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# Callyspongidic Acids: Amphiphilic Diacids from the Tropical Eastern Pacific Sponge *Callyspongia* cf. *californica*

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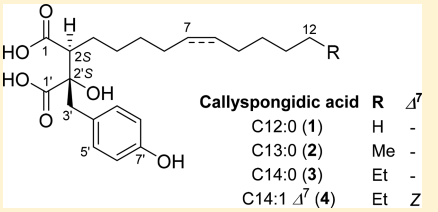
Supporting Information

**ABSTRACT:** The first chemical study of the marine sponge *Callyspongia* cf. *californica* widely distributed along the coasts of the Tropical Eastern Pacific led to the identification of a new family of amphiphilic derivatives called callyspongidic acids. The four isolated metabolites **1**−**4** feature a hydrophilic diacid end opposed to both an aromatic moiety and a long alkyl chain. They were evaluated against a panel of pathogenic microbes and seven tumoral cell lines, displaying moderate inhibitory properties against the A2058 melanoma cell line with an $IC_{50}$ of 3.2 $\mu$M for callyspongidic acid C13:0 (**2**).




Despite very original hydrological conditions inducing the construction of unique ecosystems and endemic marine species, the marine biodiversity of the Tropical Eastern Pacific is largely underexplored compared with the Central Indo-Pacific or the Caribbean Sea. Conscious of this lack of knowledge, a national initiative has supported the first comprehensive biological and chemical inventory of marine species off the coasts of mainland Ecuador. Overall, cnidarians have been identified as the most representative organisms in the marine protected area El Pelado, Santa-Elena Peninsula, and recent reports highlighted the diversity of zoantharians in this marine area.[1−3] Unlike its counterpart across the Panama Canal, the diversity and substrate cover of marine sponges are largely reduced, and two main species were identified with sufficient biomass to undertake their first taxonomical and chemical studies.

The first species was identified as *Callyspongia* cf. *californica* due to morphological and molecular traits very similar to the corresponding species first described off the coast of Mexico.[4] In Ecuador also, most of the specimens are found growing over living corals including the scleractinian coral *Pocillopora* sp., suggesting a close relationship between the sponge and its living substrate. Sponges of the genus *Callyspongia* are known to produce a broad range of secondary metabolites, and this chemical diversity might originate from systematic discrepancies pointed out in the whole order Haplosclerida.[5] We report herein the isolation and structure elucidation of four amphiphilic lipidic tyrosine derivatives named callyspongidic acids (**1**−**4**) from the sponge *Callyspongia* cf. *californica*. Their structures were deduced from spectroscopic data including 1D and 2D NMR experiments as well as HRESIMS analyses, and compounds differ by their long alkyl chains. Compounds were tested against a panel of pathogenic microbes and seven tumoral cell lines.

The freeze-dried sponge sample (60 g) was macerated and repeatedly extracted with a mixture of $CH_2Cl_2$/MeOH (1:1) under ultrasonication. The extracts (4.6 g) were then combined and fractionated by RP-C18 vacuum liquid chromatography with solvent mixtures of decreasing polarity from $H_2O$ to MeOH and $CH_2Cl_2$. The methanolic fraction was then purified by semipreparative RP-Phenylhexyl HPLC, yielding pure compounds **1**−**4**.

Compound **1** was isolated as a yellow oil with the molecular formula $C_{21}H_{32}O_6$ as deduced from HRESIMS data with a deprotonated molecule peak at $m/z$ 379.2133 $[M - H]^-$. The $^1$H NMR spectrum of **1** evidenced the presence of a long alkyl chain due to characteristic signals at $\delta_H$ 0.90 (t, $J$ = 6.8 Hz, 3H, H-12) and 1.30 (m, from H-5 to H-10) (Table 1). A *para*-

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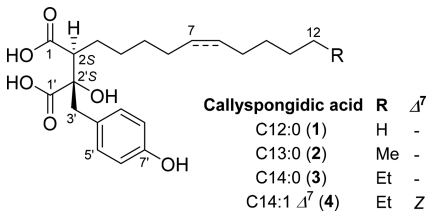


