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Bioactive Sesqui- and Diterpenoids from the Argentine Liverwort *Porella chilensis*

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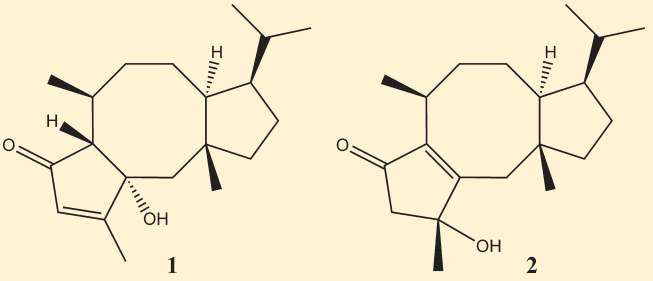
Supporting Information

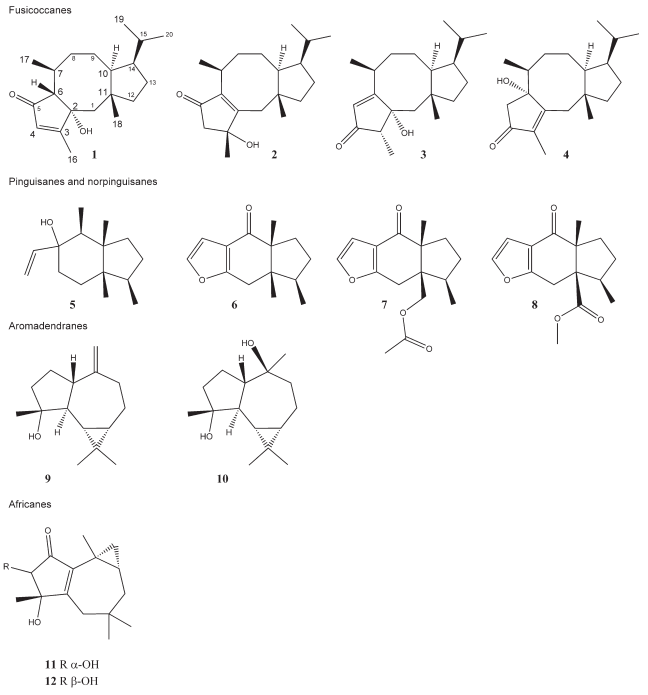
ABSTRACT: Four fusicoccane-type diterpenoids (**1**−**4**), including the new **1** and **2**; four pinguisane-type sesquiterpenoids (**5**−**8**); and two aromadendrane-type sesquiterpenoids (**9** and **10**) were isolated from an Argentine collection of the endemic liverwort *Porella chilensis*. The biofilm formation of the human pathogen *Pseudomonas aeruginosa* was inhibited by compounds **3** (53 and 47%), **9** (45 and 41%), and **10** (48 and 37%) at 50 and 5 μg/mL, respectively. Compounds **3**, **9**, and **10** also produced a slight decrease in bacterial growth and interfere with the process of quorum sensing at the same doses.



Porella, the largest genus of the family Porellaceae (Hepaticae), comprises pungent and nonpungent stem-leafy liverworts widespread in the tropical and subtropical regions of South America. Most *Porella* species are rich sources of sesqui- and diterpenoids,[1−11] many of which show interesting biological activities.[2,12] Some of the compounds from *P. chilensis* were previously found in *P. acutifolia*[6] (compound **5**), *P. densifolia*[7,8] (**6**, **8**, and **9**), *P. elegantula*[4] (**8**), *P. navicularis*[5] (**6** and **8**), and *P. recurva*[3] (**6**−**8**). There are no previous reports on fusicoccanes from the genus *Porella*.

Bacterial biofilms are complex communities of bacteria embedded in a self-produced matrix and attached to inert or living surfaces.[13] These microorganisms are more resistant to antibiotics and to the immunologic system than planktonic cells.[14] In *Pseudomonas aeruginosa*, biofilm formation, virulence factors production, and resistance to antimicrobials are regulated by the process of quorum sensing (QS).[15] Natural products, such as sesquiterpene lactones and annonaceous acetogenins, are able to alter the biofilm formation of *P. aeruginosa*.[16]

Herein, we present the first contribution to the chemistry of the liverwort *Porella chilensis* reporting four fusicoccane-type diterpenoids (**1**−**4**), including the new **1** and **2**, four pinguisane-type sesquiterpenoids (**5**−**8**), and two aromadendrane-type sesquiterpenoids (**9** and **10**).



RESULT AND DISCUSSION

The plant material was extracted with diethyl ether and then methanol. The ether extract was processed by a combination of column chromatography on silica gel, Sephadex LH-20, preparative MPLC, and RPHPLC, to furnish the new fusicoccane-type diterpenoids **1** and **2** together with the two known fusicoccanes **3** and **4**, four pinguisane-type sesquiterpenoids (**5**−**8**), and two known aromadendranes (**9** and **10**), norpinguisone (**6**) being the major compound.

