

Article

Anti-*Acanthamoeba* Activity of Brominated Sesquiterpenes from *Laurencia johnstonii*

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Abstract: Focused on our interest to develop novel antiparasitic agents, the present study was aimed to evaluate the biological activity of an extract of *Laurencia johnstonii* collected in Baja California Sur, Mexico, against an *Acanthamoeba castellanii* Neff strain. Bioassay-guided fractionation allowed us to identify the amoebicidal diastereoisomers α -bromocuparane (**4**) and α -isobromocuparane (**5**). Furthermore, bromination of the inactive laurinterol (**1**) and isolaurinterol (**2**) yielded four halogenated derivatives, (**6**)–(**9**), which improved the activity of the natural sesquiterpenes. Among them, the most active compound was 3 α -bromojohnstane (**7**), a sesquiterpene derivative which possesses a novel carbon skeleton johnstane.

Keywords: brominated sesquiterpene; marine natural products; *Laurencia johnstonii*; johnstane; 3-bromojohnstane; anti-amoeboid activity; *Acanthamoeba*

1. Introduction

Free-living amoeba (FLA) are widely distributed protozoa in the environment [1–3]. These parasites present a life cycle with two different stages: the trophozoite and the resistant phase, the cyst. Among FLA, *Acanthamoeba* genus [4] has been isolated from air, soil, water, contact lenses, air conditioning units, clinical samples, among others [5]. These parasites are able to cause pathologies in humans such as Granulomatous Amoebic Encephalitis (GAE) and Amoebic Keratitis (AK) [1–3,5]. Regarding *Acanthamoeba* infections, an early diagnosis is crucial to achieve a successful treatment [3,6]. Antimicrobial chemotherapy is the most widely used method for treating *Acanthamoeba*-caused infections. Pentamidine, azoles, sulfonamides, and possibly flucytosine, are among the most frequently used drugs in successfully treated cases of GAE, whereas topical chlorhexidine or polyhexamethylene biguanide appear to be the most effective option in cases of AK [7]. However, the existing therapies are not fully effective against these organisms mainly due to the existence of the cyst phase, and also due to the presence of strains that are resistant to the currently used anti-amoebic drugs [2,3,8].

In the past, natural products have been used for the treatment of different parasitic diseases. Artemisinin, quinine, amphotericin B, and ivermectin are examples of important antiparasitic compounds isolated from plants and microorganisms [9,10]. From 1981 to 2014, 15 small molecules were approved as antiparasitic drugs; among them, two were obtained from natural sources and five are the result of semisynthetic derivatives of natural products [11], however, none of them came from marine sources. Many other natural products of diverse molecular structures have revealed antiparasitic potency in the laboratory and, thus, represent interesting lead structures for the development of new and urgently needed antiparasitic agents.

The genus *Laurencia* is one of the richest sources of novel secondary metabolites among red algae [12–15]. Although the chemistry of *Laurencia* species has been exhaustively investigated, the biological activity of the isolated secondary metabolites has not been studied in a systematic way. Furthermore, *Laurencia* metabolites have been revealed to possess antiparasitic properties against a number of parasites and their vectors. However, no activity has been previously reported against *Acanthamoeba* [10,16].

Focused on our interest to develop novel lead structures for the development of antiparasitic agents, the present study was aimed to evaluate the biological activity of an extract of *Laurencia johnstonii* against an *Acanthamoeba castellanii* Neff strain. To the best of our knowledge, johnstonol [17] and prepacifenol epoxide [18] are the only sesquiterpenes isolated from specimens of *Laurencia johnstonii*, both with a chamigrene carbon skeleton. In this paper, we report the isolation of the known brominated metabolites laurinterol (1), isolaurinterol (2), aplysin (3), and the antiparasitic diastereoisomers α -bromocuparane (4) and α -isobromocuparane (5), with cyclolaurane, laurane and cuparane backbones. (Figure 1). In addition, transformation of (1) and (2) has yielded four structural derivatives (6–9) which improved the antiparasitic activity with respect to the natural products.

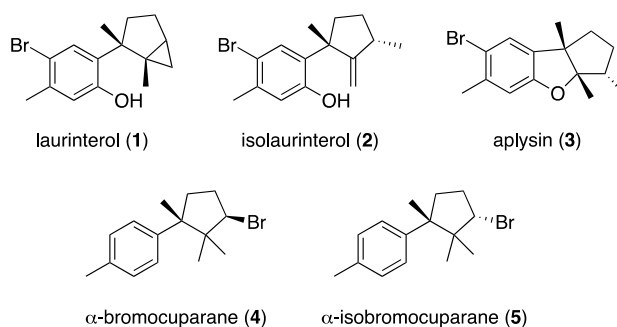


Figure 1. Structures of natural sesquiterpenes (1)–(5) isolated from *Laurencia johnstonii*.

2. Results and Discussion

2.1. Isolation and Identification of Natural Brominated Sesquiterpenes

Laurencia johnstonii was collected off the coast of the Gulf of California, Mexico. Clean and dry specimens were extracted in ethanol to give a crude extract which showed moderate anti-*Acanthamoeba* activity with an IC_{50} value of 125.14 ± 4.5 $\mu\text{g/mL}$. The bioassay-guided fractionation of the ethanolic extract by Sephadex LH-20 led us to an active fraction (SF3). This fraction was further chromatographed over a silica gel column using a step-gradient from *n*-hexane to EtOAc to give seven subfractions. The most active was SF3.1 (*n*-hexane fraction) with an IC_{50} value of 101.29 ± 0.2 $\mu\text{g/mL}$. After a separation on a silica gel open column eluted with mixtures of *n*-hexane:EtOAc, fraction SF3.1 yielded aplysin (3) [19], and the active stereoisomers α -bromocuparane (4) and α -isobromocuparane (5) [20], which showed activity against *Acanthamoeba castellanii* Neff with IC_{50} values of 90.68 ± 1.5 and 64.25 ± 1.5 $\mu\text{g/mL}$, respectively. Additionally, laurinterol (1) [21], and isolaurinterol (2) [22] were isolated from the inactive fraction SF3.2. The NMR, mass spectrometry, and optical rotation data of (1)–(5) were compared with those previously reported in the literature to confirm their structures (Supplementary Materials).

