. These FA were identified through gas chromatography coupled to mass spectrometry (GC–MS) on their methyl esters (FAME) and *N*-acyl pyrrolidide (NAP) derivatives to determine the chain length, degree and position of unsaturations.

Materials and Methods

Sponge Material

Leuconia johnstoni Carter 1871 [16] is a small-sized calcareous sponge species with an encrusting growth form (compressed lobes fused at the base, with a maximum size of 50 mm in diameter and 15 mm thick) living on subvertical rock surfaces in wave exposed sites with a distribution ranging from the British Isles to the French Brittany coasts [17]. Specimens of the intertidal calcareous sponge *L. johnstoni* were collected off the Brittany coast (North–East Atlantic, Finistère, France) near Concarneau (Plage des Dames, 47°52′N and 3°55′W). Four successive collections of specimens pooled from several rock-encrusting colonies were realized on the same site on October 2005, September 2007, March 2008 and September 2008 (through a permit granted to S. Derrien, MNHN-Concarneau). Samples were frozen immediately after collection and stored at −80 °C until further investigations. Four voucher specimens are available from the Muséum National d'Histoire Naturelle in Paris as collection number MNHN n°C2009-3, n°C2009-10, n°C2009-15 and n°C2009-12, respectively.

Extraction of Total Lipids and Preparation of Fatty Acid Methyl Esters

The extraction was based on the Bligh and Dyer method [18] as amended by Meziane and Tsuchiya [19] and included four successive steps: extraction of total lipids, saponification, methylation and purification of FA by TLC.

Briefly, frozen sponge samples were lyophilized and extracted twice with water/chloroform/methanol (1:1:2, v/v/v) mixture. The combined chloroform extracts yielded the crude total lipids.

The mixture was heated with 2 mL of methanol and 1 mL of sodium hydroxide (2 M). The saponification reaction was stopped by adding an aliquot of hydrochloric acid (35%). After two successive washes with chloroform, followed by centrifugation at 2,000 rpm for 5 min, the lipid fraction was evaporated under a nitrogen stream.

Methylation of FA was obtained by adding 1 mL of a solution of 10% of boron trifluoride (BF_3) in methanol. After 10 min at 90 °C, all FA of the total lipids were converted into the fatty methyl esters (FAME). Samples were then extracted twice by a water/chloroform (1:1, v/v) mixture and centrifuged at 2,000 rpm during 5 min. The chloroform was evaporated under nitrogen and FAME were dissolved in a chloroform/methanol (2:1, v/v) mixture, suitable for their preservation.

The lipids extracts were evaporated to dryness and recovered with three washings of 500 μL of a chloroform/methanol (98:2, v/v) mixture. Neutral and polar lipids were separated by column chromatography on silica gel microcolumns (30 × 5 mm I.D. Kieselgel 70-230 mesh, Merck) using a chloroform/methanol (98:2, v/v) mixture to elute the neutral lipids, followed by neat methanol to elute the polar lipids. Each fraction was then treated in the same way as the bulk sample.

Preparation of *N*-Acyl Pyrrolidides

N-acyl pyrrolidide derivatives (NAP) were prepared by direct treatment of the FAME with a pyrrolidine/acetic acid (10:1, v/v) mixture under reflux for 2 h [20].

Preparation of the Dimethyl Disulfide Adduct

The dimethyl disulfide (DMDS) adduct was obtained from a FAME mixture (2 mg) in dimethyl disulfide (0.48 mL) by adding a solution (0.1 mL) of iodine in diethyl ether (60 mg mL^{-1}) under agitation for 24 h at room temperature [21]. The reaction was stopped by adding hexane (6 mL), washed with a dilute solution of sodium thiosulfate (1.2 mL of a 7 mg/100 mL solution) and dried over anhydrous sodium sulfate and evaporated to dryness. The product was taken up in fresh hexane for injection onto the GC–MS.

Gas Chromatography–Mass Spectrometry Analyses

Gas chromatography coupled with mass spectrometry (GC–MS) experiments were performed on a Hewlett-Packard HP-6890 chromatograph (Agilent Technologies, Massy, France) linked to a HP-5989 spectrometer (70 eV) equipped with a HP-9000-345 integrator and using a SLB™ 5-ms 60 mm × 0.25 mm × 0.25 μm fused silica capillary column (splitless) (Supelco, Bellefonte, PA, USA). The carrier gas was helium (flow rate 1 mL/min). Injector and detector temperatures were used at 250 and 280 °C, respectively. The oven temperature was maintained at 170 °C for 4 min and then programmed from 170 to 310 °C at a rate of 3 °C/min and at 200 °C for 4 min and then programmed from 200 to 310 °C at a rate of 3 °C/min for FAME and NAP derivatives, respectively.

Mass spectral data for the two novel FA structures in the calcareous sponge *L. johnstoni*, identified as their FAME and NAP derivatives or DMDS adduct, are presented below.

2-Methyl-13-icosenoic acid methyl ester MS *m/z* (rel. intensity %) [M^+] 338 (1.3), 307 (4.0), 306 (6.1), 291 (0.7), 278 (1.2), 263 (0.5), 250 (1.2), 242 (0.4), 235 (0.4), 222 (0.3), 208 (1.4), 194 (0.7), 181 (0.5), 167 (0.8), 166 (1.0), 165 (1.1), 157 (2.2), 151 (1.2), 143 (2.3), 125 (3.8), 101 (25.8), 98 (18.1), 88 (100), 83 (22.6), 69 (34.6), 57 (16.5), 55 (46.0), 43 (20.5), 41 (26.5).

