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New Diterpenes Isolated from the Colombian Caribbean Soft Coral *Pseudoplexaura flagellosa* and Their Cytotoxic Properties

Katherine Bauer[1], Mónica Puyana[2], Leonardo Castellanos[3] and Edisson Tello[1*]

[1]*Faculty of Engineering, Bioprospecting Research Group, Universidad de La Sabana, Chía, Colombia*
[2]*Department of Biological and Environmental sciences, Universidad Jorge Tadeo Lozano, Bogotá, Colombia.*
[3]*Chemistry Deparment, Universidad Nacional de Colombia, Bogotá, Colombia*

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Abstract: Studies about secondary metabolites isolated from soft corals around the world have proven the potential of these organism as producers of compounds with a potent cytotoxic activity. In this work, we obtained the extract of *Pseudoplexaura flagellosa* collected in Santa Marta, Colombia, the cytotoxic activity of this extract, fractions and compounds was established against SiHa (ATCC® HTB-35™), MDA-MB-231 (ATCC® HTB26™), A549 (ATCC® CRM-CCL-185™), PC3 (ATCC® CRL1435), and L929 (ATCC® CCL1™) cell lines. The results showed that the extract of soft coral *P. flagellosa* has cytotoxic activity with an IC_{50} of 35.4, 72.3, 49.8 and 40.5 µg/mL against SiHa, A549, PC3 and MDA-MB-231 cell lines, respectively. Thus, this extract was repeatedly subjected to different chromatographic columns and final purification of these fractions afforded pure compounds **1** – **4**, which were elucidated by 1D and 2D NMR experiments including ^{1}H, ^{13}C, COSY, HSQC, HMBC and NOESY, and HRESIMS. In addition, Mosher method was used to establish the stereochemistry of compound **2** and chemical interconversion allow establishing the stereochemistry of compound **1**. These results let to conclude that compound **2** is a new stereoisomers of acetylated asperdiol previously reported on literature. Additionally, three analogues **5**, **6**, and **7** were synthesized from compound **1** and the cytotoxic activity of all compounds was evaluated using doxorubicin as positive control. The results showed that ***6*** (IC_{50} of 19.3, 23.7, 13.4 and 18.7 µg/mL against SiHa, A549, PC3 and MDA-MB-231, respectively) was the most active compound against all the cancer cell lines.

Keywords: *Pseudoplexaura flagellosa*; cytotoxic activity; absolute stereochemistry; stereoisomers; soft corals.

1. Introduction

One of the potential sources of compounds with cytotoxic activity are marine invertebrates, among them soft corals (Orders Alcyonacea and Gorgonacea, Class Anthozoa, Phylum Cnidaria [1]). These organisms are prolific sources of compounds, with novel structures conferring them marked biological activity such as anti-inflammatory, antiseptic, acetylcholinesterase inhibition, quorum sensing inhibition and cytotoxicity [2–8].

* Corresponding author: E-Mail: edisson.tello@unisabana.edu.co

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Studies on chemistry of soft coral around the world have proven the potential of these organisms as sources of compounds with potent cytotoxic activity. For instance, *Lobophytum michaelae* (Taiwan), *Cladiella* sp. (Indonesia), *Dichotella gemmacea* (China) and *Antillogorgia rigida* (Bahamas) have yielded compounds with high cytotoxic activity against different cell lines such as P388 (mouse lymphoma), HT-29 (colorectal adenocarcinoma), DLD-1 (colorectal adenocarcinoma), HeLa (cervix carcinoma), MCF-7 (breast adenocarcinoma), A549 (lung carcinoma) and Daoy (medulloblastoma), among others [9-12].

Although Colombia's coral reefs cover an approximate area of 2900 km^2, the search of potential applications of products derived from marine invertebrates and microorganisms has not been thoroughly evaluated [13], General calculations indicate that only 70 out of 1914 identified species of marine invertebrates have been chemical characterized or have provided technological applications [14]. Some studies in marine bioprospecting in Colombia have evaluated compounds with biological activity, focusing on medical applications to reduce degenerative processes of the skin [15] and anti-inflammatory properties [16,17]. Other studies have evaluated the antifouling potential of organic extracts from marine invertebrates [18] or evaluated mechanisms of quorum sensing inhibition in soft corals [6,19] and, cytotoxic and antiviral activity of compounds isolated from soft corals [7,8], [20–22].

