SHORT REPORT

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A New Short Chain Acetamide from the Biosphere and Bioactive Glycerolipids Extracted from the Marine Bibalve *Codakia orbicularis* (Lucinidae).

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Abstract: The marine bivalve *Codakia orbicularis*, harbors endosymbiotic bacteria located in its gill filaments. Bioassay-guided fractionation of an ethyl acetate extract of the gill tissues led to antibacterial fractions (against Gram positive and negative bacteria). The fraction eluted in AcOEt/ MeOH 90:10 displayed the greatest bioactivity. This fraction was analyzed using usual chromatographic and spectrometric methods and revealed three compounds. The first one (compound **1**) was an acetamide isolated for the first time from the biosphere. The other two were mono-glycerolipids (compound **2**) and (compound **3**) with at the position sn-2 of their glycerol monounsaturated fatty acid chains (respectively C18:1Δ^9 and C16:1Δ^9). Compounds **2** and **3** showed bacteriostatic activity against two pathogenic Gram-negative bacteria using a disc diffusion assay method. These molecules have been isolated for the first time from a bivalve. Further investigations are currently in progress, so as to understand the role of these bioactive compounds into the regulation of *C. orbicularis*' endosymbionts and/or to free-living pathogens.

Keywords: Antibacterial activity; monoacylglycol; ceramide.

1. Animal Source

Codakia orbicularis (Linné, 1758) individuals (40-60 mm shell length) were collected by hand from *Thalassia testudinum* sea-grass sediments in Guadeloupe (lat 16°09'01.7"N, long 61°33'42.9"W). These bivalves were identified by Pr. Olivier Gros (bivalve specialist).

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2. Previous Studies

Codakia orbicularis is an edible tropical bivalve belonging to the Lucinidae family. In order to assess its edibility, the first chemical study on this lucinid (whole organism without shell) has been conducted by Berg and his co-workers [1]. In their work on the bivalve, no toxins were found confirming thus its edibility. Lipids were mostly reported as it is the case for most bivalves. Some years later, other studies mainly focused on understanding how such an organism can survive into a sulfidic habitat. Biological investigations proved that this lucinid harbors a single endosymbiotic sulfur-oxidizing bacterial species located in its gill filaments [2, 3]. Flow cytometric analysis has shown that these endosymbionts maintain the duplication of their genome within the bivalve's gills without cellular division (each bacterial cell harbors up to seven copies of their genomes) [4] suggesting the existence of bacteriostatic molecules able to control bacterial cell division [5]. From that time, a new lectine called codakine, has been identified in the gills of *C. orbicularis* by crystallography and microcalorimetry but was not bioactive [6]. Few years later, a new spiro-indolothiazine named orbicularisine [7] and acetylated nucleoside derivatives [8] have been characterized by our group using both chromatographic and spectroscopic methods but no antibacterial activity has been found for none of these molecules.

3. Present Study

In order to find potential bacteriostatic molecules from the endosymbiotic gills of *C. orbicularis*, a bio-guided chemical study has been led on the gills.

Bioactivity test-Agar diffusion test [9]*:* Bacterial strains were used for heterologous bioassay: *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853, belonging to Gram negative bacteria; and *Enterococcus faecalis* ATCC 51299, *Micrococcus luteus* ATCC 10240, and *Streptococcus pneumoniae* ATCC 49619, belonging to Gram positive bacteria. Strains were cultivated on Mueller Hinton agar plate at 37°C for all bioassay experiments. Inocula were obtained using nutrient medium #1 (meat extract [5 g], agar [7.5 g], tryptic pepton [7.5 g], NaCl [2.5 g] and milliQ water [500 mL]; pH = 7) incubated at 37°C for 24 hours. One hundred microliters of a 10^{-2} dilution culture were spread on an agar plate with nutrient agar medium #1. Concerning the antimicrobial assay on Gram negative bacteria, 50 µg-amoxicillin antibiotic disc (Biomérieux, France) was used as positive control while on Gram positive bacteria, 50 µg-vancomycin antibiotic disc was used. Sterile assay discs (5 mm-diameter, Dutscher) were placed by plate after spreading. One of them received twenty microliters of ethyl acetate (solvent used to dissolve the compounds) as negative control. For the remaining sterile discs, each received twenty microliters of the prepared solution. The plates were then incubated for 24 hours at 37°C. Inhibition of a target strain was determined as positive when the diameter of the inhibition zone was at least 2 mm greater than the negative control.