2.2. Derivatization of Laurinterol (1) and Isolaurinterol (2)

Halogenation is a common biosynthetic strategy found in marine organisms as is the case of *Laurencia* species [23]. Halogen-containing natural products display a wide range of biological activities; therefore, they are interesting structures for medicinal chemistry studies. The presence of halogen substituents in many natural products also enhances their biological activities [24]. In order to evaluate structure–activity relationships of laurinterol and its congeners in antiparasitic assays, bromination of (1) and (2) were carried out to obtain their brominated derivatives. Hence, laurinterol (1) was dissolved in ethyl ether and treated with bromine. The reaction mixture was chromatographed on a silica gel column to obtain the brominated sesquiterpene derivatives (6) and (7) (Figure 2).

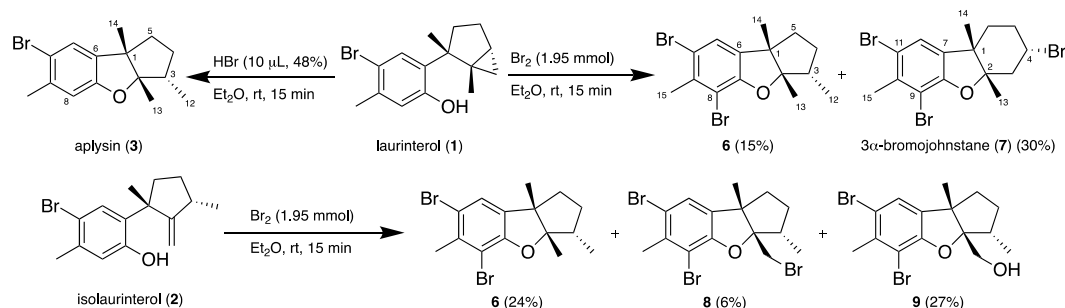


Figure 2. Bromination reaction of natural laurinterol (1) and isolaurinterol (2).

Compound (6) was obtained as a colorless oil. Its molecular formula was established by HR-EI-MS (High-Resolution Electron Impact Mass Spectrometry) analysis as $C_{15}H_{18}Br_2O$ by the presence of three molecular ions $[M]^+$ at m/z 371.9711/373.9702/375.9677 (calcd. 371.9724/373.9704/375.9683; ratio 50:100:48), indicative of two bromine atoms and six degrees of unsaturation. The structure of (6) was determined by comparison of its NMR data with those for alypsin (3) (Table 1). The main differences observed in their 1H NMR spectra were the absence of one of the two aromatic singlets of (3), H-8 (δ_H 6.59), and a deshielded aromatic methyl group H₃-15 (δ_H 2.49) for (6) compared with that of 3 H₃-15 (δ_H 2.30). These values agree with the presence of a bromine atom at C-8 in the aromatic ring as well as with the information provided by mass spectrometry.

Table 1. 1H and ^{13}C NMR data for alypsin (3) and (6) (600 MHz, 150 MHz, $CDCl_3$).

Position	Alypsin (3)		6	
	δ_C , Type	δ_H (J in Hz)	δ_C , Type	δ_H (J in Hz)
1	54.3, C		56.0, C	
2	99.8, C		100.5, C	
3	46.0, CH	1.75, ddd (12.8, 6.9, 6.5)	46.4, CH	1.79, ddd (13.3, 6.5, 6.5)
4	31.1, CH ₂	1.62, m 1.15, m	31.3, CH ₂	1.65, m 1.15, m
5	42.5, CH ₂	1.85, dd (6.4, 12.1) 1.58, d (6.2)	42.7, CH ₂	1.86, dd (12.1, 6.2) 1.64, ddd (12.1, 6.2, 6.2)
6	136.2, C		136.2, C	
7	158.1, C		156.0 ¹ , C	
8	110.8, CH	6.59, s	105.1, C	
9	136.9, C		136.0, C	
10	113.9, C		114.1, C	
11	126.5, CH	7.14, s	125.4, CH	7.11, s
12	13.0, CH ₃	1.10, d (6.8)	13.3, CH ₃	1.14, d (6.8)
13	23.1, CH ₃	1.30, s	20.4, CH ₃	1.34, s
14	23.3, CH ₃	1.29, s	23.2, CH ₃	1.32, s
15	29.9, CH ₃	2.30, s	23.5, CH ₃	2.49, s

¹ Chemical shift deduced from the HMBC experiment.

Aplysin (**3**) was easily obtained after treatment of laurinterol (**1**) with HBr (Figure 2). The specific rotations for (**1**) ($[\alpha]_D^{25} +17$) and **3** ($[\alpha]_D^{25} -49$) are consistent with those reported in the literature for both compounds (**1**): $[\alpha]_D^{25} +13$ and **3**: $[\alpha]_D^{25} -86$, for which the absolute configuration was established by X-ray crystallography [21,25]. According to this, and supported by consistent chemical shifts and coupling constants of H₃-12, H₃-13, H₃-14 and H-3 between **6** and aplysin (**3**) (Table 1), the absolute configurations of **6** are 1*S*, 2*S*, and 3*S*. Caccamese and Rinehart identified **6** as bromoaplysin in 1978 by GC-MS [26]. Later, in 2010, a patented methodology for the extraction of antiobesity sesquiterpenes from *Laurencia* species [27] refers **6** as 8-bromoaplysin, however no complete NMR data has been reported for this brominated sesquiterpene.

Compound **7** was obtained as a colorless oil. The HREIMS analysis provided four molecular ions $[M]^+$ at m/z 449.8840/451.8800/453.8798/455.8758 (calcd. 449.8829/451.8809/453.8789/455.8768; ratio 26:78:67:26) suggesting a molecular formula C₁₅H₁₇OBr₃ and six degrees of unsaturation. The structure of **7** was established based on the analysis of its spectroscopic data and comparison with those of **6**. 1D and 2D NMR spectra evidenced three methyl, three methylenes, and two methine groups (one on sp² carbon and one bearing heteroatom), in addition to five sp² and two sp³ quaternary carbons. Similar to **6**, the pentasubstituted phenyl ring was confirmed by the presence of a singlet at δ_H 7.14 (H-12) and the methyl group at δ_H 2.53 (H₃-15) in the ¹H NMR spectrum (Table 2), and the HMBC correlations from H₃-13 (δ_H 2.54, s) and H₃-14 (δ_H 1.04, s) to a quaternary carbon at δ_C 92.3 (C-2), and from H₃-14 to C-7 (δ_C 136.3) confirming the presence of the same dihydrofuran ring found in **6**. The main difference between both compounds was a COSY spin system from the diastereotopic protons H₂-3 (δ_H 2.42/1.99), sequentially coupled with the bromomethine H-4 (δ_H 3.84), methylene H₂-5 (δ_H 2.18/1.76), and methylene H₂-6 (δ_H 2.18/1.54). HMBC correlations from H₃-14 (δ_H 1.04) to C-6 (δ_C 32.8) and H₃-13 (δ_H 2.54) to C-3 (δ_C 47.6) led to the connection of this substructure within the molecule and thus established the planar structure of the rearranged sesquiterpene **7** as shown in Figure 3.