2-Methyl-13-icosenoic acid pyrrolidide MS *m/z* (rel. intensity %) [M^+] 377 (8.4), 362 (0.8), 348 (1.5), 334 (1.9), 320 (4.6), 306 (3.1), 292 (1.1), 278 (0.8), 266 (0.8), 252 (2.3), 238 (1.5), 224 (1.5), 210 (2.3), 196 (4.4), 182 (4.6), 168 (2.3), 154 (3.8), 140 (26.7), 127 (100), 113 (15.3), 98 (22.1), 83 (9.2), 70 (19.1), 55 (40.5), 43 (22.1), 41 (18.3).

Dimethyl disulfide adduct of 2-methyl-13-icosenoic acid methyl ester MS *m/z* (rel. intensity %) [M^+] 418 (3.4), 403 (1.3), 386 (2.2), 369 (1.4), 355 (0.8), 339 (1.4), 322 (2.1), 307 (0.7), 291 (2.2), 273 (28.1), 241 (27.3), 255 (2.0), 208 (4.2), 193 (4.5), 177 (3.8), 165 (4.5), 145 (37.9), 125 (16.7), 111 (31.2), 109 (26.5), 97 (50.8), 95 (36.3), 85 (36.4), 83 (50.8), 82 (43.1), 69 (80.3), 55 (100), 43 (84.8), 41 (40.1).

8,13-Octadecadienoic acid methyl ester MS *m/z* (rel. intensity %) [M^+] 294 (5.3), 263 (4.5), 241 (1.4), 222 (2.3), 220 (2.1), 199 (2.3), 180 (2.4), 177 (4.2), 164 (5.1), 150 (10.5), 149 (6.8), 137 (8.9), 135 (10.5), 123 (15.8), 109 (29.3), 95 (60.2), 87 (28.6), 81 (85.7), 74 (44.3), 69 (54.2), 67 (100), 55 (87.2), 43 (54.1), 41 (76.7).

8,13-Octadecadienoic acid pyrrolidide MS *m/z* (rel. intensity %) [M^+] 333 (6.8), 318 (0.4), 304 (0.6), 290 (1.6), 276 (1.3), 262 (1.4), 250 (1.6), 236 (3.0), 222 (6.8), 208 (3.8), 194 (4.6), 182 (7.6), 168 (6.1), 154 (4.5), 140 (7.6), 126 (64.4), 113 (100), 98 (26.5), 85 (16.0), 72 (27.4), 70 (41.7), 67 (20.4), 55 (42.4), 43 (25.8), 41 (25.1).

Results

The fatty acid (FA) composition of *L. johnstoni* was identified for four samples corresponding to four successive collections on the same geographical site, in the period 2005 to 2008 including autumn (3 replicates) and spring (1 replicate) as reported in Table 1. FA were characterized as their methyl esters (FAME) and *N*-acyl pyrrolidide (NAP) derivatives using gas chromatography-mass spectrometry (GC–MS). Identification of FA was confirmed by comparing their equivalent chain length values (ECL) and their mass spectra (MS) with those of known standards.

Forty-one FA with chain lengths between C_{14} and C_{22} were identified in the different samples, as illustrated in Table 1.

Saturated Fatty Acids

In all three samples collected in autumn from October 2005 to September 2008, similar SFA were determined whereas in the sample collected in early spring, March 2008,

Table 1 Fatty acid composition of the sponge *Leuconia johnstoni* (4 samples collected off Concarneau, France, each sample pooled from several colonies)

Fatty acids (symbol)	Abundance (wt%)				
	ECL	October 2005	September 2007	March 2008	September 2008
Saturated fatty acids					
Tetradecanoic (14:0)	14.00	0.2	0.8	1.4	1.9
4,8,12-Trimethyltridecanoic (br-13:0)	14.50	0.5	0.8	3.1	1.9
13-Methyltetradecanoic (*i*-15:0)	14.65	3.1	2.5	2.3	4.1
12-Methyltetradecanoic (*ai*-15:0)	14.72	3.6	3.6	3.2	6.3
Pentadecanoic (15:0)	15.00	0.2	0.4	0.4	0.4
14-Methylpentadecanoic (*i*-16:0)	15.63	2.0	1.3	0.7	0.8
13-Methylpentadecanoic (*ai*-16:0)	15.71	0.7	0.3	–	0.5
Hexadecanoic (16:0)	16.00	20.1	23.5	22.8	16.0
15-Methylhexadecanoic (*i*-17:0)	16.61	1.9	0.6	0.9	2.2
14-Methylhexadecanoic (*ai*-17:0)	16.69	0.7	0.3	1.4	1.1
Heptadecanoic (17:0)	17.00	0.6	0.8	0.9	0.6
15-Methylheptadecanoic (*ai*-18:0)	17.75	0.9	0.4	1.6	1.3
Octadecanoic (18:0)	18.00	9.6	13.8	14.9	10.0
17-Methyloctadecanoic (*i*-19:0)	18.69	3.4	2.7	2.9	2.1
Nonadecanoic (19:0)	19.00	0.7	0.4	–	0.5
18-Methylnonadecanoic (*i*-20:0)	19.66	1.5	0.3	1.4	0.9
Icosanoic (20:0)	20.00	1.4	1.8	1.1	1.2
Heneicosanoic (21:0)	21.00	0.6	0.4	–	0.3
Total SFA	–	51.7	54.7	59.0	52.1
Monounsaturated fatty acids					
5-Hexadecenoic (16:1)	15.80	0.5	1.0	0.3	1.4
9-Hexadecenoic (16:1)	15.87	1.8	1.8	0.9	0.7
9-Octadecenoic (18:1)	17.80	2.0	1.4	1.4	0.8
11-Octadecenoic (18:1)	17.90	4.1	3.2	2.0	0.7
13-Icosenoic (20:1)	19.75	1.9	2.5	1.5	–
2-Methyl-13-icosenoic (br-13-20:1)[a]	20.18	24.3	22.4	20.8	25.8
Heneicosenoic (21:1)[b]	20.86	1.4	1.5	1.1	–
Total MUFA	–	36.0	33.8	28.0	29.4
Di- and poly-unsaturated fatty acids					
8,13-Octadecadienoic (18:2)[c]	17.82	0.8	0.6	0.9	0.7
9,12-Octadecadienoic (18:2)	17.85	0.8	0.8	1.1	1.0
11,15-Icosadienoic (20:2)	20.30	3.1	1.9	1.4	0.8
5,8,11,14-Icosatetraenoic (20:4)	18.96	1.7	1.9	1.4	0.8
5,8,11,14,17-Icosapentaenoic (20:5)	19.20	1.8	1.5	0.9	0.7
Total DUFA and PUFA	–	8.2	6.7	5.7	4.0
Dimethylacetal					
DMA (21:2)[b]	20.75	2.9	3.2	5.9	11.8

