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Note

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Orbicularisine: A Spiro-Indolothiazine Isolated from Gills of the Tropical Bivalve *Codakia orbicularis*

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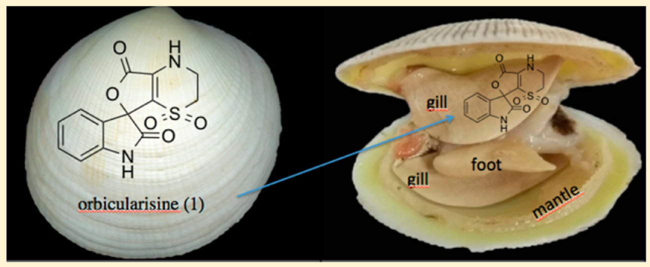
Supporting Information

ABSTRACT: A novel spiro-indolofuranone fused to a thiazine skeleton, orbicularisine (**1**), was isolated from gills of the mollusk *Codakia orbicularis*. The isolation and structure elucidation using spectroscopic evidence including mass and NMR spectroscopy are described. The final structure of **1** was supported by key HMBC correlation.



Mollusks are the second largest animal phylum on Earth constituting approximately 7% of living animals.[1] For 52 000 named species of marine mollusks described to date,[2] no more than 1000 have been subjected to chemical studies, leading to the description and characterization of about 1000 secondary metabolites.[3] Thereby, marine mollusks have become the focus of many natural products studies aimed at the isolation of new metabolites.[1] Some of the metabolites have therapeutic value, such as ziconotide, which was isolated from cone snails and is used for the treatment of chronic pain.[4] Kahalalide F (ES-285), isolated from a bivalve, has succeeded in phase I of clinical trials. Finally, dolastatin 10, described from a sea-hare, showed anticancer activity and is currently in phase II of clinical trials.[1,5] A synthetic analogue of dolastatin 10 linked to an anti-CD30 antibody, Adcetris, that is known as brentuximab vedotin, is the latest marine drug to successfully enter into the market.

We herein describe the isolation of a novel metabolite orbicularisine (**1**) from the tropical bivalve *Codakia orbicularis* living in seagrass beds. While this mollusk has been studied for years as a biological model for shallow water thio-autotrophic bacterial symbiosis,[6] as far as we are aware, no chemical studies have been conducted to date. This clam harbors intracellular sulfur-oxidizing bacteria within its gill tissues,[7] and such bacterial population is probably regulated by the host itself, using specific molecules with bacteriostatic activities.

Gill filaments were extracted with EtOAc. From this latter EtOAc extract, 20 g of yellow elemental sulfur S_8 precipitated.[8] After filtration and solvent evaporation of the supernatant, 25 g of extract was obtained. The residue was chromatographed on silica gel column using a gradient of solvent mixtures starting from hexane/EtOAc and then EtOAc/MeOH. The most antibacterial fractions according to disc agar plate method[9] were subjected to repeated separation and purification by HPLC (Sunfire C-18 columns) to provide a total of 8.1 mg of pure orbicularisine (**1**) (Figure 1).

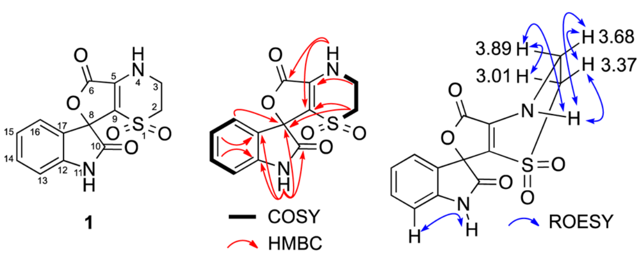


Figure 1. Structure of orbicularisine (**1**), COSY, and key HMBC and ROESY correlations.