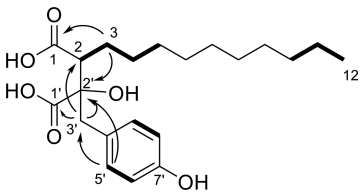

**Figure 1.** Key COSY (in bold) and HMBC (arrows from H to C) correlations for callyspongidic acid C12:0 (**1**).

disubstituted aromatic ring was also present with the two characteristic doublets at $\delta_H$ 6.54 (d, $J$ = 8.2 Hz, 2H, H-6′/8′) and 7.05 (d, $J$ = 8.2 Hz, 2H, H-5′/9′). The $^{13}$C and HSQC NMR spectra revealed the presence of two carboxyl groups at $\delta_C$ 177.5 (C-1) and 176.8 (C-1′), accounting for four oxygens, one oxygenated carbon at C-2′ ($\delta_C$ 80.4), six aromatic carbons, 12 methylene groups, and one terminal methyl. The phenolic nature of the aromatic ring was deduced from the deshielded signal of one aromatic carbon at $\delta_C$ 157.2 (C-7′), therefore leading to the last oxygen being an alcohol or an ether likely located at C-2′.

The substitution of one aromatic position by a methylene in the *para* position of the phenol functionality was evidenced by a key H-5′-7′/C-3′ HMBC correlation, with C-3′ correlating in the HSQC spectrum with the two signals of an AB system at $\delta_H$ 3.05 (d, $J$ = 13.7 Hz, 1H, H-3a′) and $\delta_H$ 2.81 (d, $J$ = 13.7 Hz, 1H, H-3b′) (Figure 1). The last oxygenated carbon was then located at C-2′ through the key $H_2$-3′/C-2′ $^2J$ HMBC correlation supported by the weak H-5′-7′/C-2′ $^4J$ HMBC correlations. Finally, both protons of the methylene at C-3′ weakly HMBC correlated with a carboxylic acid carbon at $\delta_C$ 176.8 (C-1′). The elucidation of the lipidic part of the molecule was rather straightforward starting from the spin-coupled system at position C-2. A key H-3/C-1 HMBC correlation placed the second carboxylic group at C-1, while additional $H_2$-3/C-2′ and $H_2$-3′/C-2 established a bridge between the two moieties of the molecule.

The relative configuration of **1** was assigned by comparison between the experimental and predicted chemical shifts using the DP4 probability.[6] This method has already been used for the determination of the relative configuration of a large number of natural products in the past few years.[3] Density functional theory (DFT) calculations were performed on both diastereoisomers (2*S**,2′*S** and 2*S**,2′*R**) at C-2 and C-2′. Following a conformational analysis, two major conformers were obtained for each diastereoisomer using a threshold of 1 kcal/mol. A gauge-independent atomic orbital (GIAO) calculation was realized on $^{13}$C NMR data, and the corresponding DP4 probability was calculated. All the common metrics used to compare experimental and predicted chemical shift converged (Table 2), and the relative 2*S**,2′*S**-**1** configuration was then assigned with almost no ambiguity.

Moving toward the absolute configuration, the proximity of both stereogenic centers with a chromophore suggested a successful use of electronic circular dichroism (ECD) for its determination. Three major Cotton effects (CEs) were indeed displayed in the ECD spectrum of the compound. Two CEs of alternative signs were observed at 240 and 275 nm likely

