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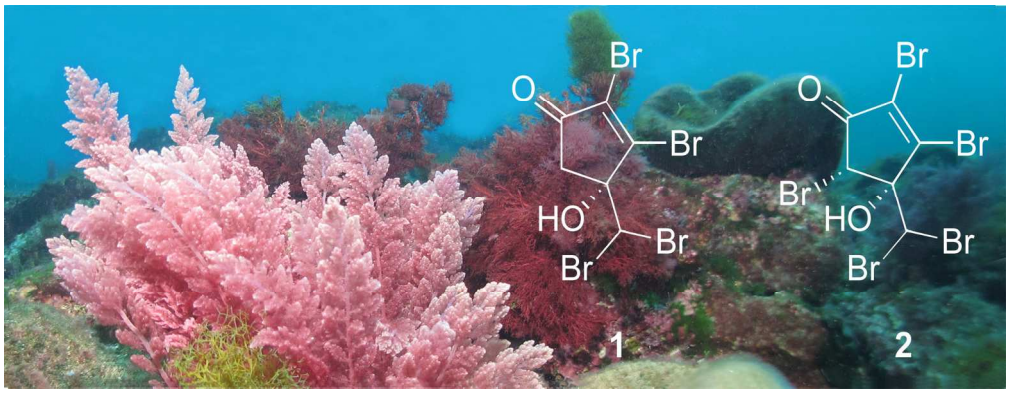

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Mahorones, Highly Brominated Cyclopentenones from the Red Alga *Asparagopsis taxiformis*

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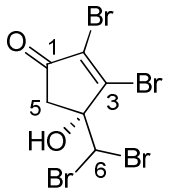
ABSTRACT

The red alga *Asparagopsis taxiformis* (Rhodophyta, Bonnemaisoniaceae) has been shown to produce a large diversity of halogenated volatile organic compounds, with one to four carbons. As the distribution of this alga may expand worldwide, we implemented a research program that aims to understand the functions of its specialized metabolome in marine ecosystems. Phytochemical investigations performed on *A. taxiformis* gametophyte stages from the Indian Ocean revealed two new highly brominated cyclopentenones named mahorone (**1**) and 5-bromomahorone (**2**). They are the first examples of natural 2,3-dibromocyclopentenone derivatives. Their structure elucidation was achieved using spectrometric methods including NMR and MS. A standardized ecotoxicological assay was used as an assessment of their role in the environment revealing high toxicities for both compounds (EC_{50} 0.16 μM for **1** and **2**). Additionally, both compounds were evaluated in antibacterial, antifungal and cytotoxicity assays. Compounds **1** and **2** exhibit mild antibacterial activities against the human pathogen *Acinetobacter baumannii*.

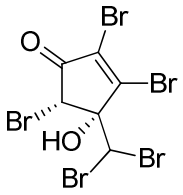
The presence of non-indigenous species in marine ecosystems has strongly increased during the last decades due to globalisation of trade exchanges and transports.[1-3] In this context, seaweeds have attracted much attention,[4-9] because of the negative impacts they can have on the environment counterbalanced by the benefits they may provide to human societies.[10-11] While a general consensus has arisen on the need to minimise these impacts and to limit them in the future, little is known about the ecological and chemical processes which trigger the proliferation of an introduced species and the impact that chemical cues may have on native species.[12] Widely distributed from tropical to temperate waters, species of the genus *Asparagopsis* (Rhodophyta, Bonnemaisoniaceae) are spreading worldwide, affecting several marine ecosystems.[5,13] The genus is composed of cryptogenic populations, and it contains only two species to date: *A. armata* Harvey and *A. taxiformis* (Delile) Trevisan de Saint-Léon. From a chemical perspective, these algae are particularly interesting due the production of a high diversity of halogenated metabolites.[14] Thoroughly studied in the 1970s, *A. taxiformis* produces low molecular weight halogenated compounds with one to four linear carbons, including methanes, ethenes, acetic acids, acetamides, propanols, propanones, propenes, acrylic acids, propylene oxide, propyl and propenyl acetates, butenols and butenones,[15-21] which exhibit an array of biological activities (Supporting Information).[22]