The molecular formula of compound **1** was suggested to be $C_{13}H_{11}N_2O_5S$ by HRESIMS, which displayed a protonated molecule at m/z 307.0391 $[M + H]^+$, appropriate for 10 degrees of unsaturations. ^{1}H NMR and HSQC spectra revealed two methylenes at δ_H 3.01 (H-2b)/3.37 (H-2a) correlated to the carbon at δ_C 48.1 (C-2) and δ_H 3.68 (H-3b)/3.89 (H-3a) correlated to δ_C 40.8 (C-3). The COSY correlations sequence between H-2, H-3, and H-4 (NH) (Figure 1) and the characteristic ^{13}C chemical shifts at δ_C 48.1 and 40.8 (Table 1) suggested a part of the thiazine SO_2–C-2–C-3–N-4.[10,11] The characteristic strong IR absorbance at 1129 cm^{-1} supported the sulfone function. Further HSQC analysis (Table 1) showed clearly an aromatic part where the protons at δ_H 7.39 (H-16), 7.36 (H-14), 7.02 (H-15), and 6.91 (H-13)

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound 1 in DMSO-d_6 (^{1}H 500 MHz, ^{13}C 125 MHz)

	orbicularisine (1)	
atom	δ_C/δ_N, type	δ_H, mult (J in Hz)
2a	48.1, CH_2	3.37, m
2b		3.01, ddd (13.6, 12.6, 4.0)
3a	40.8, CH_2	3.89, m
3b		3.68, ddd (13.8, 12.6, 2.0)
4	74.1, NH	8.47, brs
5	138.3, C	
6	165.1, C	
8	82.2, C	
9	111.2, C	
10	170.9, C	
11	133.8, NH	10.96, brs
12	142.8, C	
13	110.8, CH	6.91, d (7.6)
14	132.1, CH	7.36, dt (7.6, 1.1)
15	122.6, CH	7.02, dt (7.6, 1.1)
16	126.6, CH	7.39, d (7.6)
17	122.4, C	

were attached to carbons resonating at δ_C 126.6 (C-16), 132.1 (C-14), 122.6 (C-15), and 110.8 (C-13), respectively.

The remaining exchangeable proton signals (δ_H 10.96 (H-11)/8.47 (H-4)) were attributed by $^{1}H-^{15}N$ HSQC, as an amide (δ_N 133.8, N-11) and a secondary amine (δ_N 74.1, N-4). The UV spectrum (λ_{max} (MeOH): 210, 250, and 292 nm) suggested the presence of an indoline chromophore group.[12] Moreover, strong HMBC correlations were observed from the amide proton H-11 at δ_H 10.96 to the spiro-carbon C-8 at δ_C 82.2 and the nonprotonated carbon C-17 at δ_C 122.4. In addition, weak correlations were observed between H-11 and the nonprotonated carbons C-12 (δ_C 142.8) and C-10 (δ_C 170.9). The HMBC correlations between the protons H-14/H-15 and the carbons C-12 (δ_C 142.8)/C-17 (δ_C 122.4) suggested the indolone partial structure.[13−15] These results suggested a lactam connected to the aromatic spin system C-16−C-15−C-14−C-13. Fusion of this moiety to the remaining unassigned carbons δ_C 111.2 (C-9), 138.3 (C-5), and 165.1 (C-6) was deduced from the long-range HMBC correlations from the proton at δ_H 3.37 (H-2a) to the spiro-carbon at δ_C 82.2 (C-8) and the nonprotonated carbon at δ_C 111.2 (C-9). Likewise the proton at δ_H 3.89 (H-3a) showed correlations with the nonprotonated carbon at δ_C 138.3 (C-5). In addition H-4 of the thiazine NH showed a correlation with C-6 and confirms the orientation of the thiazine.

These latter were together attached to form a double bond and a cyclic thiazine.[16] According to Davis and co-workers, thiaplakortone displayed a double bond in a quinone−thiazine bicyclic system with similar chemical shifts.[17] In accordance with the HRMS data, the two carbonyl oxygens completed the formula. Moreover, on the basis of HMBC data, the amine proton H-4 of the cyclic thiazine showed correlations with the lactone carbonyl at δ_C 165.1 (C-6) and the nonprotonated carbons at 111.2 (C-9) and 138.3 (C-5). Thus, the cyclic thiazine was found to be fused with the furanone, which is connected to the indolone through the spiro-carbon C-8 at δ_C 82.2. IR data of compound **1** secured this assignment because the vibration frequency of this lactone was 1787 cm^{-1} corresponding to a furanone.[18] It is interesting to note that the IR spectrum confirmed the existence of the carbonyl at 1740 cm^{-1} and secondary amide and amine at 3308 cm^{-1}. The structural fragments were validated by LTQ-Orbitrap-SM^2 due to the formation of sodium fragment ions at m/z 193, 220, 257, and 285(Figure 2).[19]

