Cellular Localization of Clathridimine, an Antimicrobial 2-Aminoimidazole Alkaloid Produced by the Mediterranean Calcareous Sponge *Clathrina clathrus*

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Received March 12, 2010

Chemical investigation of the Mediterranean calcareous sponge *Clathrina clathrus* led to the isolation of large amounts of a new 2-aminoimidazole alkaloid, named clathridimine (**1**), along with the known clathridine (**2**) and its zinc complex (**3**). The structure of the new metabolite was assigned by detailed spectroscopic analysis. Clathridimine (**1**) displayed selective anti-*Escherichia coli* and anti-*Candida albicans* activities. Clathridine (**2**) showed only anti-*Candida albicans* activity, and its zinc complex (**3**) exhibited selective anti-*Staphylococcus aureus* activity. The isolation of analogues of 2-amino-imidazole derivatives from several *Leucetta* species from various sites in the Pacific Ocean and the Red Sea raises the question of their biosynthetic origin. Microscopic studies revealed abundant extracellular bacteria located in the mesohyl of the sponge, with two predominant morphotypes including spiral bacteria and long, narrow bacilli. Chemical analysis with HPLC/UV/ELSD profiles of sponge cells separated from bacteria by differential centrifugation and trypsinization of the sponge cell surface revealed that clathridine (**2**) was localized in the sponge cells.

Marine sponges are a rich source of structurally unique natural compounds, with many of them displaying a wide variety of biological activities.[1] They are also known to host a wide diversity of microorganisms that can amount up to 40−60% of the biomass of the sponge and exceed seawater concentrations by 2−4 orders of magnitude.[2−4] Recently, these associations have raised questions about the origins of the metabolites isolated from sponges.[5,6] However, despite this growing interest in marine symbiotic association research, the complex interactions between sponges and associated microsymbionts remain difficult to investigate. Thus the putative origin of natural compounds isolated from sponges is often inferred from cellular localization data.[7−12]

As part of our ongoing investigations on sponges within the framework of the French program ECIMAR (Ecologie Chimique: Indicateurs de Biodiversité et Valorisation), we studied the chemistry and microbiology of the Mediterranean calcareous sponge *Clathrina clathrus* (Schmidt, 1864) (Calcispongia, Calcinea, Clathrinida, Clathrinidae). Unlike Demospongiae, Calcispongia have been minimally studied in terms of both chemistry and microbiology. Furthermore, their relatively low microbial density and diversity, as reported for example for the Calcinea species *Pericharax heteroraphis* (Poléjaeff, 1883)[3,13] and for the Calcaronea species *Grantia compressa* (Fabricius, 1780),[2,13] might represent an advantage to better understand the origin of the secondary metabolites they produce and the putative role of their endosymbionts.

Calcareous sponges of the Calcinea subclass are known as sources of 2-aminoimidazole alkaloids with more than 40 reported compounds including clathridines,[14−17] naamidines and isonnamidines,[17−25] and naamines and isonaamines.[18,19,21,22,26−29] Some of these alkaloids have promising biological activities such as antifungal,[14,21] cytotoxic,[23−25] anti-inflammatory,[30] and antitumor activities.[31] Most of them have been isolated from species belonging to the genus *Leucetta* (Haeckel 1872), in the subclass Calcinea, order Clathrinida, and family Leucettidae in specimens collected from various geographical sites (Pacific Ocean and Red Sea). These compounds have also been isolated from nudibranchs of the genus *Notodoris*, which feed on these sponges and sequester the alkaloid pigments in their dorsal mantle.[16−18,31] To date there is no hypothesis regarding their biosynthesis, but their isolation from several *Leucetta* species from various sites raises the question of their biosynthetic origin. Currently, there is little microbiological information available on sponges belonging to the Clathrinidae family. There is only one ultrastructural report of a homogeneous population of one morphological type of Gram-negative elongated bacterium inhabiting the aquiferous canal system of *Clathrina cerebrum* (Haeckel, 1872) and of rare coiled bacteria associated with choanocytes of *Clathrina clathrus*.[33]

The 1:1 CH_2Cl_2/MeOH extract of *C. clathrus* revealed interesting broad-spectrum antimicrobial activities against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial strains and against the yeast *Candida albicans*. The HPLC/UV/ELSD/MS profile of the 1:1 CH_2Cl_2/MeOH extract revealed the presence of four metabolites belonging to the 2-aminoimidazole alkaloid family: the new clathridimine (**1**), the known clathridine (**2**), and two minor metabolites, namely, clathridine-zinc complex (**3**) and preclathridine (**4**).