In our continuous search for novel compounds with promising biological activity from Caribbean marine invertebrates, particularly soft corals, we carried out a statistical study of 42 extracts of octocorals from Colombian Caribbean against a panel of cancer cell lines. From this study, octocoral *Pseudoplexaura flagellosa* show a selective activity against cervical cancer cell line, for this reason this paper describes the isolation and identification of compounds **1**–**4** from the organic extract of *P. flagellosa* and their cytotoxic activity against a panel of human cancer cell lines (SiHa, A549, MDA-MB-231 and PC3), and a mouse fibroblast cell line (L929). Additionally, three synthetic analogues were synthesized to improve the cytotoxic activity of compound **1**.

2. Materials and Methods

2.1. General Methods

Optical rotations were measured on a Polartronic ADP440+, Bellinghan & Stanley polarimeter. NMR spectra were recorded on a Bruker Avance 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) using $CDCl_3$ as solvent unless otherwise stated, with TMS as internal standard. Chemical shifts are given in δ and coupling constants in Hertz. Mass spectra were determined in a Shimadzu LCMS-2010 instrument with a UV/Vis (SPD-10A) detector (detected at 210 nm) using a Shimadzu premier C-18 column (150 × 4.6 mm i.d.; 3 mm), with a A/B (70:30 %v/v) as eluent where A was a solution H_2O/Formic acid at 0.1% and B was a solution of ACN/Formic Acid at 0.1%; the flow rate was 0.5 mL/min. (+)-HRESIMS were obtained using a QSTAR Elite LC/MS (NI 60238).

2.2. Animal Material

Branches of the soft coral *Pseudoplexaura flagellosa* were cut off from a large colony at a depth of 20 m in Santa Marta Bay, Colombia by scuba diving. The animal material was air dried and then kept frozen until extraction. The animal was identified by the Biologist Ph.D. Mónica Puyana and a voucher was deposited in the Collection of the Institute of Natural Sciences of the National University of Colombia (ICN-MHN-PO 0257).

2.3. Isolation Procedure

The animal material (210.3 g) was cut into small pieces and repeatedly extracted with MeOH:CH_2Cl_2 (1:1 v/v) and then filtered to yield the corresponding extract (25.4 g) after rotary evaporation under vacuum. The extract was partitioned between CH_2Cl_2/H_2O (1:1 v/v) to obtain the organic fraction (11.4 g) that was subjected to flash column chromatography on silica gel (180 mm ×

50 mm) using a gradient of Hex/EtOAc/MeOH (from 100% n-hexane to 100% EtOAc and then to 100% MeOH, v/v) to yield 12 fractions (F1–F12) after concentration under vacuum. Crystals were found at the bottom of the vessel that contains fraction F8. This fraction was washed with hexane and recrystallized on EtOAc to obtain pure *compound **1*** (400 mg). Cytotoxic evaluation of these fractions showed that fractions F3 and F5 were the most active against a panel of cancer cell lines (IC_{50} values lower than 100 µg/mL) and therefore, these fractions were subjected to further separation.

F5 (1.2 g) was separated by a SiO_2 column chromatography (180 mm × 20 mm) using a gradient of Hex/EtOAc (9:1 – 100% EtOAc, v/v) providing 92 fractions of ~15 mL. These were grouped in 7 subfractions according to their TLC profile to yield subfractions F5.1–F5.7 after concentration under vacuum. Subfraction F5.5 (0.070 g) was subjected to silica gel column chromatography (210 mm × 10 mm) of increasing polarity Hex/EtOAc (9:1, 8:2, 100% EtOAc v/v) to afford 92 fractions of ~15 mL each, that were grouped in 3 subfractions (F5.5.1–F5.5.3) after concentration under vacuum. One of these, subfraction F5.5.2 (40 mg), yielded pure compound **2**.

Fraction F3 (4.3 g) was subjected to silica gel column chromatography (300 mm × 15 mm), eluting with an isocratic gradient of 400 mL Hex/EtOAc 9:1, v/v, to yield 96 fractions of ~10 mL each, that were grouped in 14 subfractions (F3.1–F3.14) after concentration under vacuum according to their TLC profile. Subfractions F3.10 and F3.11 contained pure compounds **3** (8.2 mg) and **4** (9.4 mg), respectively.