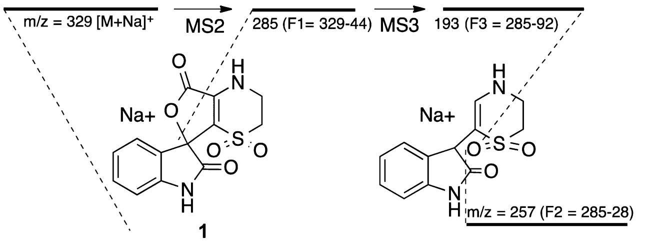

Figure 2. LTQ-Orbitrap-MS^2 and LTQ-Orbitrap-SM^3 fragmentations from the sodium adduct ion $[M + Na]^+$ of compound **1**.

At this step, all atoms had been accounted for the appropriate structure **1-A** (Figure 3). Although the regioisomer **1-B** had

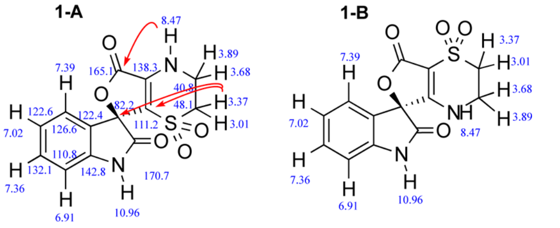

Figure 3. Discriminatory HMBC correlations corroborating the structure **1-A** for compound **1** instead of **1-B**.

been considered, the HMBC correlations from the NH of the thiazine to the lactone carbonyl carbon and the proton at δ_H 3.37 (H-2a) to the spiro-carbon at δ_C 82.2 (C-8) and the nonprotonated carbon at δ_C 111.2 (C-9) correlated to the regioisomer **1-A**.

Orbicularisine (**1**) contains one single stereocenter. The zero value of the optical rotation and the flat ECD spectrum indicated the racemic nature of the compound.

The spiro-indolothiazine skeleton of orbicularisine (**1**) could be derived from tryptophan and cysteine or their close derivatives, indole-3-propionic acid and taurine.

Orbicularisine (**1**) was subjected to various bioassays. Despite the antibacterial activity of the original fraction on Gram-negative and Gram-positive panel of bacteria, orbicularisine (**1**), which was the major compound of the fraction, was totally inactive against *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumonia*, *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 25922.

Inhibition assays against a panel of kinases including Hs_CDK2/CyclinA, Hs_CDK5/p25, Hs_CDK9/CyclinT, Hs_RIPK3, Hs_Haspin, Hs_AuroraB, Ld_TLK, Hs-Pim1, Ssc_GSK3 a/b, Lm_CK1, and Rn_Dyrk1A showed residual activities >60% for 16 μM/mL of orbicularisine (**1**). Finally, the treatment of HCT116 colon cancer cells and U87-MG glioblastoma cancer cells with concentrations up to 100 μM showed no activity. Further bioassays using various targets are in progress. It is intriguing to speculate about the role of orbicularisine (**1**) within the host bivalve. This issue is also a central theme in the research efforts, which we will continue by additional chemical and biological investigations.