The current report describes the isolation and purification of compounds **1**−**3**, the structure elucidation of clathridimine (**1**), and the antimicrobial activities of **1**−**3**. Furthermore, due to the detection of an abundant bacterial community within the sponge, morphologically characterized with scanning (SEM) and transmission (TEM) electron microscopy, we investigated the cellular localization of these secondary metabolites. Chemical profiles of populations enriched either in bacteria or in cell types of *C. clathrus*, separated by differential centrifugation and trypsinization of the sponge cell surface, are presented.

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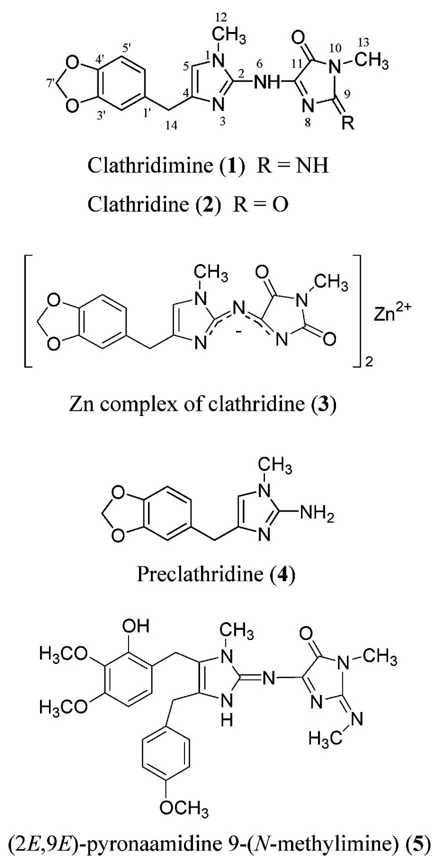
10.1021/np100175x © 2010 American Chemical Society and American Society of Pharmacognosy
Published on Web 06/30/2010



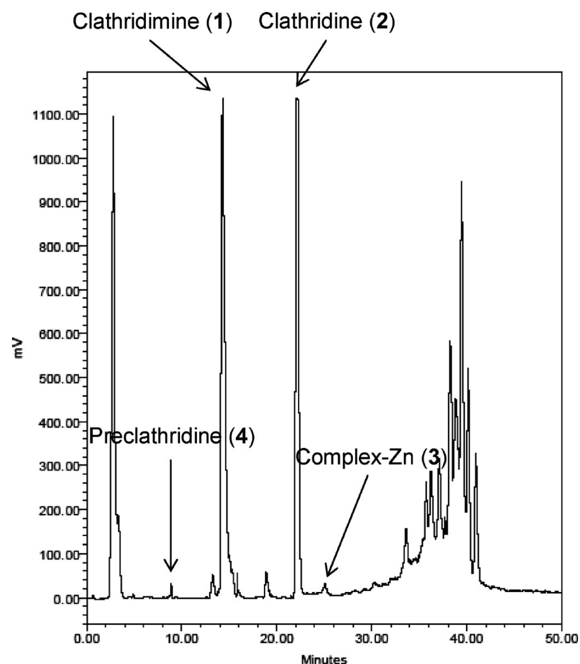


Figure 1. HPLC/ELSD chromatogram of the CH_2Cl_2/MeOH extract of *Clathrina clathrus*.

Results and Discussion

The HPLC/ELSD chromatogram of the 1:1 CH_2Cl_2/MeOH extract (Figure 1), after desalinization on a C18 SPE column, revealed the presence of two major compounds, **1** and **2**. Successive chromatographies of the extract on reversed-phase columns led to the isolation of three 2-aminoimidazole alkaloids, **1**−**3**, belonging to the clathridine family, as first suggested by the UV profiles. The structures of these natural products were determined by mass analysis and 1D and 2D NMR spectroscopic studies. Preclathridine (**4**), previously isolated from the nudibranch *Notodoris gardineri*,[16] was also observed in the sponge extract, as deduced from LC/MS experiments, but the molecule was present in an amount too small to be isolated.