To better understand the mechanisms associated with the possible proliferation of these algae, we initiated a chemical ecological study that explores the role of secondary metabolites in their interaction with native species. In the present part of the study our objectives were: i) to isolate and characterize the secondary metabolites produced by this alga; ii) to assess the ecotoxicological activities of fractions and pure compounds from this alga; iii) and to evaluate their potential as therapeutic agents due to the large biomass of algae available. Unexpectedly, the phytochemical study of specimens of *A. taxiformis* collected in the Indian Ocean led to the

isolation of two new highly brominated cyclopentenones, named mahorone (**1**) and 5-bromomahorone (**2**). We report herein the isolation, the structure elucidation, and also the ecotoxicological as well as antiinfective and antitumor activities of these compounds.



mahorone (**1**)



5-bromomahorone (**2**)

RESULTS AND DISCUSSION

Specimens of *A. taxiformis* were collected off the coast of Mayotte (Indian Ocean). Samples were kept frozen, freeze-dried and ground before extraction by MeOH/CH_2Cl_2 (1:1) and sonication. The resulting extract was then fractioned by reversed-phase (C_{18}) vacuum liquid chromatography using solvents of decreasing polarities (H_2O, MeOH, CH_2Cl_2). The chemical profile of the methanolic fraction showed a very promising UHPLC-DAD-ELSD profile, evidencing major compounds with UV profiles unknown for this alga. A succession of HPLC purification steps on this fraction resulted in the isolation of both new compounds **1** and **2**.

The isotopic pattern in the (–)-HRESIMS spectrum of **1** was indicative of a pentabromo-derivative with the most intense peak at *m/z* 506.6090, which was in accordance with a molecular formula $C_6H_5Br_5O_2$ if associated with the most common $[M-H]^-$ ion observed in (–)-ESIMS. The very low H/C ratio and the presence of a large number of heteroatoms made the structure elucidation highly challenging by NMR. Indeed, the ^{1}H NMR spectrum of **1**, first performed in CD_3OD, indicated three signals for a total integration of three, two of them corresponding to an AB system at δ_H 3.33 (d, J = 18.5 Hz, 1H, H-5a) and 2.77 (d, J = 18.5 Hz, 1H, H-5b) and a singlet at δ_H 6.06 (1H, H-6) (Table 1). The ^{13}C NMR and HSQC spectra confirmed the presence

of six carbons corresponding to the following signals: δ_C 194.9 (C, C-1) assigned to a ketone function; δ_C 131.8 (C, C-2) and 161.2 (C, C-3) assigned to a tetrasubstituted double bond; one saturated methylene, one saturated methine, and one deshielded and saturated quaternary carbon. The lack of vicinal and longer range proton-proton coupling information led us to rely on HMBC correlations to assess the structure of **1** (Figure 1). The proton signals corresponding to the C-5 AB methylene system were HMBC correlated to the other five carbons of the molecule while the proton signal of the C-6 methine was only correlated to C-3, C-4, and C-5. These data suggested that the C-5 methylene, the C-1 ketone and the C-2/C-3 unsaturation were placed within a cyclic system while the C-6 methine was expected to be exocyclic. In this case, the C-6 methine should be substituted on the resulting five-membered ring. An acyclic system would not lead to H-6/C-3 coupling while, in the case of a six-membered ring system, H-5 and C-3 would be ^4J coupled and would not exhibit an intense HMBC correlation as observed. Additionally, H-6 would be expected to couple to four carbons and, in our case, only three couplings are observed. Among the several possibilities still existing for the arrangement around this ring, the H-5a/C-6 and H-5b/C-6 together with the H-6/C-5 HMBC correlations were only consistent with a substitution of the C-6 methine at the C-4 quaternary carbon, one of the positions α to the C-5 methylene. Finally, the H-6/C-3 HMBC correlation allowed us to rule out a last possibility for the endocyclic α,β-unsaturated ketone, and the ketone was unambiguously placed at C-1, the second position α to the C-5 methylene. Among the five bromine atoms identified by HRMS, two of them were placed on the C-2/C-3 tetrasubstituted double bond, which was in agreement with the detected chemical shifts of the corresponding ^{13}C atoms.[23]

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for **1** and **2** in CD_3OD.

	mahorone (**1**)			5-bromomahorone (**2**)	
position	δ_C, mult.	δ_H, mult. (*J* in Hz)	HMBC (H→C)	δ_C, mult.	δ_H, mult.
1	194.9, C			192.1, C	
2	131.8, C			130.4, C	
3	161.2, C			159.9, C	
4	84.0, C			82.8, CH	
5a	46.3, CH_2	3.33, d (18.5)	1, 2, 3, 4, 6	50.9, CH	5.29, s
5b		2.77, d (18.5)			
6	50.7, CH	6.06, s	3, 4, 5	49.3, CH	6.20, s