2.4. Preparation of R- and S-MPA Esters

To determine the absolute stereochemistry at C-2 of ***2***, 5 mg of ***2*** were treated with *S*-MPA in 1 mL of dry DCM with DCC 1.1 equivalent and DMPA in catalytic amounts for 48 h at room temperature to obtain the *S*-MPA ester at C-2. The reaction mixture was filtered and purified in a silica gel chromatographic column (10 mm x 50 mm) eluting with a gradient from 9:1 to 8:2 of n-hexane/EtOAc to obtain the pure *S*-MPA ester of *compound **2***. To obtain the *R*-MPA ester of ***2*** it follows the same procedure described above, the only difference was the use of *R*-MPA instead *S*-MPA reactant.

*Compound **1**:* colorless crystal; $[\alpha]^{20}_{D}$ -40.5 (c 1,0 $CHCl_3$); IR 3450, 2950, 1650, 1450, 1100 cm^{-1}; the molecular formula was established as $C_{20}H_{32}O_3$ by HRESIMS *m/z*: 343.2240 $[M+Na]^+$ (calculated for $C_{20}H_{32}O_3Na$, 343.2249) and the NMR data (Table 1).

*Compound **2**:* yellow crystal; $[\alpha]^{20}_{D}$ +57.2 (c 2,0 $CHCl_3$); IR 3500, 2950, 1720, 1650, 1450, 1050 cm^{-1}; the molecular formula was established as $C_{22}H_{34}O_4$ by HRESIMS *m/z*: 385.2345 $[M+Na]^+$ (calculated for $C_{22}H_{34}O_4Na$, 385.2385) and the NMR data (Table 1).

*Compound **3***: colorless oil; $[\alpha]^{20}_{D}$ +117.7 (c 1,0 $CHCl_3$); the molecular formula was established as $C_{20}H_{34}O_3$ by HRESIMS *m/z*: 369.2412 $[M+Na]^+$ (calculated for $C_{20}H_{34}O_3Na$, 369.2406) and the NMR data (Table 1).

*Compound **4**:* colorless oil; $[\alpha]^{20}_{D}$ +150.5 (c 1,2 $CHCl_3$); the molecular formula was established as $C_{20}H_{30}O_2$ by HRESIMS *m/z*: 325.2151 $[M+Na]^+$ (calculated for $C_{20}H_{30}O_2Na$, 325.2143) and the NMR data (Table 1).

2.5. Preparation of Synthetic Analogues

*2.5.1. Acetylation of **1** and **2** with Acetic Anhydride*

The acetylation of ***1*** (0.05 mmol) and ***2*** (0.14 mmol) were carried out with anhydride acetic (1.5 and 10.4 mmol, respectively) in dry DCM and 10 µL of HCl for 48 h at room temperature. Then, the reaction products were purified in a chromatographic column of SiO_2 eluting with a gradient from 95:5 to 84:16 of Hex/EtOAc obtaining the pure *compound **5*** (quantitative products).

*2.5.2. Oxidation of **1** with PCC*

The oxidation of ***1*** (0.11 mmol) was carried out with PCC (0.17 mmol) in dry DCM at room temperature for 3 h. The reaction was quenched with water and then filtered to be purified in a chromatography column eluting with a gradient from 9:1 to 1:1 of Hex/EtOAc to obtain the synthetic analogues ***6*** (0.035 mmol) and ***7*** (0.022 mmol).

2.6. Cytotoxicity Assays

Cell lines MDA-MB-231 human breast epithelial cells (ATCC® HTB26™) and L929 (ATCC® CCL1™) were incubated in RPMI medium supplemented with 1% (v/v) L-glutamine, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin, and 1% (v/v) streptomycin. SiHa, human cervix epithelial cells (ATCC® HTB-35™), human lung cell line A-549 (ATCC® CRM-CCL-185™) and human prostate epithelial cell lines PC3 (ATCC® CRL1435), were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotic agents (penicillin 120 IU/mL and streptomycin 100 IU/mL) and 10% fetal bovine serum.

Cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The effect of extracts, fractions and pure compounds was established by MTT assay [23] with modification in the time of exposure of cell lines to fractions or compounds using doxorubicin as positive control. Doxorubicin is often used as a chemotherapeutic for aggressive cancers [24–26]. Cells were grown to approximately 1×10^5 cells/well in 96 well plates and then incubated with various concentrations of extracts, fractions, natural compounds and synthetic analogues, diluted in less than 1% of DMSO (10, 25, 50, 100 and 200 µg/mL) at 37 °C with 5% CO_2 for 48 h. Cell viability was measured with an ELISA microplate reader at wavelength of 570 nm and 50% inhibitory concentration (IC_{50}) was calculated [27].