Table 2. NMR data for 3 α -bromojohnstane (**7**) (600 MHz, 150 MHz, CDCl₃).

Position	3 α -Bromojohnstane (7)	
	δ_C , Type	δ_H (J in Hz)
1	48.1, C	
2	92.3, C	
3	47.6, CH ₂	β : 2.42 dd (3.6, 12.9) α : 1.99 dd (12.6, 12.9)
4	45.8, CH	3.84 dddd (3.6, 3.6, 12.3, 12.6)
5	34.2, CH ₂	β : 2.18 dddd (3.2, 3.5, 3.6, 12.9) α : 1.76 dddd (3.5, 12.3, 12.3, 12.6)
6	32.8, CH ₂	β : 1.54 ddd (3.5, 12.3, 12.9) α : 2.18 ddd (3.2, 3.5, 12.9)
7	136.3, C	
8	155.3, C	
9	107.6, C	
10	136.6, C	
11	115.5, C	
12	124.6, CH	7.14 s
13	19.7, CH ₃	1.54 s
14	26.7, CH ₃	1.04 s
15	23.3, CH ₃	2.53 s

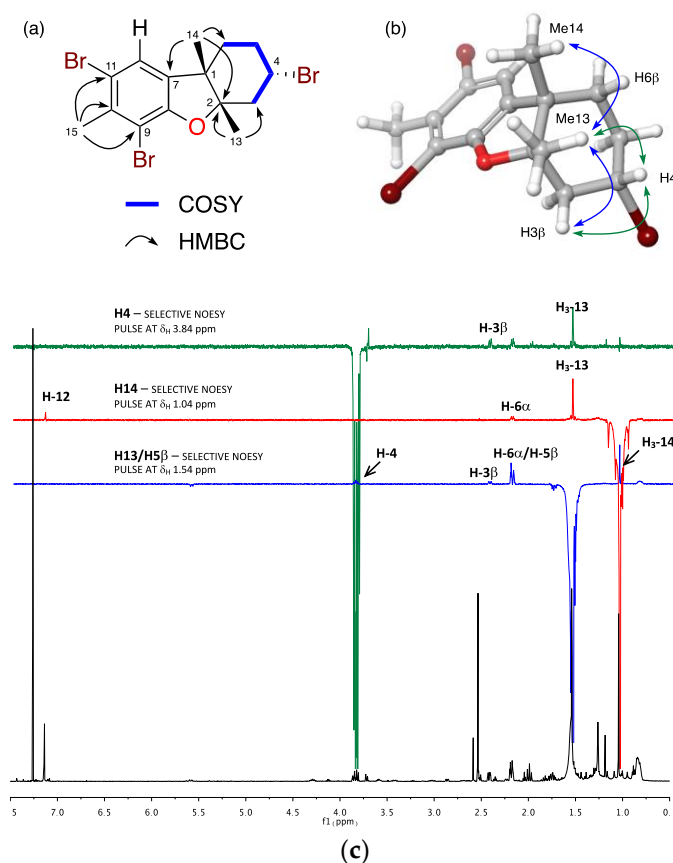


Figure 3. (a) Selected COSY and HMBC correlations, (b) key-NOE correlations and (c) 1D-NOE experiments of 3α-bromojohnstane (7).

NOE correlations observed from H₃-13 to H₃-14 and H-4, and from H-4 to the diastereotopic proton H-3β located all these protons on the same face of the molecule. Since the absolute configuration of laurinterol (1) has been confirmed and configuration at C-1 is conserved in 7, it is possible to establish the absolute configuration of its chiral centers as 1*S*, 2*S*, and 4*S*.

In 1972, Yamada et al. reported the synthesis of 2,3-dimethyl-3-(5-bromo-2-methoxy-4-methylphenyl)-cyclohexane from an anisole derivative as a key intermediate to obtain aplysin (3) and other related sesquiterpenes [28]. Also, synthetic efforts to obtain the sesquiterpene cuparene through radical cyclization exclusively yielded 1-(1,2-dimethylcyclohexyl)-4-methylbenzene [29].

It is noteworthy that, even though chemical investigations on the prolific genus *Laurencia* have yielded over 500 sesquiterpene metabolites belonging to more than 50 carbon frameworks [16], so far none has been reported to possess that of 7. Thus, for this carbon backbone we propose the name johnstane and the name 3α-bromojohnstane for 7 (Figure 4).

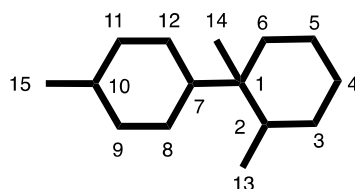


Figure 4. Johnstane carbon skeleton and numbering system.

In the same way, isolaurinterol (2) was dissolved in ethyl ether and treated with bromine to afford 6, and the known sesquiterpenes 8,10-dibromoisoaplysin (8) [30] as well as the 13-hydroxy derivative 9, also obtained after bromination of debromoaplysinol [31].

2.3. Antiparasitic Effect Against *Acanthamoeba Castellanii* Neff Strain

As well as in the case of natural sesquiterpenes 1–5, the anti-amoeboid activity of the brominated sesquiterpene derivatives 6–9 was tested against *Acanthamoeba castellanii* Neff (Inhibitory Concentrations (IC₅₀) shown in Table 3). Moreover, their toxicity (Cytotoxic Concentrations (CC₅₀) at 24 h, Table 3) against murine macrophages J774.A1 (ATCC # TIB-67) at 24 h was evaluated as previously described [32,33].

Table 3. Effect of *Laurencia johnstonii* ethanolic extract and 1–9 against *Acanthamoeba castellanii* Neff (IC₅₀) and murine macrophage J774.A1 (CC₅₀). * Reference compounds.