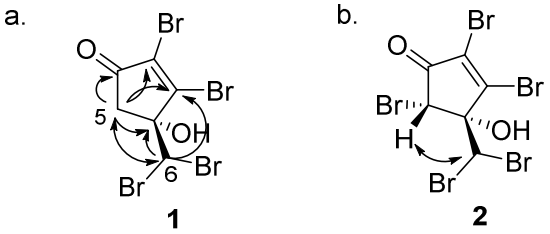
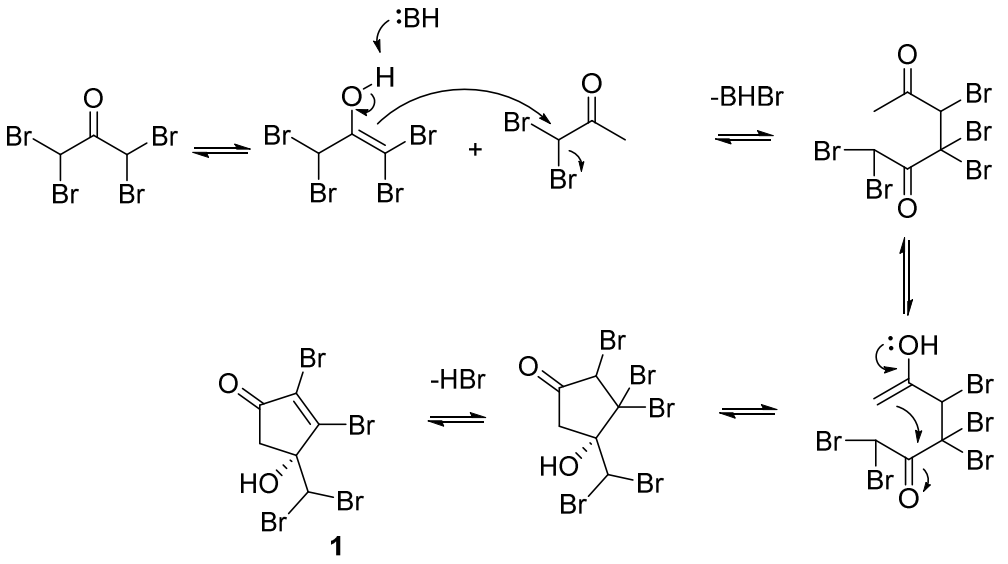


Figure 1. a. Key HMBC (H→C) correlations for **1**; b. key NOESY (double headed arrow) correlation for **2.**

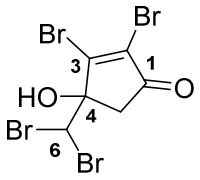
In order to determine whether the resulting bromines and alcohol were placed on the C-4 quaternary carbon or the C-6 methine, we first relied on a possible metabolic pathway leading to these compounds (Scheme 1). Indeed, chemical shifts modeling would not lead to any definitive conclusion on the position of the substituents at C-4 and C-6 due to close electronic effects of these substituents.



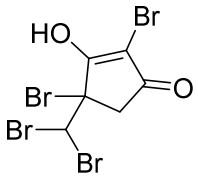
Scheme 1. Biogenetic hypothesis for mahorone (**1**).

The six carbons of **1** suggested that the construction of the skeleton could originate from the condensation of two brominated acetones, as these derivatives have already been reported from this alga.[15,18,24] The enol reactivity of the ketone would be involved, first through the connection of the second ketone partner by nucleophilic substitution, and secondly through the cyclization by an aldol-type reaction. Consequently, this hypothesis allowed us to propose the presence of the alcohol at C-4 and two bromines at C-6. In order to ascertain the position of the substituents on the cycle, we performed ^{13}C NMR modeling for the three possible isomers a, b and c (Table 2). Data obtained for **1** fit best with theoretical values of a, even more when considering values obtained for a compound already reported in the literature with bromines at C-2 and C-3.[23]

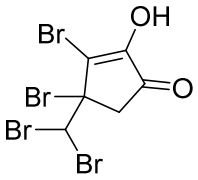
Table 2. Comparison between Experimental and Theoretical ^{13}C NMR Chemical Shifts for isomers **a**, **b** and **c** of **1**.