Compound **2** was obtained as yellow crystals. Its molecular formula $C_{16}H_{15}N_5O_4$ was obtained from its ESIMS, which showed the pseudomolecular ion $[M + H]^+$ at *m/z* 342.1189, indicating the presence of 12 unsaturations in the molecule. Detailed examination of the spectroscopic data readily established its identity as clathridine, an antifungal compound previously isolated from *C. clathrus*.[14]

Compound **1** was obtained as a yellow-orange solid. ESIMS showed the pseudomolecular ion $[M + H]^+$ at *m/z* 341.1354, which differed from that of clathridine (**2**) by only one dalton, and led to the molecular formula $C_{16}H_{16}N_6O_3$, implying the presence of 12 unsaturations in the molecule. Comparison of its ^{1}H NMR and ^{13}C NMR spectroscopic data with those of clathridine **2** confirmed strong similarities between the two compounds. Compound **1** possessed a similar piperonyl unit, as indicated in its ^{1}H NMR spectrum ($CDCl_3$) with the presence of two methylene singlets at δ 3.88 and 5.93 and three aromatic protons at δ 6.72 (s) and at δ 6.70 (d, $J = 7.8$ Hz) and 6.76 (d, $J = 7.8$ Hz), which was confirmed with 2D NMR data. An additional singlet methine proton at δ 6.39 (δ_C 115.9) and singlet N-methyl protons at δ 3.67 (δ_C 32.2) and 3.17 (δ_C 25.6), supported by further resonances in the ^{13}C NMR spectrum of quaternary carbons at δ_C 147.6 and 133.9 corresponding to two fully substituted sp^2 carbon atoms and one carbonyl (δ_C 163.2), were also reminiscent of clathridine (**2**) (Table 1). Comparison of all these data indicated that in **1** one carbonyl group was replaced by one imino group. Its localization, out of the two possibilities (C-9 or C-11), was solved by the resonance at δ_C 158.5 corresponding to the carbon bearing the imino group in position C-9, as previously reported for (2*E*,9*E*)-pyronaamidine 9-(*N*-methylimine) (**5**), in which the ambiguity in the structure was resolved through X-ray single-crystal diffraction analysis.[25] Therefore, the structure of compound **1**, named clathridimine, was established as being a new 2-aminoimidazole alkaloid with an imino group at the C-9 position.

Table 1. NMR Spectroscopic Data ($CDCl_3$) for Clathridimine (**1**)

no.	δ_C, mult.	δ_H (J in Hz)	HMBC[a]
2	147.6, C		
4	133.9, C		
5	115.9, CH	6.39, s	2, 4
7	n.o.[b]		
9	158.5, C		
11	163.2, C		
12	32.2, CH_3	3.67, s	2, 5
13	25.6, CH_3	3.17, s	9, 11
14	32.8, CH_2	3.88, s	4, 5, 1′, 2′, 6′
1′	131.2, C		
2′	109.1, CH	6.72, s	14, 3′, 4′, 6′
3′	147.9, C		
4′	146.6, C		
5′	105.4, CH	6.76, d (7.8)	1′, 3′, 4′
6′	121.9, CH	6.70, d (7.8)	14, 2′, 4′
7′	101.0, CH_2	5.93, s	3′, 4′

[a] HMBC correlations, optimized for 7 Hz, are from proton(s) stated to the indicated carbon. [b] n.o.: signal not unambiguously observed.

Compound **3** was obtained as yellow crystals. Its ESI mass spectrum showed a molecular ion cluster $[M + H]^+$ at *m/z* 745, 747, and 749 (relative intensities 100, 60 and 33), clearly indicative of the presence of one zinc atom in the molecule. Examination of spectroscopic data identified compound **3** as the Zn complex of clathridine (**2**), previously described in *C. clathrus*.[14,34]

The 1:1 CH_2Cl_2/MeOH extract showed a broad spectrum of activities against the bacterial strains *Staphylococcus aureus* and *Escherichia coli* and the yeast *Candida albicans* (Table 2). The

Table 2. Antimicrobial Activities[a] of the 1:1 CH_2Cl_2/MeOH Extract of the Sponge *C. clathrus* and of Compounds **1**−**3**

	S. aureus ATCC 6538	*E. coli* ATCC 8739	*C. albicans* ATCC 66029
CH_2Cl_2/MeOH extract of *C. clathrus*	11 ± 2	15 ± 3	20 ± 1
clathridimine (**1**)	0	15 ± 2	24 ± 4
clathridine (**2**)	0	0	18 ± 1
clathridine Zn complex (**3**)	12 ± 1	0	0
kanamycin	37 ± 4	38 ± 4	
itraconazole			21 ± 1

[a] Inhibition diameter measured by the agar diffusion assay method for 1 mg per well (6 mm of diameter) for the extract and 100 μg per well for pure compounds and controls. Samples were tested in triplicate.

three 2-aminoimidazole alkaloids **1**−**3** were tested against these three microorganisms at 100 μg/well. They revealed different and selective activities. Clathridine (**2**) displayed specific antifungal activity against *C. albicans*, and its Zn complex (**3**) exhibited specific anti-*S. aureus* activity. The new clathridimine (**1**) inhibited growth of the bacterial pathogen *E. coli* and the fungal pathogen *C. albicans*. Although qualitative, these results might suggest a role for these compounds as antimicrobial chemical defense of *C. clathrus*.