2.7. Statistical Analysis

Statistical analyses were performed using a Demo License of GraphPad Prism 6® and Microsoft® Excel. All the experiments were run by triplicate and the results were expressed as mean values ± standard deviation. IC_{50} values were obtained by a nonlinear regression with $R^2 > 0.95$.

3. Results and Discussion

3.1. Structure Elucidation

The extract of *P. flagellosa* was partitioned in two phases DCM/H_2O 1:1. The organic phase was subjected to silica gel column to afford 12 fractions, which were evaluated along with the organic extract against a panel of cell lines (SiHa, A549, PC3, MDA-MB-231 and L929) using a MTT assay during 48 h. The fractions F3, F5, and F8 were the most active fractions (IC_{50} values <100 µg/mL against the cancer cell lines), for this reason they were chosen to further separation.

From fraction F8 compound **1** was isolated in form of a colorless crystal with molecular formula $C_{20}H_{32}O_3$ assigned on the basis of the mass spectrum that exhibited a peak at *m/z* 343.2240 $[M+Na]^+$ and the ^1H NMR spectrum (Table 1) allowed to establish that *compound **1*** structure is the known compound asperdiol (Figure 1), first isolated from a soft coral of the genus *Eunicea* by Weinheimer et al. in 1977 [28]. Both genera, *Eunicea* and *Pseudoplexaura* belong to the family Plexauridae. Compound **2** was isolated from subfraction F5.5.2 as a yellow crystal with a molecular formula $C_{22}H_{34}O_4$ assigned on the basis of its mass spectrum that presented a peak at *m/z* 385.2345 $[M+Na]^+$ and its NMR data (Table 1), corresponding to a diterpene with a cembrane skeleton previously identified as asperdiol acetate (Figure 1), originally isolated from *Eunicea knighti* [29]. Although both compounds were already reported, this is the first time that they are found in a *P. flagellosa* from the Colombian Caribbean.

Table 1. NMR data for compounds **1-7** (400 MHz in $CDCl_3$, δ in ppm, *J* in Hz)

	1		**2**		**3**	**4**	**5**	**6**	**7**
No.	**δC**	**δH, mult, *J* (Hz)**	**δC**	**δH, mult, *J* (Hz)**	**δH, ult, *J* (Hz)**	**δH, mult, *J* (Hz)**	**δH, mult, *J* (Hz)**	**δH, mult, *J* (Hz)**	**δH, mult, *J* (Hz)**
1	50.8, d	2.05, m	50.3, d	2.04, m	1.96, m	2.10, m	2.20, m	2.55, dd*(9.9,3.0)*	2.28, m
2	68.5, d	4.49, dd (*7.0, 6.4)*	68.3, d	4.49, m	2.37, m	2.12, m	5.67, dd(*8.9,3.5*)	-	4.69, t(*7.4*)
2'					1.83, m	2.71, m			
3	128.8, d	5.44, d(*7.9*)	132.2, d	5.47, d(*7.9*)	5.54, t (*7.5*)	6.49, dd (*9.8, 5.0)*	5.40, d (*8.6*)	7.01, s	6.41, d (*7.9*)
4	139.7, s	-	135.4, s	-	-	-	-	-	-
5	26.0, t	2.28, m	26.1, t	2.38, m	2.31, m	2.34, dt (*10.2, 2.5*)	2.47, m	1.77, m	1.98, m
5'					2.24, m	2.45, m	2.33, m	1.62, m	1.35, m
6	26.8, t	1.47, m	26.5, t	1.47, m	1.47, m	1.47, m	1.87, m	2.19, m	2.26, m
6'		1.82, m		1.81, m	1.88, m	1.96, m	1.49, m	1.98, m	
7	65.0, d	2.68, dd(*5.8, 4.5*)	64.0, d	2.69, dd(*6.0, 4.5*)	2.73, dd (*7.4, 3.4*)	2.66, dd (*7.1, 4.3*)	2.80, m	3.30, dd(*10.1, 2.7*)	2.60, m
8	60.4, s	-	60.4, s	-	-	-	-	-	-
9	37.7, t	1.31, m	37.4, t	1.31, m	1.43, m	1.46, m	1.94, m	2.35, m	1.91, m
9'		2.01, m		2.00, m	2.00, m	1.88, m	1.45, m	1.72, m	1.55, m
10	24.2, t	2.18, m	24.0, t	2.18, m	2.16, m	2.13, m	2.20, m	1.85, m	2.18, m
10'					1.62, m			2.01, m	2.12, m
11	124.8, d	5.12, t(*6.9*)	124.7, d	5.12, t(*6.6*)	5.15, t (*6.7*)	5.22, t (*6.5*)	5.16, m	4.98, m	5.14, t (*6.8*)
12	135.6, s	-	135.4, s	-	-	-	-	-	-
13	36.3, t	2.00, m	36.0, t	1.99, m	2.14, m	2.07, m	2.16, m	3.38, ddd (*13.0,11.0,3.6*)	1.83, m
13'					2.20, m	2.15, m	2.03, m		1.62, m
14	28.2, t	1.50, m	27.9, t	1.50, m	1.44, m	1.56, m	1.60, m	2.16, m	2.58, m
14'		1.74, m			1.54, m	1.65, m		1.67, m	2.26, m
15	145.9, s	-	145.9, s	-	-	-	-	-	-
16	22.5, q	1.76, s	22.4, q	1.75, s	1.65, s	1.70, s	1.75, s	1.64, s	1.78, s
17	113.9, t	4.76, s	113.8, t	4.75, s	4.58, br s	4.66, br s	4.85, br s	4.89, s br	4.79, s
17'		4.95, s		4.95, s	4.71, br s	4.79, br s	4.64, br s	4.82, sbr	5.01, s
18	65.9, t	4.06, m	67.1, t	4.49, m	4.53, d (*12.3*)	9.34, s	4.55, d(*13*)	9.45, s	9.41, s
18'					4.42, d *(12.3*)		4.45, d *(13.6*)		
19	16.8, q	1.19, s	16.6, q	1.19, s	1.19, s	1.16, s	1.25, s	1.45, s	1.18, s
20	16.0, q	1.61, s	15.8, q	1.61, s	1.58, s	1.59, s	1.60, s	0.91, s	1.63, s
21			134.3, s	-	-		-		
22			20.9, q	2.07, s	2.04, s		2.07, s		
23							-		
24							2.05, s		