Sample	IC ₅₀ (µg/mL)	IC ₅₀ (µM)	CC ₅₀ (µg/mL)
Crude extract	125.14 ± 4.5		n/d
1	>100		23.65 ± 2.3
2	>100		7.25 ± 0.7
3	>100		323.69 ± 12.0
4	90.674 ± 1.529	322.41 ± 5.44	33.04 ± 4.2
5	64.251 ± 3.492	228.46 ± 12.42	85.34 ± 10.9
6	24.559 ± 1.105	65.64 ± 2.95	32.880 ± 3.125
7	18.804 ± 0.198	41.51 ± 0.44	62.341 ± 2.589
8	22.818 ± 1.896	50.37 ± 4.19	70.365 ± 3.245
9	29.937 ± 2.918	76.74 ± 7.48	74.931 ± 2.769
Chlorhexidine *	1.526 ± 0.45	3.02 ± 0.89	6.64 ± 0.35
Voriconazole *	0.33 ± 0.1	0.94 ± 0.29	2.64 ± 0.27

As shown in Table 3, all brominated derivatives (6–9) improved the activity of the natural sesquiterpenes (1–5). Monobrominated compounds in the aromatic ring, 1–3, were inactive, thus suggesting that dibromination of the aromatic moiety is relevant to obtain anti-amoeboid activity. On the other hand, natural diastereoisomers 4 and 5, which only differ in the configuration of the bromine atom attached to C-3 of cyclopentane ring, showed a differentiated activity. It is interesting to note that an α -oriented bromine substitution at C-3 in 5 increases the activity against *A. castellanii* while decreases the toxicity, a structural feature also found in compound 7. 3 α -bromojohnstane (7) was the most active molecule against *A. castellanii* with an IC₅₀ value of 41.51 µM, and one of those with lower toxicity values against murine macrophages among the assayed compounds.

3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations were measured in CH₂Cl₂ on a PerkinElmer 241 polarimeter (Waltham, MA, USA), by using a sodium lamp operating at 589 nm. NMR spectra were recorded on a Bruker AVANCE 500 MHz or 600 MHz, as required. NMR spectra were obtained dissolving samples in CDCl₃ (99.9%) and chemical shifts are reported relative to solvent (δ_{H} 7.26 and δ_{C} 77.0 ppm) and TMS as internal pattern. Bruker AVANCE 600 MHz instrument is equipped with a 5 mm TCI inverse detection cryoprobe. Standard Bruker NMR pulse sequences were utilized. HR-ESI-MS (High-Resolution ElectroSpray Ionization Mass Spectrometry) and HR-EI-MS (High-Resolution Electron Impact Mass Spectrometry) data were obtained on a Waters LCT Premier XE Micromass (Manchester, UK) and VG-AutoSpec Micromass (Manchester, UK) spectrometers, respectively. IR spectra were recorded on a Bruker IFS66/S (Ettlingen, Germany) equipped with an ATR accessory using CH₂Cl₂ solutions. EnSpire[®] Multimode Reader (Perkin Elmer, Walt, MA, USA) using absorbance values of Alamar Blue[®] reagent. TLC (Thin layer chromatography) (Merck, Darmstadt, Germany) was visualized by UV light (254 nm) and spraying with cobalt chloride reagent (2% in sulfuric acid, 10%) and heating.

3.2. Biological Material

Laurencia johnstonii was collected by hand during June–July 2015 off the coast of Baja California Sur, Mexico (24°21'10.8" N, 110°16'58.8" W). A voucher specimen (code 13-003) was deposited at the Herbarium of the Laboratory of Marine Algae of the CICIMAR (Centro Interdisciplinario de Ciencias Marinas, Mexico). This seaweed was identified to genus and species level by Dr. Rafael Riosmena Rodríguez (Universidad Autónoma de Baja California Sur) using taxonomic keys for future reference.

3.3. Extraction and Isolation

Specimens of *Laurencia johnstonii* were cleaned of sand and epiphytes, and dried. Dried alga was crushed using a blender and extracted with EtOH for three days at 25 °C under gentle agitation. The EtOH was replaced (3 × 1200 mL), and the combined extracts were filtered through a Whatman no. 4 filter paper. Solvent was removed using a rotatory vacuum evaporator. 10.0 g of the resulting extract were chromatographed in Sephadex LH-20 (Sigma, St. Louis, MO, USA) (500 × 70 mm, CH₃OH, 100%) to obtain five fractions. The active fraction SF3 was separated in flash Silicagel 0.2–0.5 mm (Sigma-Aldrich, St. Louis, MO, USA) (130 × 70 mm) using a stepwise gradient of *n*-hexane: ethyl acetate to obtain 7 fractions. Fractions SF3.1 (100% *n*-hexane) and SF3.2 (95% *n*-hexane) were separated on a normal phase open column (Silicagel, 0.2–0.5 mm, 300 × 50 mm, using a stepwise gradient of *n*-hexane: ethyl acetate) to yield pure compounds **5** (10.5 mg), **4** (7.2 mg) and **3** (45.9 mg) from SF3.1 and compounds **3** (10.2 mg), **2** (8.1 mg) and **1** (55.7 mg) from SF3.2. (Supplementary Materials, Scheme S1)

3.3.1. Laurinterol (**1**)

White crystal; $[\alpha]_D^{25} +17$ (c 0.15, CH₂Cl₂); HRESIMS m/z 293.0531 [M – H][−] (calc. C₁₅H₁₈O⁷⁹Br, 293.0541), 295.0518 [M – H][−] (calc. C₁₅H₁₈O⁸¹Br, 295.0521) ¹H NMR (500 MHz, CDCl₃) δ 0.55 (1H, dd, $J = 7.9, 4.8$ Hz, H-12), 0.58 (1H, t, $J = 4.6$ Hz, H-12), 1.15 (1H, dt, $J = 8.1, 4.3$ Hz, H-3), 1.28 (1H, m, H-5), 1.32 (3H, s, H-13), 1.41 (3H, s, H-14), 1.66 (1H, dd, $J = 12.3, 8.0$ Hz, H-4), 1.95 (1H, tdd, $J = 12.3, 8.1, 4.4$, H-4), 2.09 (1H, dd, $J = 13.2, 8.1$ Hz, H-5), 2.29 (3H, s, H-15), 5.26 (1H, br, s, 7-OH), 6.61 (1H, s, H-8), 7.61 (1H, s, H-11); ¹³C NMR (125 MHz, CDCl₃) δ 16.2 (C-12), 18.6 (C-13), 22.2 (C-14), 23.5 (C-15), 24.4 (C-3), 25.3 (C-4), 29.6 (C-2), 35.9 (C-5), 114.9 (C-10), 118.8 (C-8), 132.3 (C-11), 134.0 (C-6), 135.9 (C-9), 153.3 (C-7).