Light microscopic, SEM, and TEM studies revealed that *C. clathrus* contained numerous extracellular bacteria dispersed in the mesohyl of the sponge body wall (Figure 2B). Bacterial abundance was estimated to be $(2.2 \pm 0.7) \times 10^8$ bacteria/g sponge wet weight ($n = 3$ specimens) as compared to a total of $(1.5 \pm 0.7) \times 10^8$ sponge cells/g sponge wet weight ($n = 3$ specimens) counted in Dapi-DNA-stained dissociated sponge suspensions. Choanocytes were the major (70%) sponge cell types in *C. clathrus*. Intracellular bacteria were not detected ultrastructurally in sponge cell types. Two preponderant morphological types of extracellular bacteria were localized in the mesohyl: curved, spiral bacteria (Figures 2C and D) and long, narrow rods (Figures 2E and F). These bacteria were sometimes observed in close proximity to the sponge cell surface (Figure 2D).

In order to determine the cellular localization of these secondary metabolites, sponge cells were separated from bacteria via differential sedimentation of dissociated sponge suspensions (Figure 3). Cell pellets from centrifugation at three different speeds were lyophilized and extracted with CH_2Cl_2/MeOH, and each extract was analyzed by HPLC/UV/ELSD chromatography and by LC/MS experiments. Microscopic observations showed that this successive pelleting method was appropriate to obtain fractions enriched either in sponge cells (heavy fraction, C1) or in bacteria (bacterial fraction, C3) (Figure 4). The heavy fraction, C1, contained mostly sponge cells (choanocytes, a few larger spherulous cells, and undetermined sponge cell types) (Figure 4 panel C1) with remaining bacteria closely associated with the cell surface. The light fraction, C2, contained mostly bacteria with some remaining sponge cells (Figure 4 panel C2). The bacterial fraction, C3, contained exclusively bacteria and potential cell debris (Figure 4 panel C3). Heavy fraction C1 was enriched in sponge cells by a factor of 9 compared to light fraction C2 and by a factor of 30 compared to bacterial fraction C3. Trypsinization of the sponge cell surface allowed improved separation of sponge cells from extracellular bacteria that may be closely associated with the sponge cell surface, as proven by the low amount of bacteria in the pellet $C1_{tryp}$ (containing 10 times more sponge cells than bacteria) compared to the high enrichment of the pellet $C3_{tryp}$ in bacteria with almost no sponge cells.

The results were unequivocal and showed that clathridimine (**1**) was found only in the CH_2Cl_2/MeOH extract of the whole sponge before dissociation and was totally absent from the other extracts (Figure 5). These results suggested that clathridimine (**1**) is a genuine compound produced by the sponge *C. clathrus* and may be transformed into clathridine (**2**) during the cell fractionation procedure. This suggestion was supported by hydrolysis of clathridimine (**1**) into clathridine (**2**) overnight by addition of water in excess. Therefore, due to its instability in water, the origin of clathridimine (**1**) could not be determined. Clathridine (**2**) was localized in the sponge cell fractions (C1 and $C1_{tryp}$) and not in the bacterial fractions (C3 and $C3_{tryp}$) (Figure 5). Traces of clathridine (**2**) were also detected in fraction C2, due to the presence of some remaining sponge cells, and in both fraction C3 and supernatant S, which might be explained by lysis of sponge cells during mechanical dissociation and centrifugation (Figure 5). The enrichment in sponge cells was linked to the increase in clathridine content, as shown by the comparative size of peaks on HPLC chromatograms. The clathridine-zinc complex (**3**) was localized, through LC/MS experiments, only in the supernatant, suggesting its possible excretion.

