Eight-Membered Cyclic 1,2,3-Trithiocane Derivatives from *Perophora viridis*, an Atlantic Tunicate

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Two novel sulfur derivatives, one of which is a glycoside, containing substituted 1,2,3-trithiocane cycles were isolated from *Perophora viridis* which was collected off the Atlantic coast of North Carolina. They were characterized by spectroscopic methods, mainly ^1H NMR, ^{13}C NMR, IR and UV spectroscopy and mass spectrometry. The compounds gave positive results in the brine shrimp toxicity assay and the sea urchin test.

Introduction

Marine tunicates belonging to the class Ascidiacea have been the subject of extensive chemical and biological investigations. Numerous indole alkaloids,[1,2] tyrosine derivatives,[3] guanines,[4] pyrazine alkaloids[5] and piperidine alkaloids[6] have been isolated and identified from tunicates. With the continued chemical interest in these marine organisms, an increasing number of non-nitrogen containing metabolites have also been isolated, for example longithorols[7,8,9] and diterpenes.[10] Tunicates have also proved to be a rich source of a broad spectrum of sulfur-containing natural products with fascinating structures and different biological activities.[11,12] The first compound in this series was varacin,[13] and other such compounds include lissoclinotoxins[14,15] and trithianes.[16,17]

Results and Discussion

In the course of our program devoted to the search for new compounds from marine animals,[18,19] we found that the extract from *Perophora viridis* collected near Cape Hatteras (NC, USA) exhibited considerable biological activity. Lyophilized animals were extracted with a CH_2Cl_2/MeOH mixture according to the procedure of Bligh and Dyer.[20] The upper (water/methanol) phase was subjected to chromatography on Sephadex LH-20, and two fractions that contained sulfur (color reactions according to Kates[21]) were subjected to semipreparative RP-HPLC, leading to the isolation of two major compounds. One compound was isolated as an opaque amorphous solid and showed significant UV absorbances at 258 nm (*cisoid* diene) and 205 nm (α,β-unsaturated acid). In the IR spectrum, diagnostic peaks were observed for a hydroxyl group (3550 cm^{-1}) and an α,β-unsaturated acid (1705 cm^{-1}).

Compound **1** shows a molecular ion at $m/z = 360.0884$, compatible with the formula $C_{16}H_{24}O_3S_3$. The presence of a 1,2,3-trithiocane ring in **1** was suggested by the observation of three sequential losses of ^{32}S in the low resolution mass spectrum, i.e. 360 $[M^+]$, 328 $[M^+ - S]$, 296 $[M^+ - S_2]$ and 264 $[M^+ - S_3]$. The ^1H NMR spectroscopic data for **1** are compiled in Table 1. The ^1H-^1H connectivities of compound **1**, H-8′ to H-7′ and H-6′ to the methyl on C-6′, were elucidated by DQF-COSY. The relative 5′,6′-*trans* and 6′,7′-*trans* configurations were deduced from the vicinal coupling constants $J_{5',6'} = 10.2$ Hz and $J_{6',7'} = 10.4$ Hz (see Table 1). These relative stereochemistries, which were further confirmed by NOESY correlations of H-5′/H-2, H-9′/H-6′, H-9′/H-3 and H-7′/H-10′ (see Figure 1), are compatible with the relative stereochemistry of compound **1**.

The absolute configuration at C-7′ in **1** was determined to be *R* by the modified Mosher method.[22,23] The $\Delta\delta = \delta_S - \delta_R$ values in the trithiocane of the MTPA ester of **1** are shown in Figure 1. The positive Δδ values for the hydrogen atoms oriented on the right side of the MTPA plane and the negative Δδ values for those located on the left side of the MTPA plane in the 7-hydroxy-trithiocane derivative point to the *R*-configuration at C-7′.

The ^{13}C NMR spectrum shows a total of 16 carbon resonances indicating 16 total carbons. By comparing the ^{13}C NMR spectroscopic data, two terminal methylene units were recognized in the 6-carbon side chain (C-1′′ to C-6′′).