3.3.2. Isolaurinterol (**2**)

Colorless amorphous solid; $[\alpha]_D^{25} -46$ (c 0.14, CH₂Cl₂); HRESIMS m/z 293.0536 [M – H][−] (calc. C₁₅H₁₈O⁷⁹Br, 293.0541), 295.0528 [M – H][−] (calc. C₁₅H₁₈O⁸¹Br, 295.0521) ¹H NMR (500 MHz, CDCl₃) δ 1.21 (3H, d, $J = 7.0$ Hz, H-12), 1.42 (1H, ddd, $J = 12.8, 8.2, 6.6$ Hz, H-5), 1.46 (3H, s, H-14), 1.59 (1H, dt, $J = 12.9, 7.1, 7.1$ Hz, H-4), 2.05 (1H, ddt, $J = 12.8, 8.5, 7.0, 7.0$ Hz, H-3), 2.20 (1H, ddd, $J = 13.0, 8.1, 6.7$ Hz, H-4), 2.31 (3H, s, H-15), 2.85 (1H, ddt, $J = 9.1, 6.9, 6.9, 2.3, 2.3$ Hz, H-5), 4.94 (1H, d, $J = 2.2$ Hz, H-13), 5.11 (1H, d, $J = 2.2$ Hz, H-13), 5.56 (1H, br, s, 7-OH), 6.73 (1H, s, H-8), 7.45 (1H, s, H-11); ¹³C NMR (125 MHz, CDCl₃) δ 21.2 (C-14), 22.2 (C-15), 27.8 (C-12), 31.2 (C-4), 37.6 (C-3), 39.1 (C-5), 49.8 (C-1), 106.9 (C-13), 115.5 (C-10), 120.4 (C-8), 131.2 (C-11), 132.7 (C-6), 137.2 (C-9), 153.0 (C-7), 165.4 (C-2).

3.3.3. Aplysin (**3**)

Colorless needles; $[\alpha]_D^{25} -49$ (c 0.19, CH₂Cl₂); HRESIMS m/z 293.0551 [M – H][−] (calc. C₁₅H₁₈O⁷⁹Br, 293.0541), 295.0529 [M – H][−] (calc. C₁₅H₁₈O⁸¹Br, 295.0524). ¹H and ¹³C NMR data (Table 1).

3.3.4. α-Bromocuparane (**4**)

White amorphous solid; $[\alpha]_D^{25} +21$ (c 0.19, CH₂Cl₂); HREIMS [M – H]⁺ m/z 279.0744 (calc. for C₁₅H₂₀⁷⁹Br, 279.0748), 282.0723 (calc. for C₁₅H₂₀⁸¹Br, 282.0728), [M – Br]⁺ m/z 201.1640 (calc. for C₁₅H₂₁, 201.1643); ¹H NMR (500 MHz, CDCl₃) δ 0.62 (3H, s, H-13), 1.09 (3H, s, H-12), 1.43 (3H, s, H-14), 1.96 (1H, m, H-5), 2.24 (1H, m, H-4), 2.27 (1H, m, H-5), 2.32 (3H, s, H-15), 2.52 (1H, ddt, $J = 14.3, 9.3, 5.2$,

H-4), 4.07 (1H, t, $J = 9.4$ Hz, H-3), 7.09 (4H, m, H-7, H-8, H-10, H-11); ^{13}C NMR (125 MHz, CDCl_3) δ 20.7 (C-15), 20.9 (C-13), 22.4 (C-12), 25.0 (C-14), 33.1 (C-4), 36.4 (C-5), 47.4 (C-2), 48.4 (C-1), 62.0 (C-3), 127.3 (C-7, C-11), 128.1 (C-8, C-10), 135.4 (C-9), 143.9 (C-6).

3.3.5. α -Isobromocuparane (5)

Colorless oil; $[\alpha]_{\text{D}}^{25} +37$ (c 0.14, CH_2Cl_2); HREIMS $[\text{M}]^+ m/z$ 280.0826 (calc. for $\text{C}_{15}\text{H}_{21}^{79}\text{Br}$, 280.0827), 282.0801 (calc. for $\text{C}_{15}\text{H}_{21}^{81}\text{Br}$, 282.0806), $[\text{M} - \text{Br}]^+ m/z$ 201.1649 (calc. for $\text{C}_{15}\text{H}_{21}$, 201.1643); ^1H NMR (500 MHz, CDCl_3) δ 0.65 (3H, s, H-13), 1.10 (3H, s, H-12), 1.28 (3H, s, H-14), 1.61 (1H, m, H-5), 2.20 (1H, m, H-4), 2.33 (3H, s, H-15), 2.51 (1H, dtd, $J = 14.2, 9.5, 6.5$ Hz, H-4), 2.71 (1H, td, $J = 12.6, 6.4$ Hz, H-5), 4.46 (1H, t, $J = 9.2$ Hz, H-3), 7.12 (1H, d, $J = 8.1$ Hz, H-10), 7.13 (1H, d, $J = 8.1$ Hz, H-8), 7.27 (2H, d, $J = 8.1$ Hz, H-7, H-11); ^{13}C NMR (125 MHz, CDCl_3) δ 20.6 (C-15), 20.8 (C-13), 22.2 (C-12), 25.3 (C-14), 31.2 (C-4), 33.5 (C-5), 47.7 (C-2), 48.4 (C-1), 63.6 (C-3), 126.5 (C-7, C-11), 128.5 (C-8, C-10), 135.4 (C-9), 143.0 (C-6).

3.4. Transformation of Natural Sesquiterpenes 1 and 2

Laurinterol (**1**, 8.0 mg, 0.027 mmol) was dissolved in ethyl ether (5 mL) and bromine (100 μL , 1.95 mmol) was added. The reaction was left under magnetic stirring for 15 min at room temperature, after which the solvent was evaporated in vacuo. The reaction mixture was purified on a silica gel 0.2–0.5 mm (Sigma-Aldrich, St. Louis, MO, USA) column (10 $\text{O} \times$ 50 mm) using a step gradient of *n*-Hex/EtOAc (100:0–95:5) to yield 8-bromoaplysin (**6**, 1.49 mg, 14.8%) and **7** (3.45 mg, 29.6%). Similarly, isolaurinterol (**2**, 5.0 mg, 0.017 mmol) was treated with bromine (100 μL , 1.95 mmol) in the same experimental conditions. After evaporation of the solvent, the reaction mixture was purified on a silica gel column (10 $\text{O} \times$ 50 mm; step gradient of *n*-Hex/EtOAc (100:0–95:5)) to give compound **6** (1.32 mg, 23.5%), **8** (0.38 mg, 6%) and **9** (1.81 mg, 27.3%).