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Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 1 and 2 ($CDCl_3$)[a]

1				2			
H	δ_H	C	δ_C	H	δ_H	C	δ_C
1a	1.85 d (15.3)	1	51.1	1a	2.49 d (14.1)	1	37.2
1b	1.95 d (15.3)	2	82.6	1b	2.73 d (14.0)	2	169.9
4	5.77 q (1.0)	3	178.1	4a,b	2.54 s	3	76.1
6	2.50 d (10.2)	4	128.5	7	2.60 m	4	50.8
7	2.01 m	5	209.0	8a	1.59 m	5	205.0
8a	1.64 m	6	54.2	8b	2.00 m	6	146.2
8b	2.28 m	7	30.5	9a,b	1.68 m	7	31.3
9a	1.44 m	8	36.3	10	2.01 m	8	31.9
9b	1.72 m	9	19.4	12a	1.24 dd (11.3, 6.5)	9	26.3
10	2.23 m	10	48.6	12b	1.69 m	10	43.2
12a	1.38 m	11	43.9	13a	1.46 m	11	44.4
12b	1.56 m	12	45.9	13b	1.57 m	12	40.3
13a,b	1.37 m	13	23.4	14	1.94 m	13	24.9
14	1.50 m	14	49.3	15	1.74 m	14	46.3
15	1.80 m	15	28.3	16	1.43 s	15	28.6
16	2.07 d (1.0)	16	13.3	17	1.05 d (6.9)	16	28.4
17	1.34 d (6.8)	17	20.7	18	1.01 s	17	21.5
18	0.95 s	18	24.4	19	0.84 d (6.7)	18	22.9
19	0.80 d (6.7)	19	19.4	20	0.87 d (6.5)	19	20.3
20	0.90 d (6.6)	20	23.9			20	24.4

[a] Coupling constants (J in Hz) are given in parentheses. Signals indicated as m were unresolved multiplets or overlapped. ^{13}C NMR assignments were confirmed by HSQC and DEPT measurements.

The HREIMS spectrum of **1** gave a molecular ion peak at m/z 304.2395 (calcd 304.2402), consistent with the molecular formula $C_{20}H_{32}O_2$, which accounted for five degrees of unsaturation. In the ^{1}H NMR spectrum (Table 1), signals for five methyls, three of them secondary (Me-17, Me-19, and Me-20) and two tertiary (Me-18 and Me-16), a methylene AB system (H-1), and an olefinic proton (H-4) strongly suggested a close structural similarity to the known fusicoccanes **3** and **4**. The COSY spectrum revealed an allylic coupling between Me-16 and H-4 (δ 5.77), indicating the location and olefinic nature of the latter. In addition, the cross-peaks between H-4 and C-2, C-3, C-5, C-6, and C-16 in the HMBC spectrum (Figure 1) indicated the location of the double bond in the cyclopentanone ring. ^{13}C NMR resonances at δ 209.0 and 82.6 were assigned to the only two oxygenated carbons by comparison with spectroscopic data reported for fusicoccanes **3**[17] and **4**.[18] Configurational features of compound **1**, as depicted (Figure 1), were established by comparison of the NMR traces of the cyclooctane and cyclopentane rings of **1** with those of the known fusicoccanes **3** and **4**. Additional support for the relative configuration was provided by NOESY correlations between Me-18 and H-1β, H-6 (β-oriented), H-9β, H-12β, H-13β, Me-17, and Me-19, indicating that they are all on the β-face of the molecule. The relative configurations at C-6 and C-7 (Figure 1) were consistent with the observed coupling constant (J = 10.2 Hz) between H-6 (β-oriented) and H-7 (α-oriented) as shown in Table 1. PCmodel (v6.0) calculations for the minimum energy conformer indicated a dihedral angle of 147.3° for the HβC(6)−HαC(7) moiety, with a calculated J-value of 9.1 Hz. In addition, the minimum energy conformation structure calculated with the

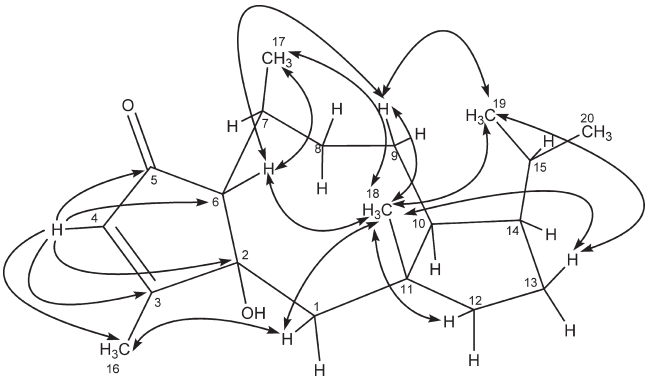

Figure 1. Partial NOEs (↔) and key HMBC correlations (→) observed for compound **1**.