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Table 1. ^1H and ^{13}C NMR signals of compound **1**

	^1H	^{13}C
1	–	170.0
2	6.05 (d, J = 15.5 Hz, 1 H)	120.1
3	7.04 (dd, J = 15.5, 9.1 Hz, 1 H)	151.2
4′	–	45.8
5′	2.48 (dd, J = 10.2, 9.1 Hz, 1 H)	52.1
6′	1.71 (ddd, J = 10.4, 10.2, 6.6 Hz, 1 H)	35.6
7′	3.44 (ddd, J = 10.4, 10.4, 3.5 Hz, 1 H)	75.7
8′	ax 2.70 (dd, J = 13.5, 10.4, Hz, 1 H) eq 2.79 (dd, J = 13.5, 3.5 Hz, 1 H)	38.4
9′	1.08 (d, J = 6.6 Hz, 1 H)	14.8
10′	1.84 (s, 3 H)	21.2
1′′	1.54 (m, 1 H), 1.37 (m, 1 H)	36.1
2′′	2.06 (m, 2 H)	30.4
3′′	–	149.2
4′′	6.37 (dd, J = 16.9, 10.8 Hz, 1 H)	138.5
5′′	5.07 (d, J = 10.8 Hz, 1 H) 5.15 (d, J = 16.9 Hz, 1 H)	114.4
6′′	4.88 (s, 1 H), 5.01 (s, 1 H)	109.7

One of these, δ_H/δ_C = 5.07, 5.15/114.4 (C-5′′), displays classical[24] *cis* and *trans* couplings of 10.8 and 16.9 Hz, respectively, to δ_H/δ_C = 6.37/138.5 (C-4′′). Conversely, the other terminal methylene, δ_H/δ_C = 4.88, 5.01/109.7 (C-6′′), does not display any splitting, and is thus connected to a quaternary carbon, C-3′′. Furthermore, HMBC correlations between H-6′′ and C-4′′, and H-5′′ and C-3′′, place these two ethylene units adjacent to each other. Also, position C-2′′ was determined to be adjacent to C-3′′ from HMBC correlations between H-2′′ and C-4′′, H-4′′ and C-2′′, and H-6′′ and C-2′′. Position C-1′′ was then evident on the basis of the COSY correlations between H-1′′ and H-2′′.

The ^1H NMR spectrum contains a series of coupled resonances spanning the chemical shift region from δ = 1.08 to 3.44 ppm. A DEPT spectrum confirmed that these protons are associated with one methyl, one methylene and three methine groups. DEPT, COSY and HMQC analysis identified the partial structure (YCH_2-HCX-HCR-HCR^1-YCR^2-CH_3), with Y probably being S, on the basis of the associated ^{13}C NMR shifts (δ = 38.4 and 45.8 ppm, respectively). The COSY data observed are as follows: H-7′/H_2-8′, H-6′; H-6′/H-7′, H-5′; H-5′/H-6′, H-9′. The protons of each methine (methylene) are not equivalent and show a complicated coupling in the ^1H NMR spectrum, which indicates that this partial structure is integrated into an eight-membered ring.

These correlations, and ChemDraw 3D modeling, led to the elucidation of the complete stereochemistry, including the chirality, of compound **1**, as illustrated in Figure 1. Consequently, the structure of **1** was determined to be (2*E*,4′*R*,5′*S*,6′*S*,7′*R*)-3-{7′-hydroxy-4′,6′-dimethyl-4′-(3′′-methylenepent-4′′-enyl)-[1′,2′,3′]-trithiocan-5′-yl}acrylic acid.

Compound **2** shows a molecular ion (determined by HRFABMS) at *m*/*z* = 708.2240, compatible with the formula $C_{30}H_{48}N_2O_9S_4$. In the same manner as described above, the planar constitution of **2** was determined by DQF-COSY and ^1H-^1H decoupling experiments to be a derivative of trithiocane. The relative 5′,6′-*trans*, 6′,7′-*trans* and 7′,8′-*cis* configurations were deduced from the vicinal coupling constants of H-5′−H-8′ See Table 2 and Figure 2). The (*E*)-3′′′′-thiomethylacrylate ester was assigned using a combination of spectroscopic data. The IR band at 1655 cm^{-1} and UV absorption at 257 nm are in a good agreement with literature values.[25] The HMBC experiment shows a correlation from the thiomethyl signal (H-4′′′′) at δ = 2.28 ppm to the olefinic carbon signal at δ = 147.8 ppm (C-3′′′′), which was correlated to the H-3′′′′ signal at δ = 7.24 ppm in the HSQC spectrum. Further HMBC correlations from the H-3′′′′ proton signal to signals at δ = 113.4 ppm (C-2′′′′) and δ = 165.4 ppm (C-1′′′′) completed the assignment. The $J_{2'''',3''''}$ coupling constant of 14.5 Hz and the ROESY correlation from C-4′′′′ to H-2′′′′ defined