**Table 1. $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Data for 1−4 in $CD_3OD$**

| | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| no. | $\delta_C$, type | $\delta_H$, mult. ($J$ in Hz) | $\delta_C$, type | $\delta_H$, mult. ($J$ in Hz) | $\delta_C$, type | $\delta_H$, mult. ($J$ in Hz) | $\delta_C$, type | $\delta_H$, mult. ($J$ in Hz) |
| 1 | 177.5, C | | 177.5, C | | 177.6, C | | 178.0, C | |
| 2 | 54.7, CH | 2.82, m | 54.8, CH | 2.82, m | 54.7, CH | 2.82, m | 54.7, CH | 2.82, m |
| 3 | 29.1, $CH_2$ | a: 1.89, m | 29.2, $CH_2$ | a: 1.86, m | 29.1, $CH_2$ | a: 1.88, m | 29.1, $CH_2$ | a: 1.89, m |
| | | b: 1.41, m | | b: 1.39, m | | b: 1.38, m | | b: 1.41, m |
| 4 | 28.8, $CH_2$ | a: 1.37, m | 28.9, $CH_2$ | a: 1.36, m | 28.8, $CH_2$ | a: 1.36, m | 28.8, $CH_2$ | a: 1.37, m |
| | | b: 1.26, m | | b: 1.26, m | | b: 1.24, m | | b: 1.26, m |
| 5 | 30.4, $CH_2$ | 1.30, m | 30.5, $CH_2$ | 1.33, m | 30.5, $CH_2$ | 1.32, m | 30.0, $CH_2$ | 1.32, m |
| 6 | 30.7, $CH_2$ | 1.29, m | 30.7, $CH_2$ | 1.30, m | 30.5, $CH_2$ | 1.32, m | 27.9, $CH_2$ | 2.03, m |
| 7 | 30.5, $CH_2$ | 1.30, m | 30.8, $CH_2$ | 1.30, m | 30.7, $CH_2$ | 1.30, m | 130.5, CH | 5.34, m |
| 8 | 30.5, $CH_2$ | 1.29, m | 30.5, $CH_2$ | 1.32, m | 30.8, $CH_2$ | 1.29, m | 131.1, CH | 5.35, m |
| 9 | 30.7, $CH_2$ | 1.29, m | 30.5, $CH_2$ | 1.32, m | 30.7, $CH_2$ | 1.30, m | 28.1, $CH_2$ | 2.02, m |
| 10 | 33.1, $CH_2$ | 1.28, m | 30.8, $CH_2$ | 1.29, m | 30.5, $CH_2$ | 1.33, m | 30.9, $CH_2$ | 1.35, m |
| 11 | 23.7, $CH_2$ | 1.32, m | 33.1, $CH_2$ | 1.26, m | 30.8, $CH_2$ | 1.29, m | 30.4, $CH_2$ | 1.30, m |
| 12 | 14.4, $CH_3$ | 0.90, t (6.8) | 23.7, $CH_2$ | 1.32, m | 33.1, $CH_2$ | 1.26, m | 32.9, $CH_2$ | 1.28, m |
| 13 | | | 14.4, $CH_3$ | 0.90, t (6.9) | 23.7, $CH_2$ | 1.31, m | 23.7, $CH_2$ | 1.32, m |
| 14 | | | | | 14.4, $CH_3$ | 0.90, t (6.8) | 14.4, $CH_3$ | 0.91, t (6.8) |
| 1′ | 176.8, C | | 176.9, C | | 176.8, C | | 177.2, C | |
| 2′ | 80.4, C | | 80.4, C | | 80.3, C | | 80.6, C | |
| 3′ | 44.0, $CH_2$ | a: 3.05, d (13.7) | 44.0, $CH_2$ | a: 3.05, d (13.7) | 44.0, $CH_2$ | a: 3.05, d (13.7) | 44.0, $CH_2$ | a: 3.06, d (13.7) |
| | | b: 2.81, d (13.7) | | b: 2.81, d (13.7) | | b: 2.81, d (13.7) | | b: 2.80, d (13.6) |
| 4′ | 128.1, C | | 128.2, C | | 128.2, C | | 128.4, C | |
| 5′/9′ | 132.5, CH | 7.05, d (8.2) | 132.5, CH | 7.05, d (8.4) | 132.5, CH | 7.06, d (8.4) | 132.5, CH | 7.06, d (8.3) |
| 6′/8′ | 115.7, CH | 6.64, d (8.2) | 115.7, CH | 6.64, d (8.4) | 115.7, CH | 6.64, d (8.5) | 115.6, CH | 6.64, d (8.4) |
| 7′ | 157.2, C | | 157.2, C | | 157.2, C | | 157.1, C | |



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**Table 2. Comparison between Experimental and Predicted $^{13}C$ NMR Chemical Shifts**

| | $R^2$ | MAE[a] | RMSD[b] | DP4 |
|---|---|---|---|---|
| 2*S**,2′*S**-**1** | 0.997 | 5.2993 | 2.2129 | 100% |
| 2*S**,2′*R**-**1** | 0.995 | 5.6169 | 2.659 | 0% |

[a]Mean absolute error. [b]Root-mean-square deviation.

originating from the aromatic ring. A very good match was finally observed between the time-dependent (TD) DFT calculated spectrum of the 2*S*,2′*S* enantiomer and the experimental one (Figure 2), finalizing the spatial structure of **1**.

Compound **2** was isolated as a yellow oil of molecular formula $C_{22}H_{34}O_6$ as indicated by HRESIMS measurement, and **2** is therefore a homologue of **1**. The $^1H$ NMR spectrum of **2** was superimposable to that of **1**, suggesting that the additional methylene was inserted into the long alkyl chain. Other NMR data confirmed this assumption but also the same 2*S**,2′*S** relative configuration as the one found for **1**. The same 2*S*,2′*S* absolute configuration was deduced from similar specific rotations for both compounds.