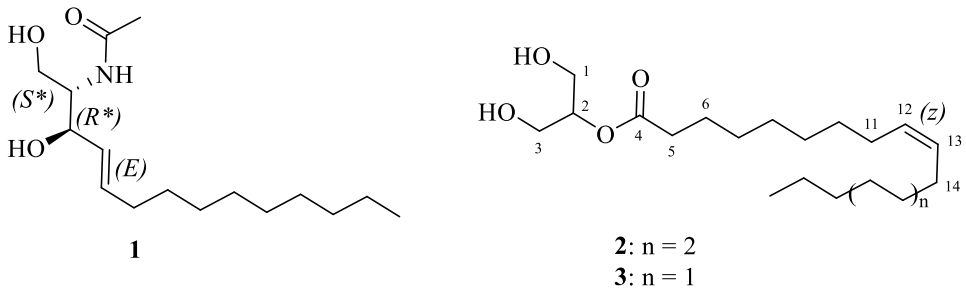
Bio-guided chemical study: Gill filaments of *C. orbicularis* were extracted with ethyl acetate, yielding twenty-five grams of crude extract. This crude extract proved to be active against two bacterial strains, *Escherichia coli* ATCC 35218 (Gram -) and *Micrococcus luteus* ATCC 10240 (Gram +), with inhibition halos of 15 mm and 10 mm respectively (see supplementary material, figure S19). The bioactive crude extract was chromatographed on silica gel column using a gradient of solvent mixtures starting from hexane/ethyl acetate followed by ethyl acetate/methanol to methanol. The fractions eluted with AcOEt/MeOH 90:10 V/V were the most active against the bacteria species mentioned earlier and were subjected to purification by HPLC. This latter purification led to the isolation of 0.5 mg of compound **1**, 2.5 mg of compound **2** and 2.3 mg of compound **3** (Figure 1). The structure of compounds **1**-**3** were elucidated by the current spectroscopic methods.

*Compound **1***: white solid; HRESIMS *m/z* 286.2390 $[M+H]^+$ (calc. for 286.2377; $C_{16}H_{31}NO_3$); ^{1}H NMR (MeOD, 600 MHz) δ 5.69 (1H, td, *J* = 7.8/ 7.8/ 14.7 Hz, H-5), 5.47 (1H, dd, *J* = 7.3/ 14.7 Hz, H-4), 4.06 (1H, t_{large}, *J* = 7.3 Hz, H-3), 3.86 (1H, dd, *J* = 6.4/ 10.8 Hz, H-2), 3.67 (2H, m, H-1), 2.04 (2H, m, H-6), 1.95 (3H, s, H-16), 1.38 (2H, m, H-7), 1.29 (12H, m, H-8 to H-13), 0.90 (3H, t, *J* = 7,3 Hz, H-14); ^{13}C NMR (MeOD, 150 MHz) δ 172.9 (C, C-15), 134.6 (CH, C-5), 131.0 (CH, C-4), 73.4 (CH, C-3), 62.2

(CH_2, C-1), 56.9 (CH, C-2), 33.1 (CH_2, C-6), 30.3 (CH_2, C-7 to C-12), 23.7 (CH_2, C-13), 22.6 (CH_3, C-16), 14.3 (CH_3, C-14).