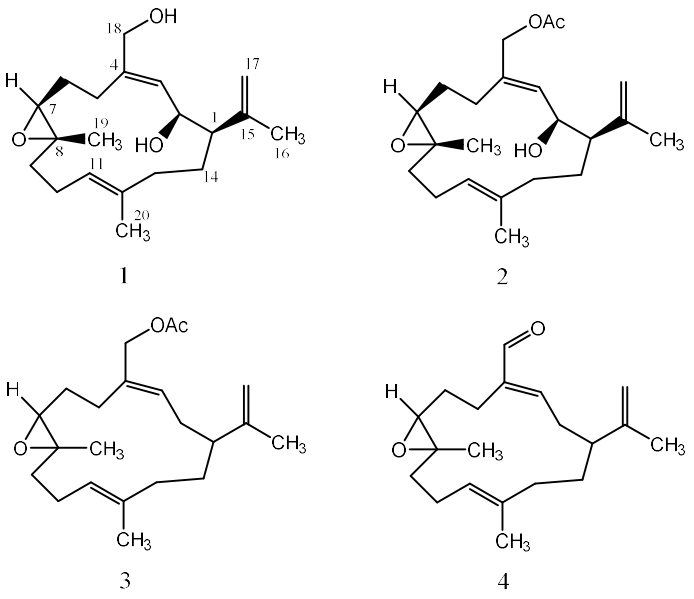

Figure 1. Structure of compounds **1**–**4**

Compound **3** was isolated as a colorless oil with a molecular formula $C_{22}H_{34}O_3$ assigned on the basis of HRESIMS spectrum that exhibited a peak at *m/z* 369.2412 $[M+Na]^+$ and the NMR data (Table 1). The NMR data revealed that ***3*** is a diterpene with a cembrane skeleton. The ^{1}H NMR spectrum let establish two olefin shifts at δH 5.15 (br t, 1H, *J*= 6.7 Hz), 5.54 (br t, 1H, *J*= 7.9 Hz), which have an *E* configuration based on the steric compression principle; and one more olefin shift at 4.58 (s, 1H) and 4.71 (s, 1H) which are two singlets corresponding to the terminal methylene; one signal at δH 2.73 (dd, 1H, *J*= 7.4, 3.4 Hz) corresponding to the methine where the epoxide is; two diastereotopic signals at 4.42 (d, 1H, *J*= 12.3 Hz) and 4.53 (d, 1H, *J*= 12.3 Hz) consistent to the methylene bearing the acetate group, and one signal at δH 2.04 (s, 1H) corresponding to the methyl of the acetate group. The NMR data and comparing it to the literature allowed us to establish that planar structure of ***3*** correspond to that of knigthol acetate [29].