FA identified as traces: *SFA (ECL)* 16-Methylheptadecanoic (17.64), 19-Methylicosanoic (20.66), 16-Methyloctadecanoic (18.79), Docosanoic (22.00); *MUFA* 13-Methyl-4-tetradecenoic (14.32), 10-Hexadecenoic (15.90), 15-Methyl-5-hexadecenoic (16.40), 6-Octadecenoic (17.54), 14-Octadecenoic (17.94), 9-Icosenoic (19.30); *DUFA* 13, x-Docosadienoic (21.80)(not previously characterized in a calcareous sponge)

[a] Not previously reported in nature

[b] Undetermined unsaturations

[c] Not previously characterized in a calcareous sponge

ai-16:0, 19:0 and 21:0 were lacking. The linear SFA hexadecanoic acid (16:0) and octadecanoic acid (18:0) were always determined as dominant. They represented between 16.0 and 23.5% and between 9.6 and 14.9% of the total FA + DMA, respectively. The minor linear SFA (20:0) was observed at similar levels (between 1.1 and 1.8%) in the four samples. In contrast, the shortest length SFA (14:0) was identified between 0.2 and 1.9% depending on sample. Additional linear SFA were also observed at noticeable levels of the total FA and DMA in all samples (less than 0.5% for 15:0 and less than 1% for 17:0).

Furthermore, *L. johnstoni* also contained a high proportion of branched SFA including *iso* (*i*) and *anteiso* (*ai*) FA. The diversity of *iso* FA was similar to those of *anteiso* FA (*i*/*ai* -15:0 to *i*/*ai*-19:0) excepted for *i*-18:0, *ai*-19:0 and *i*-20:0, only identified as traces. The isoprenoid-type 4,8,12-tridecanoic acid (br-13:0) was also identified from 0.5 to 3.1% of the FA + DMA (ECL value of the FAME = 14.50).

Monounsaturated Fatty Acids

Monounsaturated FA (MUFA) represented from 28.0 to 36.0% of the total FA + DMA in the sponge. Surprisingly, an unusual MUFA was present in all samples with an aliphatic chain of 20 carbons (ECL value of 20.18). The mass spectrum (MS) of its methyl ester (FAME) revealed a molecular ion $[M]^+$ at *m*/*z* 338 corresponding to a $C_{20:1}$ FA structure (Fig. 1).

In the MS of its FAME, the fragment ion at *m*/*z* 88 (C_3 fragment + H) as the base peak and the major ion at *m*/*z* 101 could remind a McLafferty rearrangement of a FA ethyl ester. But, the presence in the MS of the relatively intense fragment ion at *m*/*z* 306 ($[M-31]^+$) indicated that the compound was obviously a FAME. These key observations allowed to identify this compound as a MUFA with a methyl group at C-2, which was also supported by the MS of its NAP derivative, showing the molecular ion at *m*/*z* 377 (Fig. 2).

The MS of its pyrrolidide derivative displayed the expected McLafferty ion at *m*/*z* 113, while the base peak was present at *m*/*z* 127, confirming the 2-methyl branching nature of this MUFA. This observation was in accordance with the occurrence of an elevated peak at *m*/*z* 140, as depicted in the Lipid Library, for a 3-methyl branched NAP. In addition, the gap of 12 amu between fragment ions at *m*/*z* 278 and *m*/*z* 266 suggested localization of the double bond between carbons C-13 and C-14 of the aliphatic chain. In order to confirm this hypothesis, the dimethyl disulfide (DMDS) adduct of this 2-methyl MUFA was prepared as previously described by Imbs et al. [21]. Analysis of the MS of the corresponding DMDS adduct showed the presence of characteristic fragments at *m*/*z* 145, 213, 241 and 273 (Fig. 3), confirming unambiguously the localization of the unsaturation between C-13 and C-14. However its configuration could not be determined.