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EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations $[\alpha]_D$ were measured using an Anton Paar MCP-300 polarimeter. Electronic circular dichroism (ECD) experiments were performed on a Jasco J-810 spectropolarimeter. The IR spectrum was recorded on a PerkinElmer BX Fourier transform infrared spectrometer. One- and two-dimensional NMR spectra were recorded on a Bruker Avance 500 MHz or a Bruker Avance 600 MHz (TXI 1.7 mm probe) (CNRS-ICSN). The chemical shifts are relative to the residual signal solvent (CD_3OD: δ_H 3.31; δ_C 49.20; DMSO-d_6: δ_H 2.5; δ_C 39.5). High-resolution mass spectra were obtained on a Waters LCT Premier XE spectrometer in electrospray ionization mode by direct infusion of the pure compounds. Fragmentations were conducted in electrospray and nanospray ionization mode on a Thermo Scientific LTQ-Orbitrap connected with a chromatographic system Dionex Ultimate 3000. Column chromatography (63 × 3 cm) was performed on silica gel (Merck silica gel, 70−230 mesh ASTM), and flash chromatography was performed on Redisep R_f (220 g, 69-2203-422 TELEDYNE Isco). These were carried out using hexane, EtOAc, and MeOH. Thin layer chromatography (TLC) was performed on commercial TLC plates (precoated Kiesegel 60 F254 TLC, 20 × 20 mm, thickness 0.25 mm, Merck) and was visualized under UV (254 and 366 nm). Analytical HPLC analyses were performed using Waters Alliance 2695 separation module equipped with a mass spectrometer (Waters ZQ 2000 with a single-quadrupole and electrospray ionization source), ELS detector (Waters 2420), and photodiode array detection DAD (Waters 996). Semipreparative HPLC was performed using an Auto Prep system (Waters 600 controller and Waters 600 pump, equipped with a Waters 996 photodiode array detector).

Biological Material. Adult *Codakia orbicularis* (Linné, 1758) individuals (40−60 mm shell length) were collected in May 2015 by hand from *Thalassia testudinum* seagrass sediments in Guadeloupe (lat 16°9′0.596″N, long 61°33′41.797″W). The fresh material was identified by one of the authors (O.G.). Fresh gills were dissected and stored at −20 °C until chemical extraction.

Extraction and Isolation of Compound 1. Gills (3 kg) were extracted with EtOAc at room temperature under agitation for 2 days. The EtOAc solution was stored at 4 °C for 12 h. The residue formed by precipitation, which is pure elemental sulfur S_8, was separated from the EtOAc solution by filtration. The EtOAc fraction was evaporated under reduced pressure then under nitrogen purge. The residue (25 g) was fractionated by chromatography on a silica gel column using a solvent mixture of increasing polarity (EtOAc−hexane, EtOAc, EtOAc−MeOH, and MeOH), to yield 16 fractions (F_1−F_{16}). Fractions F_1−F_{12} were eluted with a hexane−EtOAC gradient (20−100%) and fractions F_{13}−F_{16} with a EtOAc−MeOH gradient (10−100%). All 16 fractions were tested on *Micrococcus luteus* ATCC 10240 (Gram-positive bacterium) and *Escherichia coli* ATCC 35218 (Gram-negative bacterium) for qualitative antimicrobial activity using the disc diffusion assay, and nine of them showed encouraging (up to 18 mm diameter) bioactivity. Fraction F_{14} (70 mg, eluted with EtOAc−MeOH/90−10%) was the most active. The HPLC profile of F_{14} was recorded using a stepwise gradient of Milli-Q H_2O and 2−100% MeCN, both with 0.1% HCOOH (flow rate = 1 mL/min; Waters Sunfire C-18 column, 150 × 4.6 mm, 5 μm). Semipreparative HPLC of this fraction (Waters Sunfire C-18 column, 150 × 10 mm, 5 μm; flow rate = 4.5 mL/min) with the same transposed gradient, gave five subfractions F_{14-1} (10 mg), F_{14-2} (10.7 mg), F_{14-3} (11.8 mg), F_{14-4} (17,4 mg), and F_{14-5} (6.8 mg). The major compound in F_{14-1} at retention time 12.52 min was detected and was slowly precipitated to give 3.9 mg of the pure compound **1** (noted $F_{14-1res}$). Semipreparative HPLC in the same conditions described above was performed on the supernatant of F_{14-1} and led to 1.9 mg of the pure compound **1**. Fraction F_{14-2} gave equally 0.7 mg of **1**. Similar protocol applied to the next fraction F_{15} (184 mg, eluted with EtOAc−MeOH/80−20%) led to 1.6 mg of **1**. Finally, a total of 8.1 mg of this compound (**1**) named orbicularisine was isolated.