Compound **4** was isolated from fraction F3.11 as a colorless oil. The HRESIMS of ***4*** exhibited a peak at *m/z* 325.2151 $[M+Na]^+$ and the NMR spectroscopic data (Table 1) established a molecular formula of $C_{20}H_{30}O_2$ implying the existence of six degrees of unsaturation. The ^{1}H NMR data shown that ***4*** is structurally similar to ***3***, the main difference being the signal at δH 9.33 (s, 2H) located in the aldehyde group which replaces the acetate group in ***3***, receiving the name of knightal isolated for the first time by Tello et al.in 2009[29] from a *Eunicea knighti* collected in the Colombian Caribbean. These four compounds were previously reported, being compounds **1**, **2**, **3** and **4** the known compounds asperdiol, asperdiol acetate, knightol acetate and knightal, respectively. However, optical rotations for compounds **1** (-40.5), ***2*** (+57.2), ***3*** (+117.7) and ***4*** (+150.5) are opposite to those reported in the literature, suggesting that these compounds are stereoisomers [29].

To establish the stereostructures of the compounds isolated from *P. flagellosa*, the modified Mosher's method was used to prepare the *R*- and *S*-MPA esters at C-2 of compound **2**. The chemical shifts difference values (Figure 2) were obtained by comparing the ^{1}H NMR spectrum of *R*- and *S*-MPA esters (See Supporting Information S13, S14, and S15). Thus, the absolute configuration for C-2 was established as *S*. The configuration of C-1, C-7 and C-8 were established based on NOE correlations observed in the NOESY experiment (Figure 3). The relationship between H-2 with H-1 and H-3 let establish an *R* configuration in C-1; the cofacial signals of H-3 and H-18, H-18 and H-6, and the crosspeak between H-6 and H-19, and H-7 and H-9 (see supporting information S8), let establish a trans correlation between H-6 and H-9 confirming a *S* configuration in C-7 and C-8. All the above, let to establish the absolute stereostructure of compound **2** as (+)-(1*R*, 2*S*, 7*S*, 8*S*, 3*E*, 11*E*)-18-Acetoxycembra-7,8-oxa-3,11,15(17)-trien-2-ol a new stereoisomer of the known asperdiol acetate [29]. To establish the stereostructure of **1,** compounds **1** and ***2*** were acetylated to obtain the

diacetylated at C-2 and C-18 compound **5** (Table 1). Finally, comparing the ^{1}H NMR data (Table 1) and the optical rotation of both compounds **5**, it allowed to confirm that the stereochemistry of ***1*** and ***2*** at C-1, C-2, C-7, and C-8 are the same, being the compound **1** the (-)-(1R, 2S, 7S, 8S, 3E, 11E)-Cembra-7,8-oxa-3,11,15(17)-trien-2,18-diol a stereoisomer of the known asperdiol [28]. The stereochemistry of compound **3** and **4** could not be stablished due the amount obtained of these compounds, however, tentatively should be the same for compounds **1** and **2**.

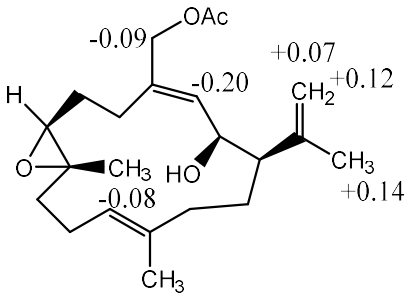

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Figure 2. Chemical shifts differences between *R* and *S*-MPA esters of compound **2**

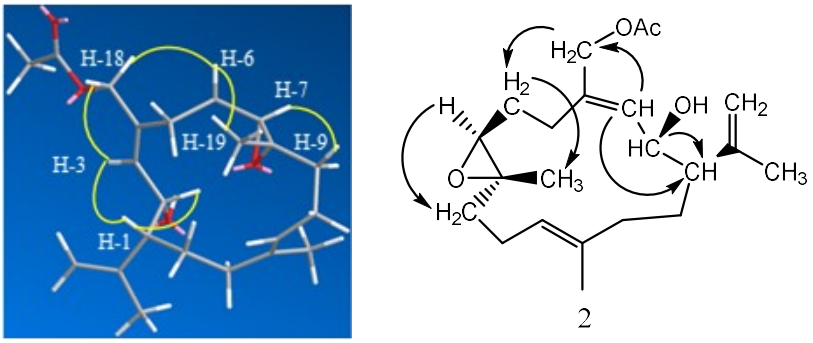

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Figure 3. NOESY correlations of compound **2**