This MUFA was unequivocally identified as 2-methyl-13-icosenoic acid (2-Me-13-20:1). Its occurrence in all samples of *L. johnstoni* collected at the same geographical site at different seasons is noteworthy. Indeed, this unusual 2-methyl FA represented 22.4% and 24.3% in autumn (September 2007 and October 2005 respectively) and 20.8% of the total FA + DMA of specimens collected in spring (March 2008). However in winter (February 2006 and 2007) the sponge lipid profile was dominated by SFA and the abundance of this unusual 2-Me-13-20:1 dropped to respectively 7.8 and 9.8% of FA + DMA, although it still dominated the sponge MUFA (data not shown, publication in preparation).

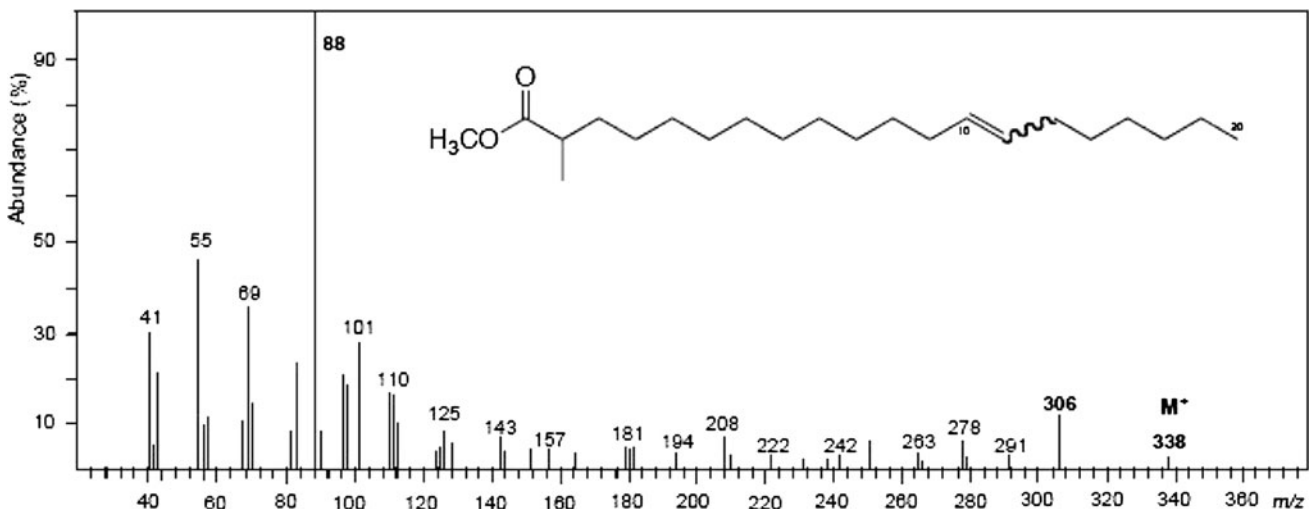

Fig. 1 Mass spectrum of 2-methyl-13-icosenoic acid methyl ester from *L. johnstoni*

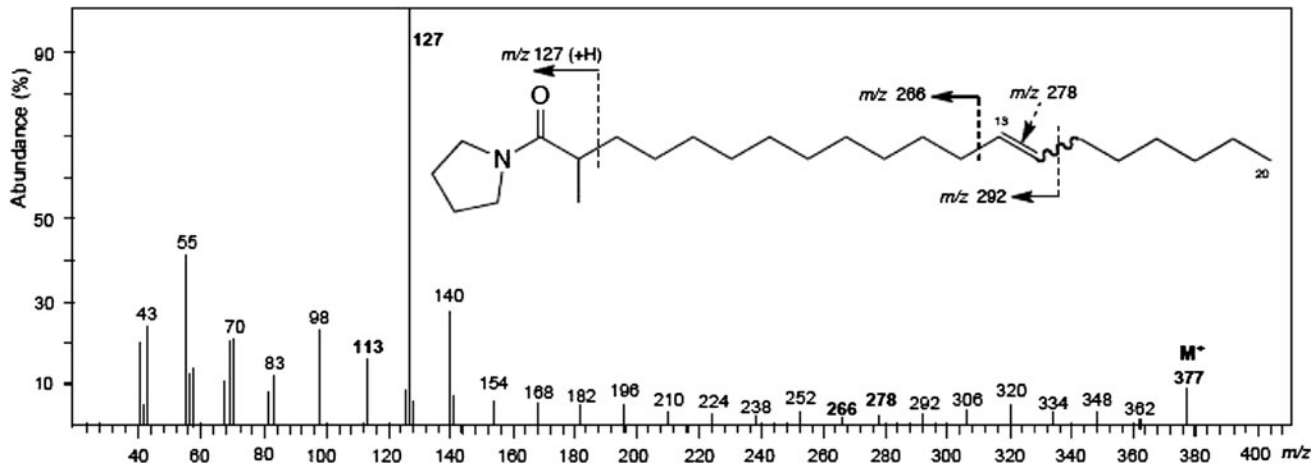

Fig. 2 Mass spectrum of 2-methyl-13-icosenoic acid pyrrolidide derivative from *L. johnstoni*