In conclusion, a new 2-aminoimidazole alkaloid, named clathridimine (**1**), along with the known clathridine (**2**) and its zinc complex (**3**), has been isolated from the Mediterranean calcareous sponge *C. clathrus* with specific activities against various microorganisms. Although clathridimine (**1**), unstable in water, could not be localized, clathridine (**2**) proved to be associated with the sponge cells through differential centrifugation. However, this experiment provides only circumstantial evidence because the hypothesis of transport of clathridine from one cell type to another cannot be excluded.[37] Future studies will investigate the ecological role of these compounds against environmental fungi and bacteria.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a UVIKON 930 spectrometer. IR spectra were recorded on a FT-IR Shimadzu 8400 S spectrometer. ^1H NMR 1D and 2D spectra (COSY, HSQC, HMBC, NOESY) were obtained on a Bruker AVANCE 400. HSQC and HMBC experiments were acquired at 400.13 MHz using a $^1H-^{13}C$ dual probehead. The delay preceding the ^{13}C pulse for the creation of multiple quanta coherences through several bounds in the HMBC was set to 70 ms. HMBC spectra were optimized for 7 Hz coupling. Mass spectra were recorded on an API Q-STAR PULSAR I from Applied Biosystem. LC/MS analyses were carried out with a Jupiter column (Phenomenex, 5 μm, C18, 300A, 150 × 1.0 mm). The extract was desalted on a SPE Strata C18-E column (55 μm, 70A). Semipreparative (Gemini C6-phenyl, 10 × 250 mm) and analytical (Gemini C6-phenyl, 3 × 250 mm) reversed-phase HPLC were performed with an Alliance apparatus (model 2695, Waters) equipped with a photodiode array detector (model 2998, Waters), an evaporative light-scattering detector (model Sedex 80, Sedere), and the software Empower.

Animal Material. Specimens of *Clathrina clathrus* were collected by scuba diving in the northwestern Mediterranean Sea (Marseille, France) from July 2007 to November 2009 between 10 and 15 m depth. A voucher specimen is available from the Museum National d'Histoire Naturelle in Paris as Porifera collection number C2010-1.

Extraction and Isolation of Compounds 1, 2, and 3. Freshly collected animal (wet weight: 40 g) was lyophilized (4.2 g), stored frozen, and further extracted with 1:1 CH_2Cl_2/MeOH (3 × 20 mL, sonication for 15 min) at room temperature. The 1:1 CH_2Cl_2/MeOH extract was concentrated under reduced pressure to yield a dark brown, viscous oil (208.1 mg), which was chromatographed on a C18 SPE column in a vacuum chamber (H_2O, H_2O/MeOH, 1:3, MeOH, CH_2Cl_2, 100 mL of each). The fraction that eluted with H_2O/MeOH, 1:3 (47.9 mg), was subjected to semipreparative reversed-phase HPLC (Gemini C6-phenyl 10 × 250 mm) with increasing amounts of CH_3CN/0.1% formic acid in H_2O/0.1% formic acid as eluent (flow rate: 5 mL/min, wavelength: 254 and 280 nm) and afforded clathridimine (**1**) (4.6 mg, 0.11% of sponge dry weight), clathridine (**2**) (2.8 mg, 0.07% of sponge dry weight), and the zinc complex of clathridine (**3**) (1.4 mg, 0.03% of sponge dry weight).

Clathridimine (1): yellow-orange solid; UV (EtOH) λ_{max} (log ε) 284 (3.73), 375 (3.84) nm; IR (dry film) ν_{max} 3240, 1775, 1740, 1694, 1620, 1559, 1489, 1446, 1397, 1303, 1246, 1114, 1038, 926 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS m/z 341.1354 $[M + H]^+$ (calcd for $C_{16}H_{17}N_6O_3$, 341.1356).