Additionally, three synthetic analogues were obtained from ***1*** and were identified through the analysis of ^{1}H NMR spectra. Compound **5** was obtained as a product of acetylation of ***1*** and ***2*** (Figure 4), the ^{1}H NMR data showed two different signals at δH 2.07 (s, 3H) and 2.05 (s, 3H) belonging to the methyls of acetyl groups that were expected to be in C-2 and C-18. Compound **6** was obtained by an oxidation of both hydroxyl groups of ***1*** at C-2 and C-18 (Figure 5). The ^{1}H NMR data (Table 1) let establish the presence of one aldehyde group at C-18 with the signal at δ_H 9.45 (s, 1H), and the absence of any hydrogen signal at C-2 indicated the presence of a ketone group as expected from the reaction. Finally, the mono-keto compound **7** was a subproduct of the same reaction (Figure 5), the ^{1}H NMR data let to establish the presence of one aldehyde signal at δH 9.41 (s, 1H) which was assigned to C-18. All the remaining signals were similar to the precursor **1.**

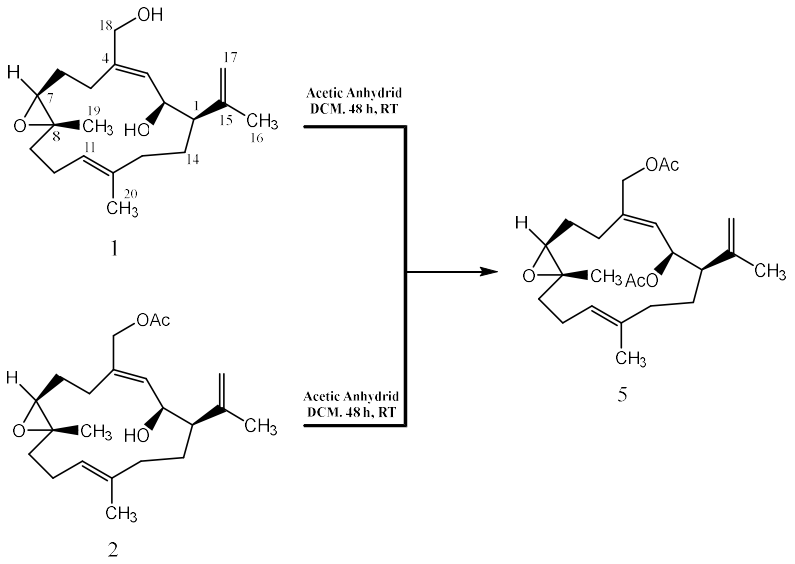

Figure 4. Acetylation of **1** and **2** with acetic anhydride

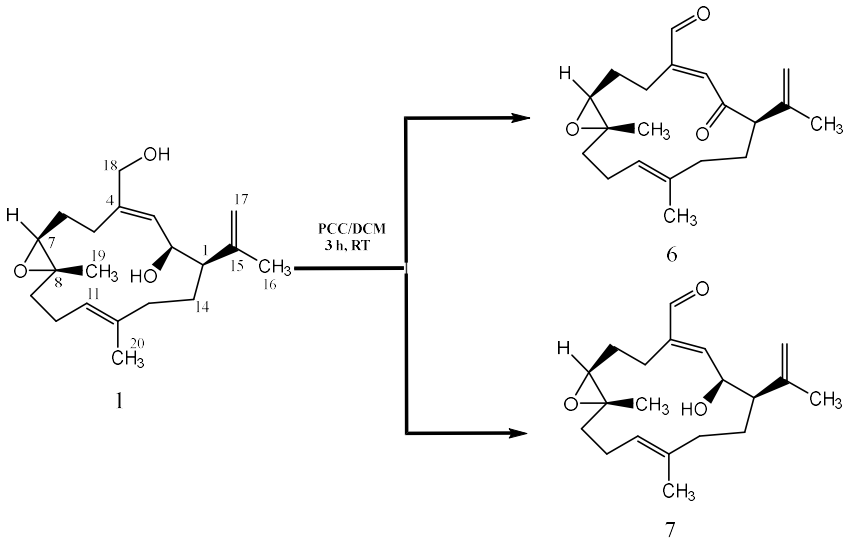

Figure 5. Oxidation of **1** with PCC

3.2. Cytotoxicity Activity

Cytotoxic assays of the crude extract, fractions and compounds **1**–**7** was carried out against a panel of four cancer cell lines (PC3, A549, MDA-MB-231, and SiHa), and one fibroblast cell line (L929) (Table 2) using the MTT assay. The extract of *P. flagellosa* showed IC_{50} values between 30-80 µg/mL against the panel of cell lines mentioned above. Fractions F3, F5 and F8, which contained the isolated compounds, also showed cytotoxic activity between 30-40 µg/mL, establishing a correlation activity among the extract and fractions.