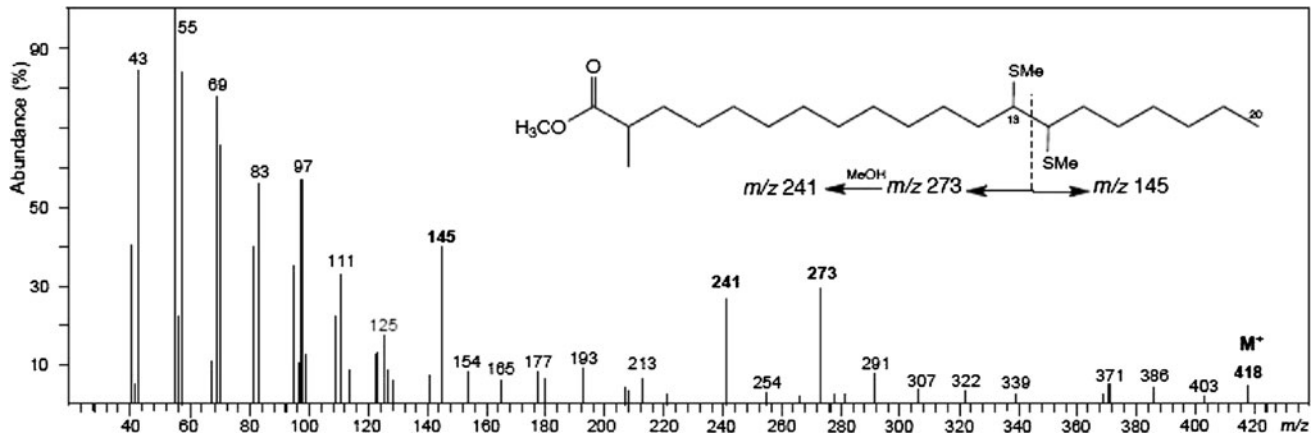

Fig. 3 Mass spectrum of 2-methyl-13-icosenoic acid dimethyl disulfide derivative from *L. johnstoni*

It appeared to us that the peak representing the new 2-methyl-13-icosenoic acid was highly represented in the polar phospholipid fraction.

Two additional hexadecenoic-type MUFA, 5-hexadecenoic and 9-hexadecenoic, were also identified in low amounts (0.3–1.4 and 0.7–1.8%, respectively) as well as two octadecenoic-type MUFA: 9-octadecenoic (0.8–2.0%) and 11-octadecenoic (0.7–4.1%). 13-icosenoic (1.5–2.5%) and heneicosenoic acid (1.1–1.5%) were only identified in three specimens out of four.

In addition, six MUFA were identified as traces: 13-methyl-4-tetradecenoic, 10-hexadecenoic, 15-methyl-5-hexadecenoic, 6-octadecenoic, 14-octadecenoic, 9-icosenoic (Table 1).

The two monomethyl branched MUFA, 13-methyl-4-tetradecenoic and 15-methyl-5-hexadecenoic, were readily identified from their GC mobilities (ECL of 14.32 and 16.40, respectively) and from their corresponding NAP derivatives MS spectra. The MS of the NAP of 13-methyl-4-tetradecenoic displayed the molecular ion at *m/z* 293 (15:1 FA structure), as well as fragment ions at *m/z* 139 and 152, and an intense fragment ion at *m/z* 166 (53%) indicating a double bond between C-4 and C-5, as shown in the Lipid Library [22]. Furthermore, a very weak ion at *m/z* 264 indicated an *iso*-branched FA. Similarly, the MS of the NAP of 15-methyl-5-hexadecenoic displayed the molecular ion at *m/z* 321 (17:1 FA structure), and fragment ions at *m/z* 140 and 153, indicating a double bond between C-5 and C-6. In this case, the *iso*-branched was indicated by a weak peak at *m/z* 292.

Polyunsaturated Fatty Acids

Four DUFA were observed, in low amounts (<2%).

The DUFA, eluted just before the 9,12-octadecadienoic (0.8–1.1%), had an ECL value of 17.82 as methyl ester and showed in its MS a molecular ion at *m/z* 294, indicating a 18:2 acid structure. The MS of its NAP showed a molecular ion at *m/z* 333 (18:2). The two double bonds were located by the 12 amu gaps between the peaks at *m/z* 182 (C-7) and

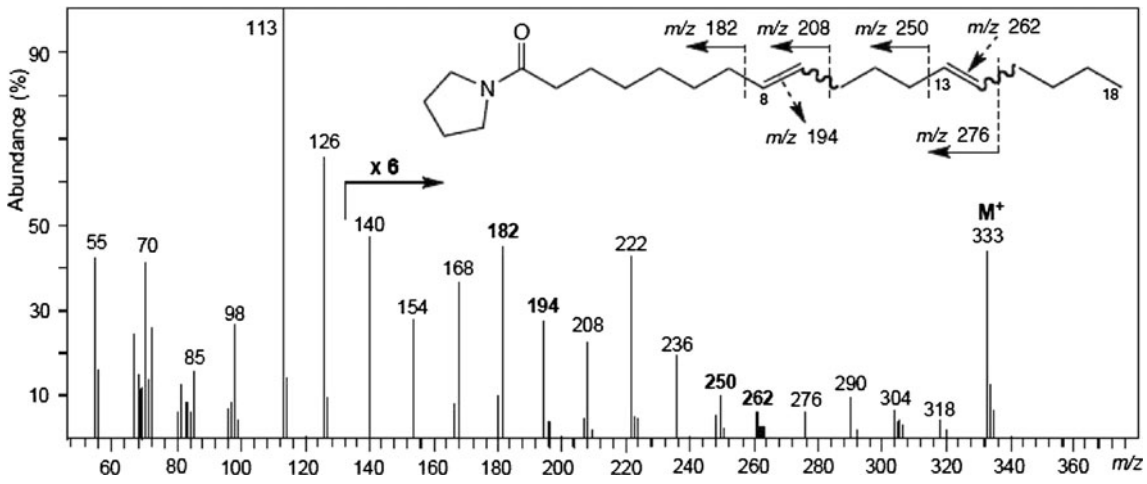

Fig. 4 Mass spectrum of 8,13-octadecadienoic acid pyrrolidide derivative from *L. johnstoni*

m/z 194 (C-8) and between the peaks at *m/z* 250 (C-12) and *m/z* 262 (C-13), indicating Δ8-Δ13 unsaturations. Major diagnostic fragmentations of FAME and NAP of 8,13-octadecadienoic acid under electron impact are given in the "Materials and Methods" section. This DUFA 8,13-octadecadienoic acid occurred in low abundance in all samples with values ranging from 0.6 to 0.9% of total FA + DMA (Fig. 4).