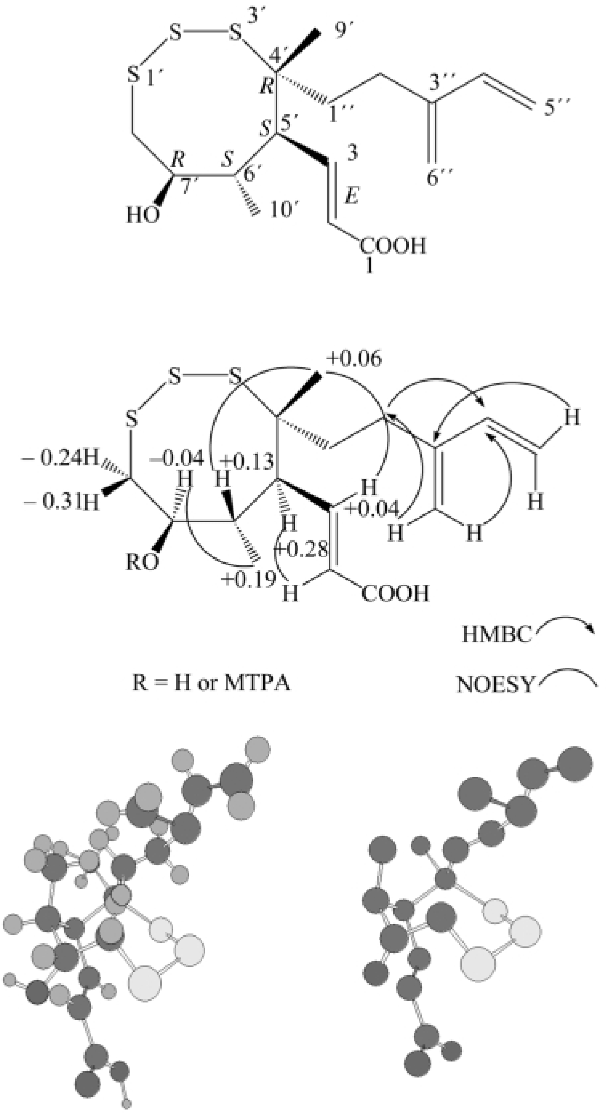

Figure 1. Structure of the new sulfur derivative **1** from *Perophora viridis* collected off the Atlantic coast of North Carolina; the HMBC correlations and ChemDraw 3D-MM2 models (with and without of hydrogens) of compound **1** are included

Table 2. ^{1}H and ^{13}C NMR signals of compound **2**

	^{1}H	^{13}C
1	–	165.9
2	6.07 (d, J = 14.9 Hz, 1 H)	123.5
3	7.10 (dd, J = 14.9, 9.7 Hz, 1 H)	147.2
4′	–	44.8
5′	2.42 (dd, J = 11.3, 9.7 Hz, 1 H)	47.2
6′	2.02 (ddd, J = 11.3, 8.8, 6.5 Hz, 1 H)	23.9
7′	3.17 (dd, J = 8.8, 3.8 Hz, 1 H)	78.4
8′	4.41 (d, J = 3.8 Hz, 1 H)	80.2
9′	1.07 (d, J = 6.5 Hz, 1 H)	13.6
10′	1.52 (s, 3 H)	22.5
1′′	1.58 (m, 1 H), 1.27 (m, 1 H)	35.4
2′′	2.06 (m, 2 H)	30.8
3′′	–	149.1
4′′	6.24 (dd, J = 17.1, 10.5 Hz, 1 H)	138.5
5′′	5.23 (d, J = 17.1 Hz, 1 H) 5.02 (d, J = 10.5 Hz, 1 H)	114.3
6′′	4.98 (s, 1 H), 4.88 (s, 1 H)	109.6
1′′′	3.25 (m, 2 H)	41.8
2′′′	1.55 (m, 2 H)	28.6
3′′′	1.56 (m, 2 H)	28.9
4′′′	3.27 (m, 2 H)	39.5
1′′′′	–	165.4
2′′′′	5.89 (d, J = 14.5 Hz, 1 H)	113.4
3′′′′	7.24 (d, J = 14.5 Hz, 1 H)	147.8
4′′′′	2.28 (s, 3 H)	15.6
1′′′′′	4.37 (d, J = 1.2 Hz, 1 H)	101.3
2′′′′′	3.69 (dd, J = 1.2, 2.5 Hz, 1 H)	70.9
3′′′′′	3.23 (dd, J = 2.5, 9.0 Hz, 1 H)	73.7
4′′′′′	3.34 (t, J = 9.0 Hz, 1 H)	67.4
5′′′′′	3.12 (ddd, J = 9.0, 9.0, 5.7 Hz, 1 H)	77.1
6′′′′′	3.67 (dd, J = 11.4, 9.0 Hz, 1 H) 3.48 (dd, J = 11.4, 5.7 Hz, 1 H)	61.6