Table 2. Cytotoxicity of compounds **1**–*7* against a panel of four cancer cell lines

	IC_{50}(µg/mL)				
Compound	**SiHa**	**A549**	**PC3**	**MDA-MB-231**	**L929**
1	>100	92.8	84.9	>100	>100
2	85.6	64.0	34.2	>100	>100
3	>100	>100	>100	>100	>100
4	>100	>100	54.28	52.7	68.7
5	45.7	28.9	21.1	54.2	>100
6	19.3	23.7	13.4	18.7	55.1
7	67.3	>100	88.3	>100	>100
Organic Extract	35.4	72.3	49.8	40.5	>100
Doxorubicin	5.0	0.5	1.3	>100	0.8

The table 2 show how the functional groups of each compound have a significant role in the cytotoxic activity allowing inferring a structure-activity relationship. It can be observed between *compound **1*** and ***2*** how the presence of the acetate group increases the activity against all the cell lines, but having only the acetate group at C-18 without the hydroxyl group in C-2 decreases significantly the activity against all the cell lines as is shown for *compound **3***. Although *compound **4*** was not active against all the cancer cell lines, the presence of the aldehyde group in C-18 increases the activity compared with *compound **3***, who does not have also the hydroxyl group in C-2. Also, the presence of the aldehyde in ***4*** makes the compound the most toxic having cytotoxic activity against the L929 cell line, while the other three compounds did not show activity against this fibroblast cell line. Consequently, this brief structure-activity relationship analysis suggests that the presence of an aldehyde group in C-18 and a hydroxyl group in C-2 will increase the activity against all the cancer cell lines.

Therefore, synthetic analogues that presents this type of combination of functional groups were synthesized to try to increase the activity of its precursors. Thus, *compound **5*** which have two acetyl groups as substituents in C-2 and C-18 was more active than ***1*** and ***2*** (its precursors), confirming that the presence of acetyl groups in this type of molecules increases the cytotoxic activity against the cancer cell lines evaluated in this work. Additionally, *compound **6*** was the most cytotoxic compound showing IC_{50} values between 10 and 25 µg/mL for all the cell lines, attributing this activity to the aldehyde group located at C-18 and the ketone group at C-2, suggesting that the presence of this type of functional groups in this kind of molecules increases the activity in a wide range. In contrast, compound **7**, which have an aldehyde group in C-18 and a hydroxyl group in C-2 did not show an increase of activity.

Although the extract of *P. flagellosa* showed potential activity under 50 µg/mL, the compounds isolated from the extract where not as active as the extract, suggesting that the cytotoxic effect of this extract could be a result of a synergistic or additive effect of all active compounds present.

On the other hand, the compound named asperdiol, the diasteroisomer of *compound **1***, was previously proven to have cytotoxic potential against the leukemia cancer cell line P-388 with an IC_{50} of 6 µg/mL and HeLa cell line KB (human cervix carcinoma cell line) with an IC_{50} 24 µg/mL [28]. However, the diastereoisomer ***1*** isolated from *P. flagellosa* did not show significant activity against the SiHa cell line at 48 h, this could be attributed to the difference in the stereochemical configuration.

It was possible to conclude that the extract of *P. flagellosa* allowed the isolation of four cembranoids, they are stereoisomers of the compounds asperdiol, asperdiol acetate, knightol acetate and knightal, previously reported in literature. Although the organic extract of *P. flagellosa* showed potent activity against the SiHa cell line, the isolated compounds did not show a significant activity at 48 h. However, *compound **4*** showed activity against the PC3 and MDA-MB-231 cell lines, and ***2*** showed the most promising activity of the natural compounds with the lowest IC_{50} values for all the four cancer cell lines evaluated.

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Supporting Information

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ORCID
Katherine Bauer: 0000-0002-2795-2517
Mónica Puyana: 0000-0001-7600-3118
Leonardo Castellanos: 0000-0001-5248-800X
Edisson Tello: 0000-0002-6401-8555

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