Compound **3** was isolated as a yellow oil, and its HRESIMS spectrum revealed a deprotonated molecule peak at *m*/*z* 407.2429 $[M - H]^-$, suggesting a molecular formula of $C_{23}H_{36}O_6$ and therefore an additional $CH_2$ relative to **2**. Together with a $^1H$ NMR spectrum similar to those of **1** and **2**, these data were in accordance with a C14:0 saturated fatty acid connected to the 3′-(*p*-hydroxyphenyl)lactate moiety.

Compound **4** was isolated as a yellow oil with the molecular formula $C_{23}H_{34}O_6$ as established by HRESIMS analysis. Together with the MS data and the fact that most $^1H$ NMR signals were very similar to those of **3** suggested that **4** was a didehydro derivative of **3**. While **1**−**3** were shown to contain fully saturated C12 to C14 alkyl chains, the presence of two olefinic protons was highlighted by the $^1H$ NMR spectrum of **4** at $\delta_H$ 5.35 (m, 2H, H-7 and H-8). The position of the double bond was deduced from the H-6 and H-3/C-4 key HMBC correlations and from the unambiguous spin system of both olefinic protons at C-7 and C-8 in the HSQC-TOCSY NMR spectrum. The *Z* configuration of the double bond at C-7/C-8 was determined by the chemical shifts of signals corresponding to the allylic carbons at around 28.0 ppm.[7] The relative and the absolute configurations of **4** were consistent with those of **1**.

From a biosynthetic point of view, callyspongidic acids are likely to originate from the coupling of two independent moieties through an aldol-type reaction, giving rise to the key C-2/C-2′ bond. First, fatty acids of different length would lead to a nucleophilic enolate at C-2, and, on the other hand, an oxidative deamination of tyrosine would lead to 4-hydroxyphenylpyruvic acid bearing the electrophilic keto group at C-2′ (Scheme 1).

Although this transformation seems straightforward, very few natural products have been described from similar metabolic pathways. As examples, we can cite some citric acid derivatives connected to a long alkyl chain mostly isolated from fungi or other fungal itaconic acid derivatives derived from an aldol condensation between pyruvic acid and fatty acids in the same manner.[8−11] We were not able to find other fatty acid derivatives connected to an aromatic derivative such as phenylalanine or tyrosine in the literature. Interestingly, the control of the stereochemistry of the C-2/C-2′ bond would suggest the involvement of an enzymatic process. This original finding highlights the huge potential of novelty in underexplored marine ecoregions such as the Tropical Eastern Pacific, and we will continue in the exploration of the marine and chemodiversity of this region.

Compounds **1**−**4** were subjected to cytotoxic activity assays against seven cell lines (A2058, A 549, Hep G2, HT 29, MiaPaCa 2, MCF-7, and PC3). Moreover, antimicrobial activities of compounds **1**−**4** were tested against a panel of Gram-positive (methicillin resistant (MRSA) and methicillin sensitive (MSSA) *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) and fungi (*Aspergillus fumigatus* and *Candida albicans*). Only compound **2** displayed moderate cytotoxic activity against the A2058 epithelial melanoma cell line with an $IC_{50}$ of 3.2 $\mu$M, while **1**, **3**, and **4** were inactive against the same cell line ($IC_{50}$ values = 26, 30, and >30 $\mu$M, respectively). Regarding the antimicrobial assays, none of the compounds displayed any significant bioactivity at the highest concentrations tested (32 $\mu$g/mL for **1**, **3**, and **4**; 4 $\mu$g/mL for **2**).