The two other detected DUFA are 11,15-icosadienoic acid (0.8–3.1%) and 13,x-docosadienoic acid (traces). Complete identification of the latter failed due to its very small amount.

Two other PUFA 5,8,11,14-icosatetraenoic acid (0.8–1.9%) and 5,8,11,14,17-icosapentaenoic acid (0.7–1.8%) were also observed.

Dimethylacetals

Several DMA were detected at trace levels in the lipid fraction of *L. johnstoni*. However, one of them was detected at a significant amount in all samples, accounting for 11.8% of total FA + DMA in September 2008. MS of this dominant DMA (ECL = 20.75), showed characteristic fragment ions at *m/z* 75 ($[(CH_3O)_2–CH]^+$) [23] and *m/z* 322 corresponding to $[M-31]^+$, indicating a C_{21} saturated fatty aldehyde with two unsaturations.

Discussion

By contrast with siliceous Demosponges and Hexactinellids, only a few reports have characterized the lipid composition of Calcisponges [8–10]. To obtain further insight into the FA content of Calcisponges, we selected *Leuconia johnstoni* as a calcareous sponge model belonging to the chemically poorly studied Calcaronea subclass. We investigated its FA composition comparing four specimens collected at the same site in a time series over 4 years.

Out of the forty-one identified FA, twenty-two were saturated FA (SFA), including eighteen major SFA represented between 52.1 and 59.0% of the total FA + DMA. In addition to the known linear SFA, *i/ai* C_{14}–C_{22}, typical FA of bacteria, were observed in all *L. johnstoni* specimens at significant levels ranging from 9 to 16% of total FA + DMA. Previous studies had reported that *i/ai* FA were found as minor compounds or traces in terrestrial eukaryotes but in high amounts in some marine eukaryotes such as sponges, nudibranch molluscs, asteroidea and tunicates [9, 24, 25]. These mid-chain-branched *i/ai* FA, which are considered as membrane constituent especially in Gram-positive bacteria, have often been assigned to bacterial sponge symbionts [15, 26, 27]. Recently, methyl-branched fatty acids were proposed to be synthesized by Poribacteria [28]. Nevertheless, 16-methyl-octadecanoic acid (*ai*-19:0) which is reported as a major FA in several Calcareous sponge species mostly of the Calcinea subclass [9] was found in low amounts in *L. johnstoni*. Furthermore, although 17-methyl-nonadecanoic acid (*ai*-20:0) was previously reported as a major compound of presumably sponge cell origin in Calcispongiae [9], this FA was lacking in our samples even as traces.

Other branched FA have also been considered as bacterial markers, especially some monounsaturated FA (MUFA). In this study, thirteen MUFA were observed, among which seven had major relative abundance (28.0–36.0%). However, *iso*-15:1 and *iso*-17:1 MUFA with various positions of the double bond, which were previously reported as characteristic of sulfate-reducing bacteria of the genus *Desulfovibrio* [29, 30], were both lacking in all samples of *L. johnstoni*.

As a major result, our study led to the identification for the first time in a calcareous sponge of a new natural

MUFA identified as 2-methyl-13-icosenoic acid as the dominant MUFA. Its high relative abundance, ranging from 20.8 to 25.8% of the total FA + DMA in all four samples of *L. johnstoni,* suggests that it might play a significant role in the biology of this sponge, potentially in the function of its cellular membranes. Unfortunately, despite of its high relative abundance, the purification of this 2-methyl FA remained difficult due to the low available sponge biomass of *L. johnstoni*.

Previously, some 2-methyl branched unsaturated FA have already been isolated but only from siliceous marine sponges. In 1990, the first 2-methyl branched FA, 2-methyloctadecanoic acid was identified from the Demosponge *Plakortis halichondroides* [31]. Later, two additional 2-methyl branched MUFA, 2-Me-24:1n-7 and 2-Me-26:1n-9 were isolated in small amounts (7.1% of sponge total FA) from the Demosponge *Halichondria panicea* [21].

Further chemotaxonomic studies would be necessary to demonstrate the specificity of this new 2-methyl-13-icosenoic acid as potential biomarker for sponges of the genus *Leuconia*.

Six polyunsaturated FA (PUFA) accounting between 4.0 and 8.2% of the total FA + DMA, including the two non-methylene-interrupted FA (NMA) 8,13-18:2 and 11,15-20:2 and one additional diunsaturated FA (DUFA) 9,12-18:2, were also detected. This is the first time that the unusual 8,13-octadecadienoic acid was characterized in a calcareous sponge. Numerous NMI FA, such as 5,11-20:2, 5,13-20:2, 7,13-22:2 and 7,15-22:2 have already been identified from marine sponges [6, 7, 32]. The 11,15-icosadienoic acid NMI observed in this study was previously identified from the Caribbean Demosponge *Amphimedon complanata* and the Axinellida sponge *Pseudaxinella* cf. *lunaecharta* [7, 33, 34]. The origin of the two PUFA 5,8,11,14-icosatetraenoic acid and 5,8,11,14,17-icosapentaenoic acid, which were detected in all samples of *L. johnstoni*, remains unknown [35]. They were observed at a very low level, representing less than 2% of the total FA and DMA content. No other PUFA with 3 or more double bonds were identified.