*Compound **2**:* transparent oil; HRESIMS *m/z* 357.3009 $[M+H]^+$ (calc. for 357.3005; $C_{21}H_{41}O_4$); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.36 (2H, m, H-12 and H-13), 4.94 (1H, quin, *J* = 4.8 Hz, H-2), 3.85 (4H, d, *J* = 4.8 Hz, H-1 and H-3), 2.39 (2H, t, *J* = 7.5 Hz, H-5), 2.03 (4H, m, H-11 and H-14), 1.65 (2H, q, *J* = 7.1 Hz, H-6), 1.30 (20H, m, H-7 to H-10 and H-15 to H-20), 0.84 (3H, t, *J* = 7,3 Hz, H-21); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 174.4 (C, C-4), 130.0 (CH, C-12 and C-13), 74.9 (CH, C-2); 61.7 (CH_2, C-1 and C-3), 34.5 (CH_2, C-5), 27.3 (CH_2, C-11 and C-14), 25.0 (CH_2, C-6), 31.9 (CH_2, C-19), 29.5 (CH_2, C-7 to C-10 and C-15 to C-18), 22.7 (CH_2, C-20), 14.2 (CH_3, C-21).

*Compound **3***: transparent oil; HRESIMS *m/z* 329.2698 $[M+H]^+$ (calc. for 329.2692, $C_{19}H_{37}O_4$); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.33 (2H, m, H-12 and H-13), 4.89 (1H, quin, *J* = 4.8 Hz, H-2), 3.80 (4H, d, *J* = 4,8 Hz, H-1 and H-3), 2.35 (2H, t, *J* = 7.5 Hz, H-5), 2.00 (4H, m, H-11 and H-14), 1.62 (2H, q, *J* = 7,1 Hz, H-6), 1.29 (16H, m, H-7 to H-10 and H-15 to H-18), 0.87 (3H, t, *J* = 7.3Hz, H-19); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 174.4 (C, C-4), 130.0 (CH, C-12 and C-13), 74.9 (CH, C-2), 61.7 (CH_2, C-1 and C-3), 34.5 (CH_2, C-5), 27.3 (CH_2, C-11 and C-14), 25.0 (CH_2, C-6), 31.9 (CH_2, C-17), 29.5 (CH_2, C-7 to C-10 and C-15 to C-16), 22.7 (CH_2, C-18), 14.2 (CH_3, C-19).



1

2: n = 2
3: n = 1

Figure 1. Structures isolated in the present work.