a



b



c

	Exp.	**a**		**b**		**c**		
position	δ_C	δ_C	Δ (ppm)	δ_C	Δ (ppm)	δ_C	Δ (ppm)	Best prediction
1	194.9	199.8	4.9	209.6	14.7	201.8	6.9	a
2	131.8	117.7	14.1	102.3	29.5	130.1	1.7	c
3	161.2	157.7	3.5	181.2	20	154	7.2	a
4	84	76.7	7.3	58.2	25.8	56.6	27.4	a
5	46.3	42	4.3	41.2	5.1	41.3	5	a
6	50.7	57.9	7.2	55.7	5	55.3	4.6	c

The fifth bromine atom of the molecular formula obtained by (–)-HRESIMS was consequently suspected to be an additional bromide ion obtained through an ion-molecule reaction during ionization leading to $[M+Br]^-$ and then a molecular formula of $C_6H_4Br_4O_2$ for **1**. The EIMS spectrum of **1** was fully consistent with this assumption. The ion cluster with four bromine atoms was observed at *m/z* 428 (1:4:6:4:1) which are odd values and that should then correspond to the molecular peak. The base peak at *m/z* 255 (1:2:1, $[M–CHBr_2]$) confirmed the five-membered ring and the exocyclic $–CHBr_2$ substituent. Bromide adducts are not frequent in (–)-ESIMS and they have usually been observed when halide salts are added to the solvents used.[25-26] In our case the bromide may be present in the methanolic solution just like for sodium adducts or by exchange with another molecule during the ionization process. This reactivity may originate from the absence of acidic protons in the mahorones but also because of the high polarisability of the numerous bromine atoms of the molecules.[27] In order to test this hypothesis, we decided to add salts to our samples to induce the formation of ion adducts. Positive ions were not observed by

(+)-ESIMS (adding LiCl or NH_4Cl) but when adding NH_4Cl we observed an isotopic ion at *m/z* 459 in the (–)-ESIMS spectrum. The presence of this chloride adduct then confirms our assumption (Supporting Information).

Due to the low level of flexibility for mahorone (**1**), we anticipated that the use of electronic circular dichroism could easily lead to the determination of the absolute configuration. Even if the helicity rule proposed by Snatzke is well adapted for *s-trans* α,β-unsaturated ketones,[28] some recent computational studies applied to 4-substituted 2-cyclohexenones demonstrated that molecular modeling was necessary to determine the absolute configuration in these cases.[29] We consequently decided to first perform a conformational analysis of **1** in order to assess the theoretical ECD spectrum of each calculated conformer (Supporting Information). While the conclusion was not clear for the $\pi_{C=C}$-$\pi^*_{C=O}$ transition at 288 nm, all of the calculated conformers obtained for the *S* configuration show a positive Cotton effect at 372 nm for the less intense $n_{C=O}$-$\pi^*_{C=O}$ electronic transition. The experimental data showing a negative Cotton effect for this transition were consistent with an *R* configuration for the asymmetric carbon at C-4.

The molecular formula of **2** was deduced as $C_6H_3Br_5O_2$ on the basis of the (–)-HRESIMS spectrum exhibiting an isotopic cluster centered at *m/z* 586.5183 assuming the same bromide adduct seen for **1**. In the ^1H NMR spectrum of **2** performed in CD_3OD, only two singlets were observed at δ_H 5.29 (H-5) and 6.20 (H-6)**.** Due to the additional bromine of the molecular formula and the disappearance of one proton from the methylene group of **1**, we suspected the substitution of one proton of the methylene at C-5 by a fifth bromine atom. Support was given by EIMS through the isotopic cluster centered at *m/z* 506 (1:5:10:10:5:1; 5 Br) and a base peak at *m/z* 333 (1:3:3:1; [M–$CHBr_2$]). The relative configuration around the five-membered ring was assessed using NOESY. Indeed, a clear nOe was observed between H-6 and H-5 which is consistent with a most likely *cis*-configuration between the hydroxy at C-4 and the bromine at C-

5 (Figure 1). Because the ECD spectrum of **2** presented the same Cotton effects as those encountered for **1,** the 4*R*,5*S* absolute configuration was assigned for this compound.