Furthermore, we noticed that demospongic acids, defined by Christie for the database Lipid Library as long-chain FA with a Δ5,9 unsaturation system and recently revisited as NMI FA, were totally absent in *L. johnstoni*. These NMI FA, observed in various marine sources with a large range of chain-lengths (C_{16}–C_{32}) [36] were previously reported in specimens of calcareous sponges in the genera *Clathrina* (Calcinea subclass) and *Sycon* (Calcaronea subclass) [8].

Finally, some DMA were observed, of which one was abundant, identified as 21:2 and accounting for 2.9–11.8% of the total FA + DMA.

The finding of DMA among FAME in *L. johnstoni* points to the presence of plasmalogens (1-*O*-1′-enyl-2-acyl-glycerophospholipids) in the sponge. The DMA are produced from aldehydes, released from plasmalogens, concomitantly with the FAME during the esterification step [23]. These plasmalogens are widely distributed in the animal kingdom and also occur in some anaerobic microorganisms and marine invertebrates [37]. In mammals, they could have a role in physical properties of cell membranes, storage of arachidonic acid, activation of phospholipase A_2 and cellular signalization. They are also involved in protection against oxidative stress [38]. The presence of plasmalogens was previously reported in the Demosponge *Polymastia penicillus* but their structures could not be determined due to both low amounts and co-elution with other lipids during purification [7]. However, to date, the biological role of plasmalogens in sponges is not clearly determined. We speculate that they may have similar functions as in more complex metazoan organisms including protection against oxidative stress or against attack by microorganisms.

Surprisingly, by contrast with all other FA, which were in constant relative abundance in the four specimens collected during two seasons over 4 years, detection of this DMA was variable and fluctuated between 2.9 and 11.8% without any seasonal corroboration.

In conclusion, this first investigation of the fatty acid composition of the Calcaronea species *Leuconia johnstoni* Carter 1871 identified the new 2-methyl-13-icosenoic FA in a calcareous sponge. It also revealed the occurrence at low levels of the unexpected PUFA 20:4 and 20:5 and of the DMA 21:2 although in fluctuating ratios. The nearly constant high relative abundance of the new 2-methyl-13-icosenoic FA in *L. johnstoni* suggests that this MUFA might be further explored as a potential biomarker. Further investigations on lipids and FA composition of *Leuconia* species remain to be carried out to substantiate this hypothesis.

Acknowledgments This work is part of Elodie Quévrain's Ph.D. thesis, supported by a grant from the French Ministère de l'Enseignement Supérieur et de la Recherche. The authors also thank the director and staff of the Laboratoire de Biologie Marine du Muséum National d'Histoire Naturelle, Station Marine de Concarneau, France, for providing access to facilities for regular sponge collections.

References

1. Sipkema D, Franssen MCR, Osinga R, Tramper J, Wijffels RH (2005) Marine sponges as pharmacy. Mar Biotechnol 7:142–162
2. Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR (2010) Mar Nat Prod 27:165–237
3. Ralifo P, Tenney K, Valeriote FA, Crews P (2007) A distinctive structural twist in the imidazole alkaloids from a calcareous