the *E*-geometry. The C-1′′′′ signal shows a further HMBC correlation to a signal at δ = 3.27 ppm (δ_C = 39.5, C-4′′′).

The methylthioacrylic acid and substituted acrylic acid moieties (which form an amide linkage with carbonyl C-1, as judged from the long-range correlation with H-2 in the HMBC spectrum) are linked to putrescine by amide bonds, as was evident from the 2D correlations (predominantly COSY) and their respective configurations as deduced from coupling constants and NOE data.

Acid hydrolysis of **2** yielded mannose. To determine the absolute configuration of the carbohydrate in the glycoside, the acetylated 2-butyl derivatives prepared from the glycoside were analyzed by GC.[26,27] The (+)-2-butyl-2,3,4,6-tetraacetyl-L-mannoside was eluted as two peaks with retention times of 17.32 and 17.89 minutes, respectively, which were identical with those of α- and/or β-tetraacetyl (+)-2-butyl-L-mannoside, respectively. These results revealed that **2** contains L-mannose. The sugar unit is attached at the C-7′ position, as indicated by a correlation in the HMBC spectrum between the proton signal at δ = 4.37 ppm (d, J = 1.2 Hz, 1 H, H-1′′′′′) and the carbon at δ = 78.4 ppm (C-7′). The large vicinal coupling constants, $^{3}J_{\text{H-3}''''',\text{H-4}'''''}$ =9.0 Hz and $^{3}J_{\text{H-4}''''',\text{H-5}'''''}$ =9.0 Hz, and the NOE between H-3′′′′′ and H-5′′′′′ indicated that the H-3′′′′′, H-4′′′′′ and H-5′′′′′ protons are located in an axial position. The missing observation of an NOE between the H-4′′′′′ and H-2′′′′′ protons suggested that H-2′′′′′ is oriented in an equatorial position. These data indicate that the sugar is a mannopyranoside. The β-configuration was determined by an NOE correlation from H-1′′′′′ to H-3′′′′′ and H-5′′′′′, and by a small $^{1}J_{\text{C-1}''''',\text{H-1}'''''}$ coupling constant of 158.1 Hz (<166 Hz).[28] All these experiments imply that compound **2** is (2*E*,4′*R*,5′*S*,6′*R*,7′*R*,8′*S*,2′′′′*E*)-3-{8′-hydroxy-4′,6′-dimethyl-4′-(3′′-methylenepent-4′′-enyl)-7′-(β-L-mannopyranosyloxy)-[1′,2′,3′]-trithiocan-5′-yl}-*N*-[4′′′-(3′′′′-methylsulfanylacryloylamino)butyl]-acrylamide.

Both compounds **1** and **2** show a modest activity against different microorganisms (see Table 3). They are active against *Staphylococcus aureus* and *Bacillus subtilis*, but inactive against the Gram negative bacterium *Escherichia coli*. They also give positive results in the brine shrimp toxicity assay and sea urchin test.

Although ascidians are the source of a large number of sulfur compounds, this is the first report of a naturally occurring 1,2,3-trithiocane. It is also the first report of a naturally occurring glycoside from ascidians.