Ecotoxicological activities of chromatographic fractions and pure compounds **1** and **2** were evaluated against a marine bioluminescent bacteria (*Vibrio fischeri*) using a standardized Microtox assay in order to target bioactive metabolites and hypothesize their role in the environment. Bioactivity was quantified by measuring the direct effect on the metabolism of the bacteria indicated by a decrease in light emitted after 15 min exposure. Toxicity was expressed as: i) EC_{50}, that represents the concentration (in $\mu g.mL^{-1}$) of compounds/extracts that reduces bacteria light to a half the initial light; and ii) as gamma units relative to one mg dry mass.mL^{-1} as this index appears to be relevant for ecological comparisons.[30] Interestingly, the MeOH fraction containing **1** and **2** displays the lowest EC_{50} (2.8 $\mu g.mL^{-1}$) and the maximum gamma (6.452) (Table 3). According to Martì et al., who set the threshold between toxic and non-toxic samples at 0.5 gamma units, this fraction appears as the most bioactive. As a reference, the extract of *Falkenbergia rufolanosa*, the tetrasporophytic stage of *A. armata*, presented a gamma value of 5.204, and was consequently considered as highly toxic.[30] Pure compounds were also evaluated following the same methodology and revealed high toxicities with EC_{50} values of 0.07 $\mu g.mL^{-1}$ (0.16 μM) and 0.08 $\mu g.mL^{-1}$ (0.16 μM) for **1** and **2**, respectively.[31]

Table 3. EC_{50} ($\mu g.mL^{-1}$) and Gamma Units of Fractions of *Asparagopsis taxiformis* Gametophyte Stage (Microtox Bioassay after 15 min Exposure). EC_{50} values are means ± SD (n = 3).

		EC_{50} ($\mu g.mL^{-1}$)		Gamma
Fraction	Solvent fractionating	Mean	SD	
A1	H_2O	<1000	0.0	0.000
A2	H_2O/MeOH (1:1)	6.0	0.6	0.135
A3	MeOH	2.8	0.3	**6.452**
A4	MeOH/CH_2Cl_2 (1:1)	11.6	1.4	0.012
A5	CH_2Cl_2	10.2	0.9	0.000
B1	Hexane	2.9	0.2	0.028
B2	Hexane/EtOAc (1:1)	5.8	0.9	0.081
B3	EtOAc	48.0	3.4	0.001
B4	EtOAc/MeOH	170.5	1.6	0.003
B5	MeOH	391.8	1.2	0.001

Several studies already reported the antimicrobial/antifungal activities of *A. taxiformis* extracts from medium to low polarity fractions, evidencing a large range of biological activities.[32-37] However, biological assays using single compounds from the genus remain scarce.[22,38-39] The release of brominated metabolites involved in the control of epiphytic bacterial communities has been reported from *A. armata* and *Bonnemaisonia asparagoides*.[39-40] Antibacterial and quorum sensing inhibition activities of MeOH extracts of *A. taxiformis* have also been demonstrated.[41] The high toxicities exhibited by the methanolic fraction and compounds **1** and **2** of *A. taxiformis* against a bacterial marine pathogen may be considered as a real advantage for the species. Although their release in the environment has not been demonstrated, we might anticipate their potential role in the interaction of this species with other micro- and/or macro-organisms.

At the same time, we decided to assess the pharmaceutical potential of these compounds, keeping in mind a possible biotechnological use of this widespread macroalga. Isolated metabolites **1** and **2** were thus tested against human bacterial and fungal pathogens: Gram negative bacteria (*Acinetobacter baumannii*, two strains of *Escherichia coli* 2884 and 5746,

Pseudomonas aeruginosa), Gram positive bacteria (*Staphylococcus aureus* MRSA/MSSA - methicillin resistant/sensitive strains) and fungi (*Aspergillus fumigatus* and *Candida albicans*). Mahorones **1** and **2** exhibited the strongest effect against *A. baumannii* (MIC80 of 8 and 16 μg.mL^{-1} respectively) (Table 4). Compound **1** exhibited a weak effect on *E. coli* 5746, while both compounds inhibited the cell growth of Gram positive *S. aureus* MRSA in the same range.

Table 4. Antibacterial and Antifungal MIC_{80} values of **1** and **2**.