Orbicularisine (1): yellow powder; $[\alpha]_D^{25}$ 0.0 (*c* 3.7, MeOH); UV (MeOH) λ_{max} (log ε) 210 (1.32), 292 (0.41) nm; IR (neat) ν_{max} 3308, 2934, 1788, 1740, 1663, 1622, 1474, 1293, 1181, 1129, 1029, 799, 684 cm^{-1}; for ^{1}H NMR (DMSO, 500 MHz) and ^{13}C NMR (DMSO, 125 MHz), see Table 1; HRESIMS *m/z* 307.0391 [M + H]$^+$ (calcd for $C_{13}H_{11}N_2O_5S$, 307.0389).

Bacterial Strains. A panel of bacterial strains were used for bioassays: *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, and *Streptococcus pneumonia* ATCC 49619 belong to Gram-positive bacteria, and *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 25922 belong to Gram-negative bacteria. These strains were obtained from the collection of the Pasteur Institute (Guadeloupe) and were cultivated on agar plates with Mueller-Hinton nutrient medium at 37 °C for all bioassay experiments.

Bioassay Using Disc Diffusion Assays. Individual chromatographic fractions were evaluated on *Micrococcus luteus* (ATCC 10240) and *Escherichia coli* (ATCC 35218). An agar diffusion assay modified according to Brinkhoff et al.[20] was used. 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 Milli-Q water [500 mL]; pH 7) incubated at 37 °C for 24 h. One hundred microliters of a 10^{-2} dilution culture was spread on an agar plate with nutrient agar medium #1. Concerning the antimicrobial assay on Gram-negative bacteria, 50 μg of an amoxicillin antibiotic disc (Biomérieux, France) was used as positive control, whereas on Gram-positive bacteria, 50 μg of a vancomycin antibiotic disc was used. Moreover, two sterile antibiotic assay discs (5 mm diameter, Dutscher) were placed by plate after spreading. One of them received 20 μL of EtOAc as the negative control. Fractions were solubilized by EtOAc, and the remaining sterile discs received 20 μL of the prepared solution. The plates were then incubated for 24 h at 37 °C. Inhibition zones of bacterial development around the impregnated discs containing the fractions will appear if they are active fractions. 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. The experiments were performed in three replicates from three different extractions.

Bioassay Using MIC Method in Liquid Culture. Compound **1** (2.7 mg) was dissolved in DMSO (100 μL) and then diluted with distilled H_2O (900 μL). From this solution, eight diluted solutions were prepared: 256, 128, 64, 32, 16, 8, 4, and 2 μg/mL. Microbial suspensions were prepared in sterile 96-well cell culture plates (Cat. No. 655180, CELLSTAR). Each well received 178 μL of Mueller-Hinton medium, 20 μL of one of the solutions S2−S9, and 2 μL of an overnight culture of bacteria corresponding to 0.5 McFarland[21] scale, namely, 1.5 × 10^8 CFU/mL. Two controls were prepared. The first one was used in order to know if the DMSO inhibited bacterial growth. This control was composed of 2 μL of the overnight culture bacteria (1.5 × 10^8 CFU/mL), 178 μL of Mueller-Hinton medium, and 20 μL from a solution containing 100 μL of DMSO and 900 μL of H_2O. To ensure that working conditions were sterile (no contamination by environmental bacteria), a second control was used (180 μL of Mueller-Hinton medium + 20 μL from a solution consisting of 100 μL of DMSO and 900 μL if H_2O). The same protocol was applied for each bacterial strain. The plate was incubated for 24 h at 37 °C and inspected for any inhibition (clear) or growth of bacteria (turbid).[22]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00149.

Spectroscopic data, ^{1}H, ^{13}C, NMR, COSY, HSQC, HMBC, ROSY, ^{1}H−^{15}N HSQC, ^{1}H−^{15}N HMBC, IR, UV, HRMS and EDC for orbicularisine **1** (PDF)

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Notes
The authors declare no competing financial interest.

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