Laurinterol (**1**, 10.0 mg, 0.034 mmol) was dissolved in ethyl ether (5 mL) and HBr (10 μL , 48%) was added. The reaction was left under magnetic stirring for 15 min at room temperature, after which the solvent was evaporated in vacuo. Quantitative conversion was observed to aplysin (**3**) which was purified by crystallization in *n*-hexane.

3.4.1. 8-Bromoaplysin (6)

Colorless oil; $[\alpha]_{\text{D}}^{20} -30$ (c 0.12, CHCl_3); IR ν_{max} 3264, 1652, 1315, 1103, 1022, 904 cm^{-1} ; HREIMS $[\text{M}]^+ m/z$ 371.9711 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}^{79}\text{Br}_2$, 371.9724), 373.9702 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}^{79}\text{Br}^{81}\text{Br}$, 373.9704), 375.9677 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}^{81}\text{Br}_2$, 375.9683); ^1H and ^{13}C NMR data (Table 1).

3.4.2. 3 α -Bromojohnstane (7)

Colorless oil; $[\alpha]_{\text{D}}^{20} -35$ (c 0.19, CHCl_3); IR ν_{max} 3259, 2362, 2158, 2031, 1975, 906, 800 cm^{-1} ; HREIMS $[\text{M}]^+ m/z$ 449.8840 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}_3$, 449.8829), 451.8800 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}_2^{81}\text{Br}$, 451.8809), 453.8798 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}^{81}\text{Br}_2$, 453.8789), 455.8758 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{81}\text{Br}_3$, 455.8768); ^1H and ^{13}C NMR data (Table 2).

3.4.3. 8,10-Dibromoisoaplysin (8)

Colorless oil; $[\alpha]_{\text{D}}^{20} -17$ (c 0.04, CHCl_3); HREIMS $[\text{M}]^+ m/z$ 449.8836 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}_3$, 449.8829), 451.8815 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}_2^{81}\text{Br}$, 451.8809), 453.8783 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{79}\text{Br}^{81}\text{Br}_2$, 453.8789), 455.8764 (calc. for $\text{C}_{15}\text{H}_{17}\text{O}^{81}\text{Br}_3$, 455.8768).

3.4.4. 8,10-Dibromoaplysinol (9)

Colorless oil; $[\alpha]_{\text{D}}^{20} -6$ (c 0.08, CHCl_3); HREIMS $[\text{M}]^+ m/z$ 387.9680 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}_2^{79}\text{Br}_2$, 387.9674), 389.9667 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}_2^{79}\text{Br}^{81}\text{Br}$, 389.9653), 391.9639 (calc. for $\text{C}_{15}\text{H}_{18}\text{O}_2^{81}\text{Br}_2$, 391.9633).

3.5. Cell Culture

The amoeba strain used in this study was the type strain: *Acanthamoeba castellanii* Neff (ATCC 30010) which was axenically grown in PYG medium (0.75% (*w/v*) proteose peptone, 0.75% (*w/v*) yeast extract and 1.5% (*w/v*) glucose) containing 40 µg/mL of gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

The murine macrophages J774A.1 (ATCC# TIB-67) cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂ atmosphere, was used for the cytotoxicity assays.

3.6. Anti-*Acanthamoeba* Activity

To evaluate the biological activity of the fractions and molecules, the anti-*Acanthamoeba* assays were determined using the alamarBlue[®] method as previously described [15,16]. Firstly, *Acanthamoeba* strain was seeded in triplicate on a 96-well microtiter plates with 50 µL from a stock solution of 10⁴ cells/mL. Amoebae were left to adhere to the bottom of the well for 15 min, which was checked using a Leika DMIL inverted microscope (Leika, Wetzlar, Germany). After that, 50 µL of serial dilutions of the molecules were added to the wells (1% DMSO was used to dissolve the highest dose of the compounds with no effects on the parasites). As a control, we use chlorhexidine (chlorhexidine digluconate; Alfa Aesar), which is a standard antiseptic belonging to the biguanide family of antiseptics that is commonly used in contact lens maintenance solutions; and voriconazole (Sigma) is an inhibitor of ergosterol synthesis that has been proven previously to be highly effective against clinical strains of *Acanthamoeba* [26,27]. Finally, the alamarBlue Reagent[®] (Life Technologies, Madrid, Spain) was placed into each well at an amount equal to 10% of the final volume. Then, plates containing alamarBlue[®] were incubated for 96 h at 26 °C with a slight agitation.

3.7. Cytotoxicity Test

The cytotoxicity assay was carried out using the alamarBlue[®] method as previously described [15,16]. Firstly, the macrophages were seeded in triplicate on a 96-well microtiter plates with 50 µL from a stock solution of 2 × 10⁵ cells/mL. Macrophages were left to adhere to the bottom of the well for 15 min, which was checked using a Leika DMIL inverted microscope (Leika, Wetzlar, Germany). After that, 50 µL of serial dilutions of the molecules were added to the wells. Finally, the alamarBlue Reagent[®] (Life Technologies, Madrid, Spain) was placed into each well at an amount equal to 10% of the final volume. Then, plates containing alamarBlue[®] were incubated for 24 h at 37 °C in presence of CO₂ at 5%.

3.8. Statical Analysis

Briefly, the test plates were analyzed, during an interval of time between 24 and 96 h, on an EnSpire[®] Multimode Plate Reader (Perkin Elmer, Madrid, Spain) using fluorescence, a test wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of the growth inhibition, 50% inhibitory concentration (IC₅₀ or CC₅₀) was calculated by linear regression analysis with 95% confidence limits using Sigma Plot 12.0 statistical analysis software (Systat Software). All experiments were performed three times, and the mean values were also calculated. A paired two-tailed t-test was used for analysis of the data. Values of *p* < 0.05 were considered significant.