		1	**2**	**Control**
		MIC_{80} (μg.mL^{-1})		
Gram-negative bacteria	*A. baumannii*	8	16	Rifampicin 1
	E. coli (2884)	> 32	> 32	Novobiocin 0.3
	E. coli (5746)	16	32	Novobiocin 0.1
	P. aeruginosa	> 10	> 10	Ciprofloxacin 1.5
Gram-positive bacteria	*S. aureus* MRSA	16	16	Imipenem 8
	S. aureus MSSA	> 32	> 32	Penicillin 0.01
Filamentous fungi	*A. fumigatus*	> 32	> 32	
	A. fumigatus + caspofungin enhancer	> 32	> 32	
	caspofungin enhancer	> 32	> 32	
Yeast	*C. albicans*	> 32	> 32	

Cytotoxicities of both compounds were also evaluated on an immortalized hepatocyte (Fa2N4) and several human tumor cell lines including lung (A549), liver (HepG2), colon (HT29) and breast (MCF7). Neither compound showed any inhibition of tumor cells, while compound **1** evidenced a cytotoxic effect on healthy liver cells (53 % inhibition of cell growth at 5 μM). No other study has reported cytotoxic activities of *A. taxiformis* compounds. In addition, Genovese et al. did not find any effect when studying the toxicity of extracts of *A. taxiformis* on digestive glands of mussels, *Mytilus galloprovincialis,*[36] while Zubia et al. highlighted significant cytotoxicities of MeOH/CH_2Cl_2 extracts of *A. armata* against two tumor cell lines.[42]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined using a Perkin Elmer 343 polarimeter. UV and ECD spectra were measured at 20 °C on a J-810 spectropolarimeter (Jasco), and the IR spectrum was measured on a Bruker Tensor 27 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer; chemical shifts were referenced to residual solvent signals (CD_3OD, δ_H = 3.31, δ_C = 49.0). EIMS spectra were recorded on an Agilent 6890 gas chromatograph coupled to a mass spectrometer 5973N at 70 eV. HRESIMS was performed on a LTQ Orbitrap mass spectrometer (Thermo Finnigan) in negative mode. UHPLC data were acquired on a Dionex Ultimate 3000 equipped with Ultimate 3000 RS pump, 3000 Diode Array Detector and Agilent Technologies 380-ELSD. HPLC purifications of the fractions were achieved on a Jasco LC-2000 system equipped with a PU-2087 Plus preparative pumping system and a UV-2075 Plus detector.

Collection and Identification of the Alga. *Asparagopsis taxiformis* gametophyte stages were sampled off the coast of Mayotte (Airport, 12.8213° S, 45.2901° E) in April 2011. Our chemical investigations were focused on the gametophyte stage of the algal life cycle because it represents the highest biomass in native ecosystems and this stage interacts frequently with autochthonous species such as corals and native macrophytes. Voucher specimens were deposited at the MARS Herbarium (MARS07731/Saint-Charles center at Aix-Marseille University). Fresh material was conveyed in ice to the laboratory, frozen at -20 °C and freeze-dried before grinding. Ground material was protected from moisture by adding silica gel packed into paper bags, and stored at -70 °C before extractions.

Extraction and Isolation. Dry material (185 g) was extracted three times with 500 mL MeOH/CH_2Cl_2 (1:1) and sonication (5 min). After filtration, the solutions were pooled and concentrated to dryness at 40 °C to give 17.7 g of a brown residue. The extract was first

solubilized in CH_3CN and then in CH_2Cl_2 to give fractions A and B (3.7 g and 1.9 g, respectively). Fraction A was subjected to reversed-phase vacuum liquid chromatography (VLC, non end-capped C_{18} Polygoprep 60-50, Macherey-Nagel) eluting with H_2O, H_2O/MeOH (1:1), MeOH, MeOH/CH_2Cl_2 (1:1) and CH_2Cl_2, giving fractions A1 to A5. Fraction B was subjected to normal phase VLC (diol, LiChroprep DIOL 40-63 μm, Macherey-Nagel) eluting with hexane, hexane/EtOAc (1:1), EtOAc, EtOAc/MeOH (1:1) and MeOH, giving fractions B1 to B5. Chemical analyses of A1-A5, B3-B5 were performed by UHPLC-DAD-ELSD. The purification was performed on a preparative XSELECT CSH Phenylhexyl column (19 mm x 250 mm, 5 μm, Waters). Elution rate was set at 12 mL.min^{-1}. Initial conditions, maintained during 5 min, were followed by a linear gradient of CH_3CN in H_2O (35 to 90 % for A3) over 22 min. The first purification afforded pure compound **1** (14.3 mg). Compound **2** was further purified on a semi-preparative column Synergi Fusion-RP 80A (10 mm x 250 mm, 4 μm, Phenomenex). Elution was performed at 5 mL.min^{-1} in isocratic mode at 55:45 (CH_3CN/H_2O), and this second purification afforded pure compound **2** (0.8 mg).