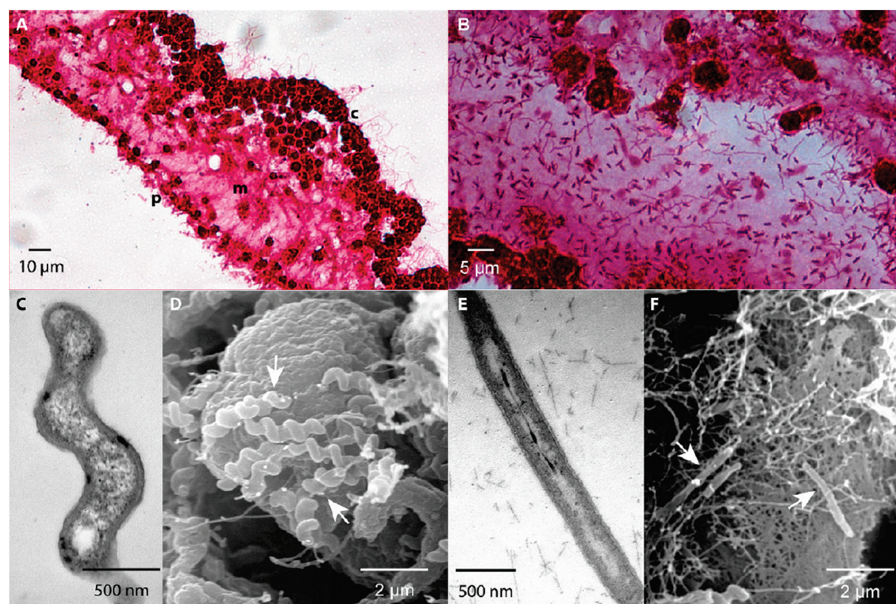


Figure 2. Light micrographs (A, B) of Gram-stained histological sections, showing ascon organization of the aquiferous system of *Clathrina clathrus* (A) with magnification of the thin mesohyl portion of the tubes containing numerous bacteria (B). Transmission electron micrographs (C and E) and scanning electron micrographs (D and F) of the two preponderant bacterial morphotypes: curved, spiral bacteria (C and D) and long, narrow rods (E and F). c: choanoderm, m: mesohyl, p: pinacoderm.

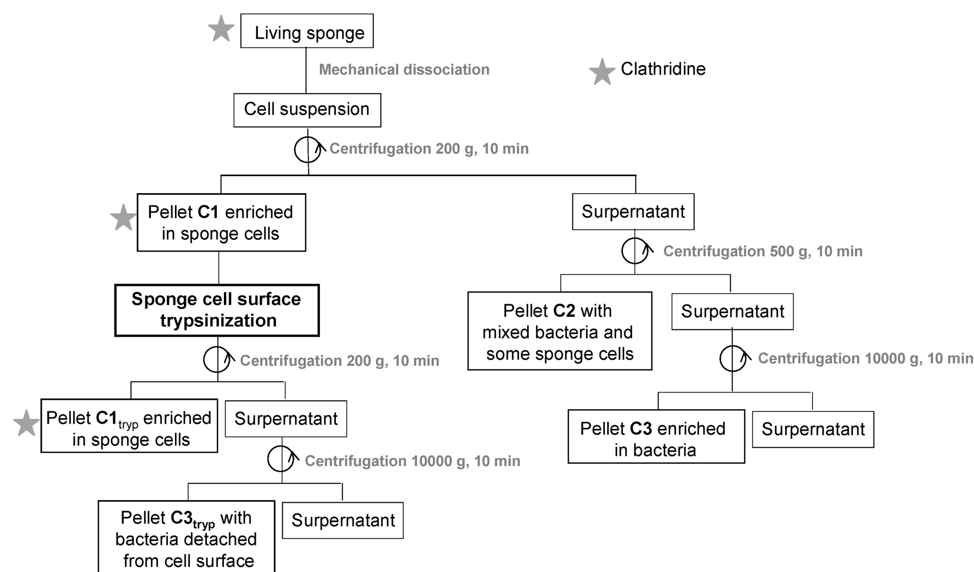


Figure 3. Localization of clathridine (**2**) in the sponge cell enriched fractions (C1, $C1_{tryp}$), obtained by differential centrifugation and sponge cell surface trypsinization.

Clathridine (2): yellow crystals; spectroscopic data matched those previously published.[13]

Zn Complex of Clathridine (3): yellow crystals; spectroscopic data matched those previously published.[13,33]

Hydrolysis of Clathridimine (1) into Clathridine (2). Compound **1** (200 μg) was treated with 20 mL of water overnight at room temperature and then analyzed by ESIMS.

Antimicrobial Assays. Assays were performed by the agar diffusion assay method. Extract (1 mg) or pure compound (100 μg) was solubilized in DMSO and deposited in a 6 mm well cut out from agar plates seeded with reference strains *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), and *Candida albicans* (ATCC 66029). Antimicrobial activity was determined by measuring the diameter of the inhibition zones after 24 h of incubation at 37 °C for *S. aureus* and *E. coli* or 30 °C for *C. albicans*. Kanamycin was used as the standard antibiotic positive control for the *S. aureus* and *E. coli* bacterial strains and itraconazole for the *C. albicans* yeast.