Compound **1** presented an exact mass of 285.2304 which suggested its molecular formula as $C_{16}H_{31}NO_3$ with two degrees of unsaturation (*m/z* 286.2390 $[M+H]^+$; calc. 286.2377 $\delta \pm 0.0013$ ppm). Its ^{1}H NMR shown olefinic and aliphatic protons including a characteristic acetyl group observed by the H-16 proton at δ_H 1.95. From the ^{13}C NMR data, it has been deduced that the only nitrogen into the structure of compound **1** was connected to the C-2 carbon at δ_{C2} 56.9 which was bounded to a proton δ_H 3.86 (H-2). ^{1}H-^{1}H COSY spectra revealed that this H-2 proton was correlated to the H-3 methine proton at δ_H 4.06 and to the two H-1 methylene protons at δ_H 3.67. According to the ^{1}H-^{13}C HSCQ spectra, the H-3 was supported by the C-3 carbon at δ_C 73.4 whereas the two H-1 by the C-1 carbon at δ_C 62.2. The chemical shifts of C-1 and C-3 carbons were characteristics of carbons linked to hydroxyl groups. The carbon C-3 at δ_C 73.4 from the 1,3-propanediol, 2-amino fragment found, have been connected via strong ^{1}H-^{13}C HMBC correlations to the olefinic proton at δ_H 5.47 (H-4) corresponding to the carbon C-4 at δ_C 131.0 (^{1}H-^{13}C HSQC data). The combined 2D NMR spectra revealed that the second carbon of the double bound, C-5 at δ_C 134.6 and wearing the H-5 proton (δ_H 5.69) , was attached to a nine-carbon alkyl chain formed by aliphatic protons (H-6 – H-14) linearly assembled. Because of the large coupling between the olefinic protons (H-4; H-5, $J_{4,5}$ = 14.7 Hz) the *E* configuration was attributed to this double bound [10]. The above results led to a sphingoid base. Otherwise the ^{1}H-^{13}C HMBC spectrum of compound **1** showed correlations between the protons at δ_H 1.95 ppm (H-16) and the C-15 carbonyl at δ_C 172.9 corresponding to an acetyl group (Figure 2). All these data shown that compound **1** was a *N*-acetylated sphingoid base. By comparing C-2 and C-3 chemical shifts with those of synthetic and natural ceramides, the relative configuration was deduced to be 2*S**, 3*R** which is a characteristic configuration in sphingosine-type bases of marine invertebrates [10, 11]. **1** is a 14-ES ceramide namely the *N*-[(*2S**, *3R**, *4E**)-3-hydroxy-2-(hydroxymethyl)-4-tridecen-1-yl]-acetamide. Natural ceramides usually occur with the configuration 2*S*, 3*R*, 4E [11]. Compound **1** is a natural ceramide formed by a C2 fatty acid and a C14 sphingosine backbone. Ceramides with short fatty acids (C2) have been only found before in mammals and are formed by an acetyl transfer from platelet-activating factor to a sphingosine [12]. The short chain acetamide (compound **1)** has been known until now only as a synthetic compound (CAS

number 2097561-20-9). Here we report the isolation of *N*-[(*2S**, *3R**, *4E**)-3-hydroxy-2-(hydroxymethyl)-4-tridecen-1-yl]-acetamide (compound **1**) for the first time from the biosphere.

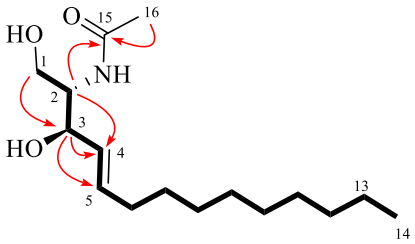


Figure 2. ^{1}H-^{13}C HMBC (arrows) and ^{1}H-^{1}H COSY (bold) correlations in compound **1**.

The molecular formula of compound **2** was found to be $C_{21}H_{40}O_4$ (Δm = 1,1 ppm) by HRESIMS which displayed a protonated molecule $[M + H]^+$ at *m/z* = 357.3009. The HRESIMS of compound **3** gave a positive ion at *m/z* = 329.2698 corresponding to the molecular formula $C_{19}H_{36}O_4$ (Δm = 1,8 ppm). Compounds **2** and **3** shown a mass difference of 28 Da equivalent to two methylene groups and both molecules had similar ^{1}H NMR spectra showing a first part corresponding to signals of unsaturated fatty acids and a second part to a glycerol moiety [13, 14]. Monoglycerolipids with an oleic fatty acid or a palmitoleic fatty acid have been already discovered but the ^{1}H NMR spectra of compounds **2** and **3** showed some differences with those of the literature for the signals of the glycerol [15, 16, 17]. The ^{1}H NMR data of compound **2** are compared to those of an α-monoglyceride of oleic acid [16] in **Table 1.**

Table 1. ^{1}H NMR spectroscopic data of compound (**2**) compared to literature's data, both in $CDCl_3$.