Mahorone (**1**): yellow amorphous solid; $[\alpha]_D^{20}$ +3 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε) 254 (3.2) nm; ECD (*c* 9.75x10^{-5} M, MeOH) λ_{max} ($\Delta\varepsilon$ M^{-1}.m^{-1}) 372 (-0.25), 288 (-0.37) nm; IR (neat) ν_{max} 3200 (br), 1728, 1582, 1212, 1045, 1022, 1000, 735 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; EIMS (70 eV) *m/z* (%) 428 [M$^+$, $^{79}Br_2{}^{81}Br_2$] (<1), 349 (4), 347 (4), 258 (3), 257 (50), 256 (6), 255 (100), 253 (53), 229 (6), 227 (13), 225 (7), 199 (4), 197 (3), 173 (3), 161 (3), 159 (3), 133 (8), 131 (10), 119 (4), 117 (5), 51 (4), 50 (3), 42 (3); (–)-HRESIMS *m/z* 506.6090 [M+Br]$^-$ (calcd for $C_6H_4Br_5O_2$, 506.6092, Δ –0.2 ppm).

5-Bromomahorone (**2**): yellow amorphous solid; $[\alpha]_D^{20}$ +2 (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ε) 254 (3.2) nm; ECD (*c* 5.64x10^{-5} M, MeOH) λ_{max} ($\Delta\varepsilon$ M^{-1}.m^{-1}) 370 (-0.59), 288 (-0.70) nm;^{1}H and ^{13}C NMR data see Table 1; EIMS (70 eV) *m/z* (%) 506 [M$^+$, $^{79}Br_3{}^{81}Br_2$] (<1), 337

(32), 335 (95), 334 (11), 333 (100), 332 (16), 331 (35), 330 (14), 268 (14), 266 (13), 255 (17), 253 (15), 251 (14), 225 (19), 223 (19), 197 (11), 187 (9), 185 (9), 175 (10), 173 (14), 133 (19), 131 (24), 79 (11), 62 (11), 61 (10), 50 (10); (–)-HRESIMS *m/z* 586.5183 $[M+Br]^-$ (calcd for $C_6H_3Br_6O_2$, 586.5177, Δ +1.02 ppm).

Ecotoxicological Assays. Microtox (Microbics) is a standardized ecotoxicological bioassay that measures the toxic effect of compounds/extracts on bioluminescent marine bacteria, *Vibrio fischeri* (NRRL B-11177 strain).[43] Tests were carried out to evaluate the toxicity of A1 to B5 fractions, as well as those of pure compounds **1** and **2**. Fractions were made up to an initial concentration of 1000 or 2000 μg.mL^{-1} (500 μg.mL^{-1} for pure compounds) using artificial seawater containing 2% acetone to assist compound dissolution. Concentrations tested were 45, 22.5, 11.25 and 5.625% of the initial concentrations after 15 min of bacteria exposure to toxins. Samples were diluted when necessary to fit apparatus recommendations. Toxicities, given as conventional gammas from Microtox, are measured as $(I_0 / I_t) - 1$ where I_0 and I_t are the intensity of the bioluminescences before and after exposure time, respectively. A linear relationship is obtained when plotting the log of gamma against the log of the tested concentrations, and permit the direct determination of an EC_{50} value (equivalent to a gamma of 1) representing the concentration of fractions (or compounds) that reduces the initial bioluminescence to 50%. However, because extract concentrations (and consecutive EC_{50}) are expressed as μg of extracts.mL^{-1} of solution independent of extraction yields, comparisons between samples/species were difficult. So, new regression curves were generated replacing original concentrations (μg extracts.mL^{-1}) by modified ones (mg DM alga.mL^{-1} of solution) as described:

Concentrations (mg DM alga.mL^{-1}) = concentration of extract (μg.mL^{-1}). dry mass of initial sample (mg) / dry mass of fraction (μg).

Using modified concentrations, mathematical relationships were generated using Statgraphics (four concentrations in triplicate, except for fraction B5 because of insufficient material), and unconventional gamma units relative to one mg of alga.mL^{-1} of solution were calculated, according to the formula.

Gamma = 10^{B} where B is the intercept of generated regressions for each fraction (Supporting Information).