4. Conclusions

To the best of our knowledge, red algae have never been evaluated as a potential source of amoebicidal agents. Furthermore, this is the first time that the anti-*Acanthamoeba* activity of the brominated sesquiterpenes α-bromocuparane (4) and α-isobromocuparane (5), isolated from *Laurencia johnstonii*, is reported. On the other hand, after chemical modification of the inactive metabolites laurinterol (1) and isolaurinterol (2), a set of brominated derivatives, 6–9, that substantially improve the activity of the natural sesquiterpenes have been obtained. The addition of a second

bromine atom in the aromatic ring of 6–9 seems to be relevant to increase the anti-amoeboid activity. Whereas all obtained derivatives possess a laurane-type framework, the most active compound, 3 α -bromojohnstane (7), possesses a different rearranged carbon skeleton, johnstane, never found among natural sesquiterpene metabolites isolated from *Laurencia* species so far. Our results suggest that *Laurencia*-based brominated sesquiterpenes could be a potential source of novel therapeutic agents against *Acanthamoeba* in the near future.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1660-3397/16/11/443/s1>. Scheme S1: Isolation process of sesquiterpenes 1–5 from *Laurencia johnstonii*; Figure S1: $^1\text{H-NMR}$ spectrum for laurinterol (1); Figure S2: $^1\text{H-NMR}$ spectrum for isolaurinterol (2); Figure S3: $^1\text{H-NMR}$ spectrum for aplysin (3); Figure S4: $^1\text{H-NMR}$ spectrum for α -bromocuparane (4); Figure S5: $^1\text{H-NMR}$ spectrum for α -isobromocuparane (5); Figure S6: $^1\text{H-NMR}$ spectrum of 8-bromoaplysin (6); Figure S7: COSY spectrum of 8-bromoaplysin (6); Figure S8: HSQC-ed spectrum of 8-bromoaplysin (6); Figure S9: HMBC spectrum of 8-bromoaplysin (6); Figure S10: ^{13}C NMR spectrum of 8-bromoaplysin (6); Figure S11: HREIMS spectrum of 8-bromoaplysin (6); Figure S12: ^1H NMR spectrum of 3 α -bromojohnstane (7); Figure S13: COSY spectrum of 3 α -bromojohnstane (7); Figure S14: HSQC-ed spectrum of 3 α -bromojohnstane (7); Figure S15: HMBC spectrum of 3 α -bromojohnstane (7); Figure S16: ^{13}C NMR spectrum of 3 α -bromojohnstane (7); Figure S17: 1D-NOE experiments of 3 α -bromojohnstane (7); Figure S18: HREIMS spectrum of 3 α -bromojohnstane (7); Figure S19: ^1H NMR spectrum of 8,10-dibromoisoaplysin 8; Figure S20: ^1H NMR spectrum of 8,10-dibromoaplysinol 9.

Author Contributions: J.J.F., A.R.D.-M., and J.L.-M. conceived and designed the experiments; S.G.-D. and E.V.-V. collected the algae and prepared extracts; S.G.-D. performed isolation, purification and chemical experiments; J.L.-M., M.R.-B. and I.S. designed and performed the anti-*Acanthamoeba* activity assays and analyzed the activity data; J.E.P. contributed to activity analysis tools and discussed activity data; J.J.F. and A.R.D.-M. analyzed the chemical data and wrote the paper. All authors contributed to the final version of the manuscript.

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References

- Marciano-Cabral, F.; Cabral, G. *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol. Rev.* **2003**, *16*, 273–307. [[CrossRef](#)] [[PubMed](#)]
- Siddiqui, R.; Khan, N.A. Biology and pathogenesis of *Acanthamoeba*. *Parasite. Vectors* **2012**, *5*, 6. [[CrossRef](#)] [[PubMed](#)]
- Lorenzo-Morales, J.; Martín-Navarro, C.M.; López-Arencibia, A.; Arnalich-Montiel, F.; Piñero, J.E.; Valladares, B. *Acanthamoeba* keratitis: An emerging disease gathering importance worldwide? *Trends Parasitol.* **2013**, *29*, 181–187. [[CrossRef](#)] [[PubMed](#)]
- Stothard, D.R.; Schroeder-Diedrich, J.M.; Awwad, M.H.; Gast, R.J.; Ledee, D.R.; Rodriguez-Zaragoza, S.; Dean, C.L.; Fuerst, P.A.; Byers, T.J. The Evolutionary History of the Genus *Acanthamoeba* and the Identification of Eight New 18S rRNA Gene Sequence Types. *J. Eukaryot. Microbiol.* **1998**, *45*, 45–54. [[CrossRef](#)] [[PubMed](#)]
- Lorenzo-Morales, J.; Khan, N.A.; Walochnik, J. An update on *Acanthamoeba* keratitis: Diagnosis, pathogenesis and treatment. *Parasite* **2015**, *22*, 10. [[CrossRef](#)] [[PubMed](#)]
- Khan, N.A. *Acanthamoeba*: Biology and increasing importance in human health. *FEMS Microbiol. Rev.* **2006**, *30*, 564–595. [[CrossRef](#)] [[PubMed](#)]
- Siddiqui, R.; Aqeel, Y.; Khan, N.A. The development of drugs against *Acanthamoeba* infections. *Antimicrob. Agents Chemother.* **2016**, *60*, 6441–6450. [[CrossRef](#)] [[PubMed](#)]
- Lorenzo-Morales, J.; Kliescikova, J.; Martinez-Carretero, E.; De Pablos, L.M.; Profotova, B.; Nohynkova, E.; Osuna, A.; Valladares, B. Glycogen phosphorylase in *Acanthamoeba* spp.: Determining the role of the enzyme during the encystment process using RNA interference. *Eukaryot. Cell* **2008**, *7*, 509–517. [[CrossRef](#)] [[PubMed](#)]