Microscopic Studies. For light microscopy, fragments of sponges were fixed and decalcified overnight after collection in Bouin fixative at pH 4 (5% acetic acid 75% saturated picric acid, and 20% formol), then washed extensively in 70% EtOH and stored at 4 °C. Fixed tissue was dehydrated in an increasing series of EtOH substituted by xylene and embedded in paraffin. Histological sections (5−6 μm, Microtome Leitz) were deparaffinized in xylene and rehydrated and were Gram stained (first stained with crystal violet for 3 min, then covered with Lugol's solution for 3 min, differentiated with 95% EtOH, and counterstained with Ziehl fuchsin for 3 min). Tissue sections were observed by light microscopy with a DMLB microscope fitted with a Leica DC300F camera, and images were acquired with the IM500 software. For transmission electron microscopy (TEM) and scanning electron microscopy (SEM), pieces of sponges were fixed immediately after collection in 2.5% glutaraldehyde in a mixture of 0.4 M cacodylate buffer and seawater (1:4:5; 1120 mOsm, pH 7.4), postfixed in 2% OsO_4 in seawater, and dehydrated through a graded EtOH series. Alternately, sponge fragments were fixed in 2.5% glutaraldehyde in Sörensen phosphate 0.1 M/sucrose 0.6 M buffer (pH 7.6) containing 10 mM $CaCl_2$ and 0.055% w/v ruthenium red, postfixed in 1% OsO4 in Sörensen buffer, and dehydrated in EtOH. For SEM, specimens

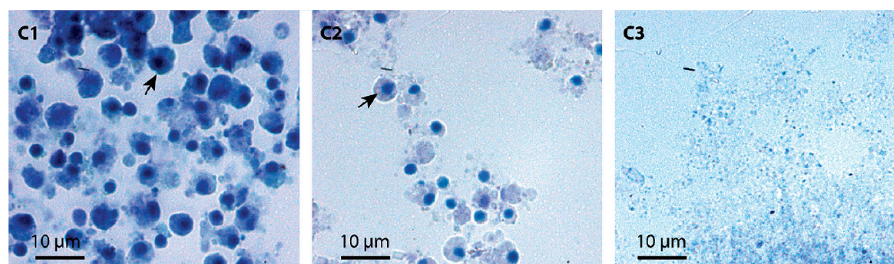


Figure 4. Cell type composition of the fractions obtained by differential centrifugation and stained with methylene blue-azur II: C1, heavy fraction (enriched in sponge cells); C2, light fraction (enriched in bacteria, with a few sponge cells); C3, bacterial fraction. Arrows point to large spherulous sponge cell types.

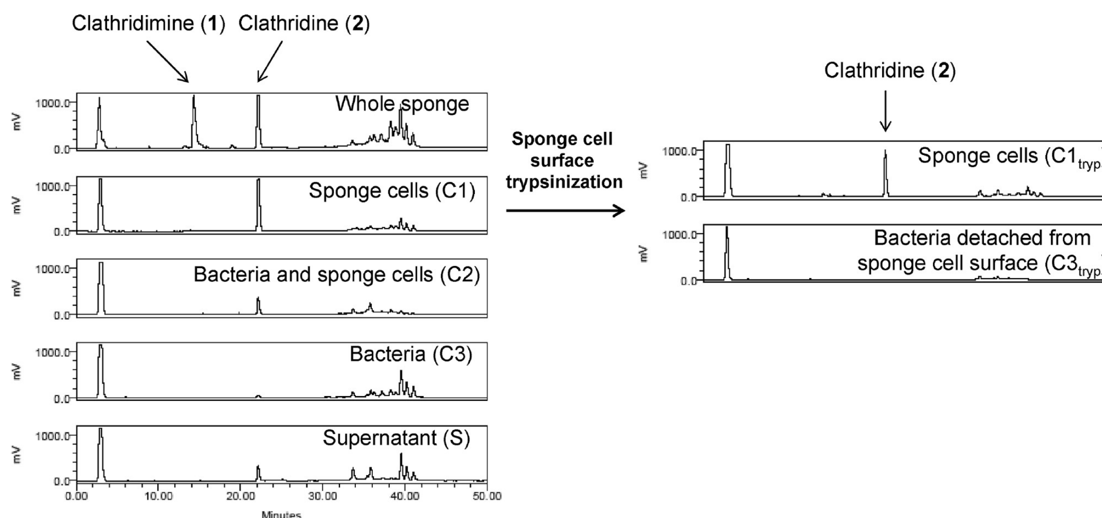


Figure 5. HPLC/ELSD chromatograms of the CH_2Cl_2/MeOH extracts (injection of 200 μg) of whole sponge and of fractions C1 (heavy sponge cells), C2 (mixed bacteria and small-sized sponge cells), C3 (bacteria), S (supernatant), $C1_{tryp}$ (heavy sponge cells), and $C3_{tryp}$ (bacteria detached from sponge cell surface).