Experimental Section

General Experimental Procedures: UV spectra were measured on a Cary 118 (Varian) apparatus in EtOH over the range 200−350 nm. A Perkin−Elmer Model 1310 (Perkin−Elmer, Norwalk, CT, USA)

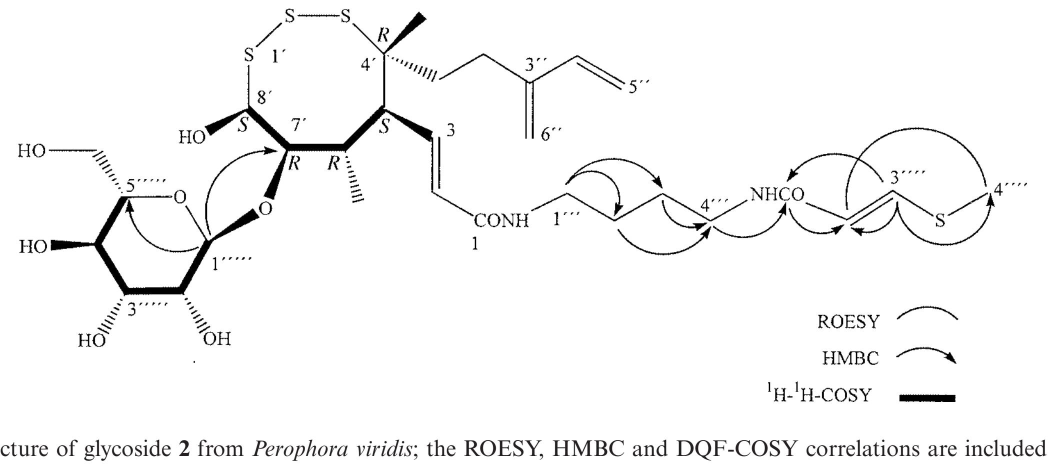

Figure 2. Structure of glycoside **2** from *Perophora viridis*; the ROESY, HMBC and DQF-COSY correlations are included

Table 3. Bioactivities of sulfur compounds **1** and **2**

Test organism	**1**	**2**
Staphylococcus aureus[a]	19.5	24.1
Bacillus subtilis[a]	9.8	18.2
Escherichia coli[a]	0	0
Saccharomyces cerevisiae[a]	0	0
Artemia salina[b] [c]	14.3	8.1
Paracentrotus lividus[c] [d]	22.5	0.2

[a] Samples (10 µg) were applied on 50.8 mm paper disks, values are diameters (mm) of inhibitory zones. [b] In µg/mL (minimum lethal doses). [c] In µg/mL (IC_{50}). [d] See Exp. Sect. for details.

IR spectrophotometer was used for scanning IR spectroscopy of compounds as neat films. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H), 125.7 MHz (^{13}C). High- and also low-resolution MS were recorded using a VG 7070E − HF spectrometer (70 eV). GC-MS of the sugar derivatives were done using a Finnigan 1020 B single-state quadrupole GC-MS instrument in the EI mode. A C_{18} reversed phase column (5 µm, 7.8 × 250 mm, Supelco, USA), was employed. A linear gradient from 40% H_2O and 60% acetonitrile to pure acetonitrile over 36 minutes, flow rate 1.7 mL/min with detection at 256 nm, was used to separate both compounds from the crude extract.

Animal Materials: The tunicate *Perophora viridis* (Verr 1871), family Perophoridae, order Phlebobranchia, superclass Tunicata were collected from old wharf pilings near Buxton (Outer Banks), NC, USA. The animals were 2.5 mm long, and nearly as wide. The colonies were 7 cm long and 8 cm wide. The habitats were creeping, finlike, translucent greenish-yellow.

Extraction and Isolation: Fresh tunicates (500 g wet weight) were put into ethanol and stored at −10 °C, concentrated under nitrogen and extracted by the methods of Blight and Dyer.[20] The total lipid extracts — viscous dark oils — were subjected separately to a Sephadex LH-20 chromatographic column separation with CH_2Cl_2/MeOH (1:5 v/v), yielding two fractions. After further separation by semi-preparative RP-HPLC (see above), the first fraction was found to be composed of compound **1**, whereas the second one contained glycoside, i.e. compound **2**.

Acid Hydrolysis of 2: The glycoside (≈1 mg) was refluxed in 2 N HCl (0.5 mL) for 2 h. The aglycone was extracted three times with EtOAc (10 mL). After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$ and lyophilized.