Present work (compound **2**)			Literature (α-monoglyceride of oleic acid; [16])	
Location	*Type*	δ_H (*J* in Hz)	*Type*	δ_H (*J* in Hz)
12,13	CH	5.36, m	CH	5.35, m
2	CH	4.94, quin (*J* = 4.8 Hz)	CH	4.21, dd (*J* = 11.7/ 4.3 Hz)
1, 3	CH_2	3.85, d (*J* = 4.8 Hz)	CH_2	3.93, m; 4.15, dd (*J* = 11.7/ 5.8 Hz)
			CH_2	3.60, dd (*J* = 11.7/ 5.8 Hz); 3.70, dd (*J* = 11.7/ 3.9 Hz)
5	CH_2	2.39, t (*J* = 7.5 Hz)	CH_2	2.35, t (*J* = 7.6 Hz)
11, 14	CH_2	2.03, m	CH_2	2.02, m
6	CH_2	1.65, q (*J* = 7.1 Hz)	CH_2	1.63, m
7-10 and 15-20	CH_2	1.30, m	CH_2	1.29, m
21	CH_3	0.84, t (*J* = 7.3 Hz)	CH_3	0.88, t (*J* = 6.8 Hz)
4	C	--------	C	--------

The analysis of both compounds was performed in deuterated chloroform. The signal differences between compound **2** and the α-monoglyceride of oleic acid from the literature have been presented in bold type in Table 1 and have been explained by the position of the fatty acid on the glycerol. In fact, the 4/1 ratio observed between the protons of the glycerol moiety at δ_H 4.94 (H-1 and H-3) and at δ_H 3.85 (H-2) proved an equivalence of these methylene and suggested a symmetry in the structure of compounds **2** and **3**. This symmetry was again validated by ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC correlations between the protons at δ_H 3.85 (H-2) and the carbon at δ_C 61.7 ($CH_{2glycerol}$). It was deduced that the fatty acid chain of compounds **2** and **3** was on the position *sn*-2 of the glycerol for both compounds' contrary to this one of the literatures which was on the position *sn*-1 (or *sn*-3). Furthermore, the multiplet at δ_H 5.36 (H-12 and H-13) indicated one unsaturation for each fatty acid and according to Liu and his co-workers [18] the chemical shifts of the associated carbons δ_C 27.3 (C-11 and C-14) confirmed an unsaturation with a Z configuration. In the literature, only D-9 mono-unsaturated fatty acids have been described as part of sn-2 substituted mono-glycerolipids isolated from plants or microalgae [19]. In addition to that, enzymatic hydrolysis of triglycerides often led to *sn*-2 mono-

extracted from a bivalve. As many kinds of glycerolipids are described to be bioactives [22], it encouraged glycerolipids [20] and the triglyceride *1*-linoleoyl-*2*-oleoyl-*3*-stearoyl-glycerol has been previously isolated among 15 g of other triacylglycerols from *C. orbicularis* by our group (data not shown). We confronted our data to those given in the literature, which led us to the identification of compound **2** as being 9-octadecenoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester and compound **3** which was identified as 9-palmitoleic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester. So far, in the marine biosphere, monoglycerolipids have only been reported from algae [21] and sponges [15] but none have been previously the evaluation of the bioactivity of compounds **2** and **3** according to the agar diffusion method described above for assessing antibacterial activity.

The highest inhibition zone was obtained against *E. coli* ATCC 35218 with a diameter of 13.5 ± 0.5 mm for compound **2** and 12.5 ± 0.5 mm for compound **3**. Both compounds revealed a moderate activity against *K. pneumonia* ATCC 700603 (10.5 ± 0.5 mm). Compounds **2** and **3** were inactive against the Gram-positive bacteria tested but they showed an antibacterial activity against two Gram-negative bacteria. These results are interesting as 90% of the bacteria found into the marine biosphere are Gram-negative bacteria [23] and the positive bacteria detected in coastal marine environments are generally faecal contaminants of human origin or potentially human pathogens [24] with a short lifespan due to the salts. Glycerolipids have already presented several bioactivities (cyclooxygenase-II inhibitor; algicidal) [16]. Up to now, the dodecylglycerol was the only antibacterial natural product described in the literature [25]. Here, compounds **2** and **3** are reported as two new bioactive glycerolipids.

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Supporting Information

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