Antimicrobial Assays. All bioassays were performed by Fundacion Medina (Granada - Spain). Antimicrobial assays were carried out against six bacteria (*Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* as Gram negative bacterial models; *Staphylococcus aureus* MSSA, *Staphylococcus aureus* MRSAas examples of Gram-positive bacteria). Positive controls were used against Gram-negative strains (rifampicin, novobiocin, ciprofloxacin for *A. baumanii*, *E. coli*, *P. aeruginosa* respectively) and Gram positive strains (imipenem, penicillin and tunicamycin for *S. aureus* MSSA, *S. aureus* MRSA respectively). Amphotericin B was used as negative controls against all strains. The liquid assay procedure was employed to measure microbial susceptibility of all strains. Antifungal assays were performed on *Candida albicans* as a yeast model and *Aspergillus fumigatus* as a filamentous fungus, using the liquid assay procedure.

In the liquid assay procedure, compounds were tested in a liquid growth medium dispensed in 96 or 384 well plates (Costar 3370 and 3680 from Corning) inoculated with a bacterial or fungal suspension. Following overnight incubation at 37 ºC, the plates were examined for visible bacterial/fungal growth as evidenced by turbidity or fluorescence. Compounds were dissolved in DMSO to a stock solution of 1 mM, and tested at the final concentration of 5 μM in triplicate in the same experiment. For *A. baumannii*, *P. aeruginosa*, *E. coli* and *S. aureus* liquid assays, 90 μL of the appropriate diluted inoculums are mixed with 8.4 μL of medium (Luria Broth) and 1.6 μL

of a 0.312 mM (DMSO 100%) stock solution of compounds per well. The *A. fumigatus* and *C. albicans* liquid assays were performed in 384-well microtiter plates: 0.5 μL per well of the compounds from a 0.5 mM solution followed by the inoculum to a final volume of 50 μL. The medium used was a modified RPMI medium: 10.4 $g.L^{-1}$ of RPMI-1640 medium (R8755 from Sigma), 6.7 $g.L^{-1}$ of Yeast Nitrogen Base (YNB) (BD – Becton, Dickinson and Company), 1.8 % (w/v) glucose and 40 mM HEPES (pH 7.1). An amphotericin B curve (0.5 to 4 $\mu g.mL^{-1}$) was used as a control. Cellular viability was scored using resazurin at the final concentration of 0.002 %.[45]

Cytotoxicity Assays. Five human cancer cell lines (A549, HepG2, HT29, MCF7, MiaPAca2) and healthy liver cells (Fa2N4) were used to evaluate cytotoxic effects according to the MTT methodology.[46] Cells were seeded at a concentration of 3×10^4 cells/well in 200 μL culture medium and incubated at 37 ºC at 5% CO_2. Compounds **1** and **2** were prepared at 1 mM in 100% DMSO. One μL of this solution was added to 199 μL of culture medium (1/200 dilution), and left in contact with the cells for 24 h at 37 ºC in a 5% CO_2 incubator. After this time, a MTT solution was prepared at 5 $mg.mL^{-1}$ in PBS 1X and then diluted at 0.5 $mg.mL^{-1}$ in MEM without phenol red. 100 μL of the MTT solution was added to each well. The plates were gently shaken and incubated for 3 h at 37 ºC in 5% CO_2 incubator. The supernatant was removed and 100 μL of DMSO 100% was added. The plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a multireader Victor at a wavelength of 570 nm. Methyl methanesulfonate MMS (8 mM) was used as positive control, and DMSO 1 % as a negative control (same concentration as compounds).

Calculations.

NMR prediction was achieved using the PERCH software (PERCH Solutions Ltd). After importation of the molecular model, geometry optimisation was conducted using Monte Carlo analysis. The most stable conformer was used to obtain the calculated chemical shifts.

ECD calculations were performed at 298 K using the Gaussian03 program package.[47] The Density Functional Theory (DFT) was used to scan the potential energy surface at the B3LYP/6-311G* level to identify the most stable conformers. TDDFT was employed to calculate excitation energy (in eV) and rotatory strength R in dipole velocity (R_{vel}) and dipole length (R_{len}) forms. The calculated rotatory strengths were simulated in ECD curve by using the software package SpecDis.[48]

Statistical Analyses. Statgraphics Centurion 15.2.11.0 was used to determine linear regressions.

Supporting Information Available. All NMR spectra, HRESIMS, EIMS and ECD spectra for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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