The identification and the assignment of the D- or L-configuration of the sugar (i.e. L-mannose) was performed by GC-MS (according to the methods of Gerwig et al.,[26] with some modifications, as described previously,[27] with SPB-1 (Supelco) column (30m × 0.25 mm I.D.).

(*S*)-MTPA Esters: To a stirred solution of ≈1.0 mg of the hydroxy compound (**1**) in 0.3 mL of dry pyridine was added 20 µL of (−)-MTPA chloride. The mixture was stirred under N_2 at room temperature for 1 h and the solvent was then removed by blowing with N_2. The residue was redissolved in 2 mL of EtOAc/hexane and filtered through a Sep-Pak silica column. After removing the solvent under vacuum, the residue was separated by reversed-phase HPLC (ODS column, 100% MeCN) to yield ≈1.0 mg of the *S* ester as a colorless gum. For ^1H NMR spectra, see Figure 1.

(*R*)-MTPA Esters: Prepared as described for the (*S*)-esters. An amount of ≈1.0 mg of compound (**1**) and 20 µL of (+)-MTPA chloride gave 0.9 mg of (*R*)-esters as a colorless gum.

The following compounds were isolated:

(2*E*,4′*R*,5′*S*,6′*S*,7′*R*)-3-{7′-Hydroxy-4′,6′-dimethyl-4′-(3′′-methylenepent-4′′-enyl)-[1′,2′,3′]-trithiocan-5′-yl}acrylic Acid (**1**) is an opaque amorphous solid (5.7 mg, i.e. 0.00114%), $[\alpha]_D^{24} = +121.5$ ($c = 0.15$, EtOH). UV (EtOH): λ_{max} (log ε) = 258 nm (3.24), 205 (1.05). IR (film): $\tilde{\nu}_{max} = 3550, 1705\ cm^{-1}$. HREIMS: *m*/*z* = 360.0884 (calcd. for $C_{16}H_{24}O_3S_3$ 360.0887); LREIMS: 360 [M^+], 328 [$M^+ - S$], 296 [$M^+ - S_2$] and 264 [$M^+ - S_3$]. NMR spectroscopic data, see Table 1.

(2*E*,4′*R*,5′*S*,6′*R*,7′*R*,8′*S*,2′′′′*E*)-3-{8′-Hydroxy-4′,6′-dimethyl-4′-(3′′-methylenepent-4′′-enyl)-7′-(β-L-mannopyranosyloxy)-[1′,2′,3′]-trithiocan-5′-yl}-*N*-[4′′′-(3′′′′-methylsulfanylacryloylamino)-butyl]acrylamide (**2**) is a pale yellow amorphous solid (4.3 mg, i.e. 0.00086%), $[\alpha]_D^{24} = +197.3$ ($c = 0.11$, EtOH). UV (EtOH): λ_{max} (log ε) = 257 nm (2.78). IR (film): $\tilde{\nu}_{max} = 3480, 1655\ cm^{-1}$. HRFABMS: *m*/*z* = 708.2240 (calcd. for $C_{30}H_{48}N_2O_9S_4$ 708.2242); NMR spectroscopic data, see Table 2.

Antibacterial Tests: The test organisms were *Bacillus subtilis, Staphyloccocus aureus Escherichia coli* and *Saccharomyces cerevisiae* (Czechoslovak Collection of Microorganisms, Brno). Antibacterial assays were carried out according to a literature procedure.[29] The amounts used were 50 µg of compound per test disk (see Table 3).

Brine Shrimp Toxicity Bioassay: The sample (≈0.05 mg) was dissolved in 50 µL of DMSO and added to a test vial of artificial seawater (3.0 mL). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a 24 h period. A positive assay was the death of all brine shrimp.

Sea Urchin Eggs Test: Sea urchin eggs and sperm were collected from mature specimens of *Paracentrotus lividus*. Soon after fertilization, the eggs were dropped onto the test media which were previously prepared from a graded concentration of compounds **1** and **2** dissolved in EtOH and filtered seawater. After allowing the test media to stand, the embryos were observed with a light microscope at certain intervals. In an inhibitory test against the development of sea urchin eggs,[30] **1** and **2** were effective at levels of $IC_{50} = 2.5$ and 0.2 µg/ml, respectively.

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