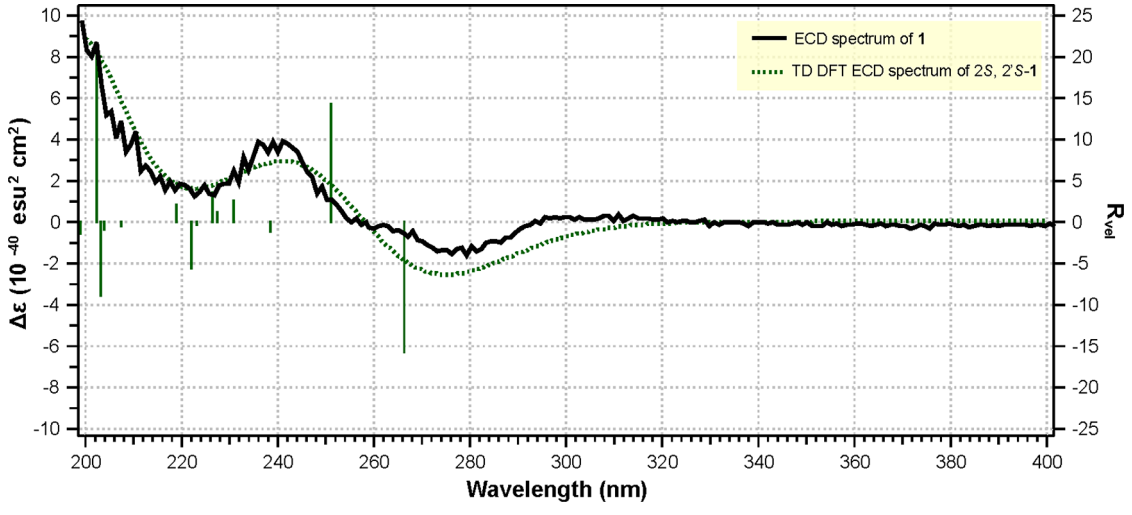


**Figure 2.** Comparison between experimental and TDDFT-calculated ECD spectra of **1**.



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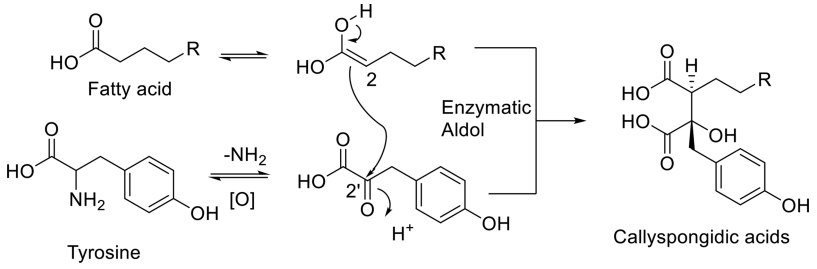
**Scheme 1. Biosynthetic Hypothesis for the Formation of Callyspongidic Acids**




## EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were recorded with a Unipol L1000 (Schmidt+Haensch) equipped with a 10 cm microcell and a sodium lamp. UV and ECD data were obtained on a Chirascan V100 spectrophotometer (Applied Photophysics). NMR experiments were performed on a 500 MHz spectrometer (Varian). Chemical shifts ($\delta$ in ppm) are referenced to the carbon ($\delta_C$ 49.0) and residual proton ($\delta_H$ 3.31) signals of $CD_3OD$. High-resolution mass spectra (HRESIMS) were obtained from an Agilent 6540 mass spectrometer. HPLC separation and purification were carried out on a Dionex Ultimate 3000 (Thermo Scientific) equipped with a variable-wavelength detector.

**Biological Material.** *Callyspongia* cf. *californica* was collected in the Marine Protected Area "El Pelado" (Bajo Laberinto, 1°56′07.92″ S/80°47′22.84″ W) off the Ecuadorian coasts, on August 25, 2015, at 5 m depth by scuba diving. A voucher sample "150825EP04-04" is kept at the Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM, San Pedro, Ecuador) (see Supporting Information for more details).

**Extraction and Isolation.** The dry sponge sample (60 g) was cut into small pieces and extracted three times with 600 mL of a mixture of MeOH/$CH_2Cl_2$ (1:1, v/v) at room temperature, yielding 4.6 g (7.7% yield from dry weight) of extract after evaporation of the solvent. The extract was fractionated by RP-C18 vacuum liquid chromatography (elution with a decreasing polarity gradient of $H_2O$/MeOH from 1:0 to 0:1, then MeOH/$CH_2Cl_2$ from 1:0 to 0:1). The methanolic fraction (F4, 414 mg) was then subjected to RP-HPLC on a semipreparative XSelect CSH Phenyl-hexyl OBD column, 19 × 250 mm, 5 $\mu$m (Waters), using a mobile phase of $H_2O$ (A) and MeCN (B), both buffered with 0.1% acetic acid. The flow rate was fixed at 3 mL/min, and the wavelength set at 210 nm. The purification method was developed on a 35 min acquisition time: 45% B for 2 min, then gradient to 65% B in 7 min, held at 65% B for 8 min, then gradient to 90% B in 6 min, back to 45% B in 1 min, and held at that percentage of B for 9 min to afford pure **1** ($t_R$ 16.0 min, 2.9 mg, $4.8 \times 10^{-3}$ % w/w), **2** ($t_R$ 18.1 min, 1.8 mg, $3.0 \times 10^{-3}$ % w/w), **3** ($t_R$ 19.4 min, 0.5 mg, $8.3 \times 10^{-4}$ % w/w), and **4** ($t_R$ 22.9 min, 14.3 mg, $2.4 \times 10^{-2}$ % w/w).