9. Kayser, O.; Kiderlen, A.F.; Croft, S.L. Natural products as antiparasitic drugs. *Parasitol. Res.* **2003**, *90* (Suppl. 2), 55–62. [[CrossRef](#)]
10. Young, R.M.; Pesce, E.R. Screening for antiparasitic marine natural products. In *Marine Biomedicine from Beach to Bedside*; CRC Press: Boca Raton, FL, USA, 2015; Chapter 9. [[CrossRef](#)]
11. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.* **2016**, *79*, 629–661. [[CrossRef](#)] [[PubMed](#)]
12. Blunt, J.W.; Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine natural products. *Nat. Prod. Rep.* **2018**, *35*, 8–53. [[CrossRef](#)] [[PubMed](#)]
13. Souto, M.L.; Manríquez, C.P.; Norte, M.; Leira, F.; Fernández, J.J. The inhibitory effects of squalene-derived triterpenes on protein phosphatase PP2A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1261–1264. [[CrossRef](#)]
14. Gutiérrez-Cepeda, A.; Fernández, J.J.; Norte, M.; López-Rodríguez, M.; Brito, I.; Muller, C.D.; Souto, M.L. Additional insights into the obtusallene family: components of *Laurencia marilzae*. *J. Nat. Prod.* **2016**, *79*, 1184–1188. [[CrossRef](#)] [[PubMed](#)]
15. Morales-Amador, A.; de Vera, C.R.; Márquez-Fernández, O.; Daranas, A.H.; Padrón, J.M.; Fernández, J.J.; Souto, M.L.; Norte, M. Pinnatifidenyne-derived ethynyl oxirane acetogenins from *Laurencia viridis*. *Mar. Drugs* **2018**, *16*. [[CrossRef](#)] [[PubMed](#)]
16. Harizani, M.; Ioannou, E.; Roussis, V. The *Laurencia* paradox: An endless source of chemodiversity. *Prog. Chem. Org. Nat. Prod.* **2016**, *102*, 91–252. [[CrossRef](#)] [[PubMed](#)]
17. Sims, J.J.; Fenical, W.; Wing, R.M.; Radlick, P. Marine natural products III. Johnstonol, an unusual halogenated epoxide from the red alga *Laurencia johnstonii*. *Tetrahedron Lett.* **1972**, *13*, 195–198. [[CrossRef](#)]
18. Faulkner, J.D.; Stallard, M.O.; Ireland, C. Prepacifenol epoxide, a halogenated sesquiterpene diepoxide. *Tetrahedron Lett.* **1974**, *15*, 3571–3574. [[CrossRef](#)]
19. Yamamura, S.; Hirata, Y. Structures of aplysin and aplysinol, naturally occurring bromo-compounds. *Tetrahedron* **1963**, *19*, 1485–1496. [[CrossRef](#)]
20. Suzuki, T.; Suzuki, M.; Kuroswa, E. α -bromocuparene and α -isobromocuparene, new bromo compounds from *Laurencia* species. *Tetrahedron* **1975**, *35*, 3057–3058. [[CrossRef](#)]
21. Irie, T.; Suzuki, M.; Kurosawa, E.; Masamune, T. Laurinterol and debromolaurinterol, constituents from *Laurencia intermedia*. *Tetrahedron Lett.* **1966**, *17*, 1837–1840. [[CrossRef](#)]
22. Irie, T.; Suzuki, M.; Kurosawa, E.; Masamune, T. Laurinterol, debromolaurinterol and isolaurinterol, constituents of *Laurencia intermedia* Yamada. *Tetrahedron* **1970**, *26*, 3271–3277. [[CrossRef](#)]
23. Wang, B.-G.; Gloer, J.B.; Ji, N.-Y.; Zhao, J.-C. Halogenated Organic Molecules of Rhodomelaceae Origin: Chemistry and Biology. *Chem. Rev.* **2013**, *113*, 3632–3685. [[CrossRef](#)] [[PubMed](#)]
24. Gribble, G.W. Biological Activity of Recently Discovered Halogenated Marine Natural Products. *Mar. Drugs* **2015**, *13*, 4044–4136. [[CrossRef](#)] [[PubMed](#)]
25. Cameron, A.F.; Ferguson, G.; Robertson, J.M. The crystal structure and absolute stereochemistry of Laurinterol. The absolute stereochemistry of aplysin. *Chem. Commun.* **1967**, 271–272. [[CrossRef](#)]
26. Erickson, K.L. Constituents of *Laurencia*. In *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J., Ed.; Academic Press: New York, NY, USA, 1983; Volume V, pp. 131–257. ISBN 978-0-12-624005-4. in reference to the original publication: Caccamese, S.; Rinehart, K.L. New compounds and activities from *Laurencia* species. In *Drugs and Food from the Sea. Myth or Reality?* Kaul, P.N., Sindermann, C.J., Eds.; University of Oklahoma: Norman, OK, USA, 1978, pp. 187–197.
27. Guo, Y.; Wang, H.; Mao, S.; Zhang, X. Extraction method of laurane-type sesquiterpenoids or their derivatives from seaweed of *Laurencia* and its application as antiobesity agent. Faming Zhuanli Shenqing CN101628855 A, 20 January 2010.
28. Yamada, K.; Yazawa, H.; Hirata, Y. The synthesis of 2,3-dimethyl-3-(5-bromo-2-methoxy-4-methylphenyl)-cyclohexene, a potentially useful intermediate for the syntheses of aplysin and related sesquiterpenes. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 587–590. [[CrossRef](#)]
29. Srikrishna, A.; Sundarababu, G. Radical cyclization strategies to terpenoids; syntheses of (\pm)- β -cuparenone, (\pm)-laurene and epilaurenes. *Tetrahedron* **1991**, *47*, 481–496. [[CrossRef](#)]
30. Yu, X.Q.; He, W.F.; Liu, D.Q.; Feng, M.T.; Fang, Y.; Wang, B.; Feng, L.H.; Guo, Y.W.; Mao, S.C. A seco-laurane sesquiterpene and related laurane derivatives from the red alga *Laurencia okamurai* Yamada. *Phytochemistry* **2014**, *103*, 162–170. [[CrossRef](#)] [[PubMed](#)]

31. Suzuki, M.; Kurata, K.; Kurosawa, E. The structure of isoaplysin, a brominated rearranged cuparane-type sesquiterpenoid from the red alga *Laurencia okamurai* Yamada. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 3981–3982. [[CrossRef](#)]
32. Martín-Navarro, C.M.; Lorenzo-Morales, J.; Cabrera-Serra, M.G.; Rancel, F.; Coronado-Álvarez, N.M.; Piñero, J.E.; Valladares, B. The potential pathogenicity of chlorhexidine-sensitive *Acanthamoeba* strains isolates from contact lens from asymptomatic individuals in Tenerife, Canary Islands, Spain. *J. Med. Microbiol.* **2008**, *57*, 1399–1404. [[CrossRef](#)] [[PubMed](#)]
33. McBride, J.; Ingram, P.R.; Henríquez, F.L. Development of colorimetric microtiter plate assay for assesment of antiicrobials against *Acanthamoeba*. *J. Clin. Microbiol.* **2005**, *43*, 629–634. [[CrossRef](#)] [[PubMed](#)]



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