dehydrated in EtOH were fractured in liquid nitrogen, critical-point-dried, sputter-coated with gold−palladium, and observed under a Hitachi S570 SEM. For TEM the pieces were embedded in Araldite or Spürr resin. Sections were cut with a Diatome 45° diamond (Ultracut microtome). Semithin sections were stained with toluidine blue 1%/borax 1% in 70% ethanol. Ultrathin sections were counterstained with uranyl acetate 2% in 50% and were observed under a Zeiss-1000 TEM and LEO 910 at 75 kV with a Hitachi H7100 transmission electron microscope equipped with a digital CCD Hamamatsu camera.

Sponge Dissociation and Cell Fractionation. Freshly collected sponges were rinsed with artificial seawater, and their epibionts were carefully discarded. Sponge fragments were mechanically dissociated by pressing through a 40 μM pore-size autoclaved plankton net in cold (4 °C) calcium and magnesium-free artificial seawater (CMF-ASW), according to Wilson.[35] The suspension was then filtered through a 40 μm mesh sterile nylon sieve to remove large spicule debris and cell aggregates.

In order to separate populations enriched either in cell types of *C. clathrus* or in bacteria, the cell suspension obtained by mechanical dissociation was centrifuged first at 200*g* for 10 min (pellet C1 enriched in sponge cells), then its supernatant at 500*g* for 10 min (pellet C2 with bacteria and some remaining sponge cells), and finally the second supernatant at 10000*g* for 10 min (pellet C3 enriched in bacteria), according to a protocol modified from Richelle-Maurer et al.[11] In order to separate extracellular bacteria potentially associated with the cell surface in the C1 heavy fraction, a trypsinization experiment was conducted. The pellet C1 enriched in sponge cells was resuspended in CMF-ASW and trypsinized (30 mg of trypsin/g of sponge wet weight) for 15 min at room temperature (20 °C). The suspension was centrifuged at 200*g* for 10 min to separate the cells ($C1_{tryp}$ enriched in sponge cells) from the supernatant, which was then centrifuged at 10000*g* for 10 min ($C3_{tryp}$ enriched in bacteria detached from sponge cell surface). The cell−bacteria separation and trypsinization experiments were repeated three times independently, with two replicates per experiment.

To check the efficiency of the cell−bacteria separation protocol, a part of the dissociated sponge suspension and of each fraction was fixed with paraformaldehyde at 3% final concentration or with glutaraldehyde 2.5% final concentration and stored at 4 °C until further processing. After elimination of the fixative by centrifugation, the cells and/or bacteria were resuspended in PBS/EtOH/Tween 20 50/50/0.1% (pH 7.4) to help dissociate small aggregates. The resulting suspensions were smeared on a glass slide, and the DNA was stained with the fluorochrome DAPI 0.1% in H_2O, or the cells were visualized either with methylene blue/azur II or Groat hematoxylin histological stains according to Martoja et al.[36] Alternately, suspensions were stained with DAPI 0.1% in PBS/EtOH/Tween 20 50/50/0.1% (pH 7.4), and sponge cells and bacteria were counted on a Petroff-Hausser slide according to the manufacturer's directions, with a DMLB epifluorescence microscope under blue light (Leica Microsystèmes SAS). The abundance of cells versus bacteria was visualized by the difference in size of marked nuclei versus marked bacterial DNA.

The remainder of the different fractions were frozen without fixation, lyophilized, extracted with 1:1 CH_2Cl_2/MeOH, and desalted on SPE C18 for HPLC chromatography (injection of 200 μg of each) and for LC/MS experiments in order to look for the presence of clathridine **2** and its analogues.

Acknowledgment. This work is part of M. Roué's thesis, within the French program ECIMAR, supported by the Agence Nationale pour la Recherche (ANR). We thank A. Blond and A. Deville (MNHN, Paris) for NMR spectra, A. Marie and L. Dubost (MNHN, Paris) for MS measurements, M. Martin for preparation of histological sections, and M. Vandervennet for her help in antimicrobial bioassays.

Supporting Information Available: NMR and ESIMS spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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NP100175X