marine sponge: isolation and characterization of leucosolenamines A and B. J Nat Prod 70:33–38
4. Hagemann A, Voigt O, Wörheide G, Thiel V (2008) The sterol of calcareous sponges (Calcarea, Porifera). Chem Phys Lipids 156:26–32
5. Thiel V, Blumenberg M, Hefter J, Pape T, Pomponi S, Reed J, Reitner J, Wörheide G, Michaelis W (2002) A chemical view of the most ancient metazoan—biomarker chemotaxonomy of hexactinellid sponges. Naturwissenschaften 89:60–66
6. Barnathan G (2009) Non-methylene-interrupted fatty acids from marine invertebrates: occurrence, characterization and biological properties. Biochimie 91:671–678
7. Denis C, Wielgosz-Collin G, Bretéché A, Ruiz N, Rabesatroa V, Boury-Esnault N, Kornprobst JM, Barnathan G (2009) New 17-methyl-13-octadecenoic acid and 3,16-docosadienoic acids from the sponge *Polymastia penicillus*. Lipids 44:655–663
8. Bergquist PR, Lawson MP, Lavis A, Cambie RC (1984) Fatty acids composition and the classification of Porifera. Biochem Syst Ecol 1(2):63–84
9. Schreiber A, Wörheide G, Thiel V (2006) The fatty acids of calcareous sponges (Calcarea, Porifera). Chem Phys Lipids 143:29–37
10. Carballeira NM, Shalabi F (1995) The rare Caribbean sponge *Leucosolenia canariensis*: phospholipid fatty acids and sterols. Lipids 30:467–470
11. Tianero MD, Hanif N, de Voogd NJ, van Soest RW, Tanaka J (2009) A new antimicrobial fatty acid from the calcareous sponge *Paragrantia* cf *waguensis*. Chem Biodivers 6:1374–1377
12. Willis RH, de Vries DJ (1997) BRS-1, a C_{30} bis-amino, bis-hydroxy polyunsaturated lipid from an Australian calcareous sponge that inhibits protein kinase C. Toxicon 35:125–1129
13. Quévrain E, Domart-Coulon I, Pernice M, Bourguet-Kondracki ML (2009) Novel natural parabens produced by a *Microbulbifer* bacterium in its calcareous sponge host *Leuconia nivea*. Environ Microbiol 11:1527–1539
14. Roué M, Domart-Coulon I, Ereskovsky A, Djediat C, Perez T, Bourguet-Kondracki ML (2010) Cellular localization of clathridimine, an antimicrobial 2-aminoimidazole alkaloid produced by the Mediterranean calcareous sponge *Clathrina clathrus*. J Nat Prod 73:1277–1282
15. Gillian FT, Stoilov IL, Tam Ha TB, Raederstorff D, Doss GA, Li HT, Djerassi C (1988) Fatty acids as biological markers for bacterial symbiont in sponges. Lipids 23:1139–1145
16. Carter HJ (1871) A description of two new calcispongiae (*Trichogypsia*, *Leuconia*). Ann Mag Nat Hist 8:1–28
17. Van Soest RWM, Boury-Esnault N, Hooper JNA, Rützler K, de Voogd NJ, Alvarez B, Hajdu E, Pisera AB, Vacelet J, Manconi R, Schönberg C, Janussen D, Tabachnick KR, Klautau M (2008) World Porifera database. Available online at http://www.marinespecies.org/porifera. Accessed 15 Oct 2010
18. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
19. Meziane T, Tsuchiya M (2002) Organic matter in a subtropical mangrove-estuary subjected to wastewater discharge: origin and utilization by two macrozoobenthic species. J Sea Res 47:1–11
20. Andersson BA (1978) Mass spectrometry of fatty acid pyrrolidides. Prog Chem Fats other Lipids 16:279–308
21. Imbs AB, Rodkina SA (2004) Isolation of 2-methyl branched unsaturated very long fatty acids from marine sponge *Halichondria panicea* and identification of them by GC–MS and NMR. Chem Phys Lipids 129:173–181
22. Christie WW. AOCS Lipid Library. http://lipidlibrary.aocs.org/ms/ms03b/index.htm Accessed 29 Mar 2011
23. Christiansen K, Mahadevan V, Viswanathan CV, Holman RT (1969) Mass spectrometry of long-chain aldehydes, dimethyl acetals and alk-1-enyl ethers. Lipids 4:421–427
24. Sargent JR, Falk-Petersen IB, Calder AG (1983) Fatty acid compositions of neutral glycerides from the ovaries of the asteroids *Ctenodiscus crispatus*, *Asterias lincki* and *Pteraster militaris* from Balsfjorden, northern Norway. Mar Biol 72:257–264
25. Carballeira NM, Shalabi F, Stefanov K, Dimitrov K, Popov S, Kujumgiev A, Andreev S (1995) Comparison of the fatty acids of the tunicate *Botryllus schlosseri* from the Black Sea with two associated bacterial strains. Lipids 30:677–679
26. Kaneda T (1977) Fatty acids of the genus *Bacillus*: an example of branched-chain preference. Bacteriol Rev 41:391–418
27. Kaneda T (1991) *Iso*- and *anteiso*-fatty acids in bacteria: biosynthesis, function and taxonomic significance. Microbiol Rev 55:288–302
28. Hochmuth T, Niederkrüger H, Gernert C, Siegl A, Taudien S, Platzer M, Crews P, Hentschel U, Piel J (2010) Linking chemical and microbial diversity in marine sponges: possible role for poribacteria as producers of methyl-branched fatty acids. Chembiochem 11:2572–2578
29. Taylor J, Parkes JR (1983) The cellular fatty acids of the sulfate-reducing bacteria, *Desulfobacter* species, *Desulfobulbus* species and *Desulfovibrio desulfuricans*. J Gen Microbiol 129:3303–3309
30. Boon JJ, de Leeuw JW, Hoek GJ, Vosjan JH (1977) Significance and taxonomic value of *iso* and *anteiso* monoenoic fatty acids and branched hydroxy acids in *Desulfovibrio desulfuricans*. J Bacteriol 129:1183–1191
31. Carballeira NM, Shalabi F (1990) Identification of naturally-occurring *trans,trans* Δ-5,9-fatty acids from the sponge *Plakortis halichondroides*. Lipids 25:835–840
32. Christie WW, Brechany EY, Marekov KL, Stefanov KL, Andreev SN (2004) The fatty acids of the sponge *Hymeniacidon sanguinea* from the Black Sea. Comp Biochem Physiol A 109:245–252
33. Carballeira NM, Restituyo J (1991) Identification of the new 11, 15-icosadienoic acid and related acids in the sponge *Amphimedon complanata*. J Nat Prod 54:315–317
34. Barnathan G, Kornprobst JM, Doumenq P, Mirallès J (1996) New unsaturated long-chain fatty acids in the phospholipids from the axinellida sponges *Trikentrion loeve* and *Pseudaxinella* cf *lunaecharta*. Lipids 31:193–200
35. Valentine RC, Valentine DL (2004) Omega-3 fatty acids in cellular membranes: a unified concept. Lipid Res 43:383–402
36. Kornprobst JM, Barnathan G (2010) Demospongic acids revisited. Mar Drugs 8:2569–2577
37. Kraffe E, Soudant P, Marty Y (2004) Fatty acids of serine, ethanolamine, and choline plasmalogens in some marine bivalves. Lipids 39:59–66
38. Kuczynski B, Reo NV (2006) Evidence that plasmalogens are protective against oxidative stress in the rat brain. Neurochem Res 31:639–656