*Callyspongidic acid C12:0 (**1**):* yellow oil; $[\alpha]^{20}_D$ −11 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 225 (3.85), 277 (3.14) nm; $^1$H NMR and $^{13}$C NMR data, Table 1; HRESIMS (−) *m*/*z* 379.2133 [M − H]$^-$ (calcd for $C_{21}H_{31}O_6$, 379.2126, Δ +1.8 ppm).

*Callyspongidic acid C13:0 (**2**):* yellow oil; $[\alpha]^{20}_D$ −9.9 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 224 (3.85), 276 (3.15) nm; $^1$H NMR and $^{13}$C NMR data, Table 1; HRESIMS (−) *m*/*z* 393.2277 [M − H]$^-$ (calcd for $C_{22}H_{33}O_6$, 393.2283, Δ −1.5 ppm).

*Callyspongidic acid C14:0 (**3**):* yellow oil; $[\alpha]^{20}_D$ −12 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 224 (3.84), 277 (3.15) nm; $^1$H NMR and $^{13}$C NMR data, Table 1; HRESIMS (−) *m*/*z* 407.2429 [M − H]$^-$ (calcd for $C_{23}H_{35}O_6$, 407.2439, Δ −2.4 ppm).

*Callyspongidic acid C14:1 Δ$^7$ (**4**):* yellow, oily solid; $[\alpha]^{20}_D$ −15 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 224 (3.84), 277 (3.19) nm; $^1$H NMR and $^{13}$C NMR data, Table 1; HRESIMS (−) *m*/*z* 405.2293 [M − H]$^-$ (calcd for $C_{23}H_{33}O_6$, 405.2283, Δ +2.5 ppm).

**Computational Details for Compound 1.** The conformational analysis has been realized using the GMMX package implemented in Gaussian 16W (Gaussian 2016) with the MMFF94 force field and a 1 kcal/mol energy threshold in order to obtain many conformers due to the high flexibility of the long chain. Two conformers were obtained for each diastereosisomer of **1**. After geometry optimization (STO-3G) and frequency calculation at the same level, NMR chemical shifts were predicted using the GIAO method at the mpw1pw91/6-311+G(2d,p) level. Both experimental and theoretical chemical shifts were compared with the DP4 probability (in-house implementation of the Java source code available at http://www-jmg.ch.cam.ac.uk/tools/nmr/). ECD spectra were calculated at the B3LYP/6-311+G(d,p) level for 20 excited states. Rotational strengths were plotted into the ECD spectrum using GaussView 6.

**Evaluation of the Biological Activities.** Compounds **1**−**4** were tested for their ability to inhibit the growth of Gram-positive (*S. aureus* ATCC29213 (MSSA) and *S. aureus* MB5393 (MRSA)) and Gram-negative bacteria (*E. coli* ATCC25922, *K. pneumoniae* ATCC700603, *P. aeruginosa* PAO1, and *A. baumannii* CL5973) and fungi (*A. fumigatus* ATCC46645, *C. albicans* ATCC64124), following previously described methodologies.[12,13] Cytotoxic activities against the human-derived cell lines A2058 (epithelial melanoma), A 549 (lung carcinoma), Hep G2 (hepatocellular carcinoma), HT 29 (colorectal adenocarcinoma), MiaPaCa 2 (pancreatic carcinoma), MCF-7 (breast adenocarcinoma), and PC3 (prostatic carcinoma) were determined as previously reported.[14]

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00683.

Biological description of the species; NMR and MS spectra of compounds **1**−**4**; calculation details (PDF)

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#K. Calabro and B. E. Chalén contributed equally to this work.


### Notes

The authors declare no competing financial interest.

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y de Invertebrados de la Reserva Marina "El Pelado" a Escala Taxonómica, Metabolómica y Metagenómica para su Uso en Salud Humana y Animal. Part of this project (Grant-Aid Agreement No. PBA/MB/16/01) was carried out with the support of the Marine Institute and was funded under the Marine Research Programme by the Irish Government. K.B.J. acknowledges NUI Galway for supporting part of their Ph.D. scholarship. We are grateful to R. Doohan (NUI Galway, Ireland) for her help in recording NMR experiments. P. Lasserre (NUI Galway) is also acknowledged for help with the bioassays and E. Hajdu for training K.B.J. in the taxonomy of sponges from Ecuador.

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