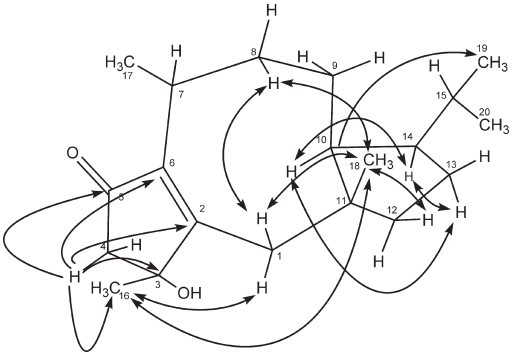

Figure 2. Partial NOEs (↔) and key HMBC correlations (→) observed for compound **2**.

Hyperchem (v8.0.6) program (54.73 kcal/mol) is consistent with that proposed in Figure 1.

The molecular formula of **2**, $C_{20}H_{32}O_2$, was deduced from its HREIMS, which showed a molecular ion peak at m/z 304.2401 (calcd 304.2402) that accounted for five degrees of unsaturation. NMR data of **2** (Table 1) showed close similarity to those of compound **1**, **3**, and **4** with five methyl signals and an AB system in the ^{1}H NMR spectrum. Differences in the chemical shifts of the olefinic carbons (δ 146.2 and 169.9) of **2** (Table 1) compared to the corresponding signals in the ^{13}C NMR spectrum of compounds **1**, **3**, and **4**, together with the absence of an olefinic proton and the HMBC correlations (Figure 2) between H-4 (δ 2.54) and both C-2 and C-6, permitted locating the double bond as depicted. On the basis of the HMBC correlations (Figure 2) observed for the cyclopentenone moiety, the location of the oxygenated groups was clearly assessed. NOESY correlations permitted the assignment of relative configurations, as shown in Figure 2. Additional evidence for the proposed structure was provided by spectroscopic data of the cyclopentenone moieties of compounds **11** and **12**, previously isolated from a liverwort of the same genus, *P. swartziana*.[19]

The absolute configuration of **2** was established on the basis of its CD spectrum, which showed a trace similar to those of the cyclopentenone sesquiterpenes **11** and **12**,[19] which share, in the cyclopentenone ring, similar structural features with compound **2**. The n → π^* transition of the carbonyl group gives rise to a weak absorption band between 310 and 350 nm for α,β-unsaturated cyclopentenones. Cotton effects were observed for these type of compounds at the mentioned wavelengths.[20,21] The CD

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spectrum of **2** showed a negative Cotton effect ($\Delta\varepsilon_{317}$ −5.11), as can be predicted by the back octant rule.[22,23] According to its NOESY spectrum, the CH_3-16 lies in the upper right (−) octant due to its pseudoaxial orientation, while the remaining sustituents surrounding the carbonyl chromophore have symmetrical partners or lie close to the carbonyl plane and, therefore, exert low "weight" to the Cotton effect. The absolute configurations of **11** and **12**, as reported,[19] were also established on the basis of the negative sign of the Cotton effect for the n → π^* transition ($\Delta\varepsilon_{336}$ −2.61 and $\Delta\varepsilon_{350}$ −0.68) for the cyclopentenone ring; therefore compound **2** as well as **11** and **12** belong to the same stereochemical series.

On the basis of biogenetical reasons, the absolute configuration of the new fusicoccanes **1** and **2** can be assumed to be similar to those of the known fusicoccanes anadensin (**3**) and fusicoauritone (**4**), whose configurations had been unambiguously determined by total synthesis.[24,25] It is important to note that in compounds **1**−**4** the location of the OH group on the α-face of the cyclopentenone ring has been supported by the results obtained in singlet-oxygen oxidation reactions of fusicoccadienes,[24] in which the $^1O_2$ attack occurs stereoselectively at the α-face exclusively, giving rise to α-epoxides in the cyclopentane ring. These transformations constitute biogenetic-type syntheses of oxygenated fusicoccanes.

The known compounds anadensin (**3**),[17] fusicoauritone (**4**),[18] pinguisenol (**5**),[26] norpinguisone (**6**),[26] norpinguisone acetate (**7**),[3] norpinguisone methyl ester (**8**),[27] *ent*-spathulenol (**9**),[1,28] and *ent*-4β,10α-dihydroxyaromadendrane (**10**)[29] were identified by their spectroscopic features in comparison with literature data. Anadensin (**3**), isolated from the liverwort *Anastrepta orcadensis*,[17] was the first fusicoccane isolated from bryophytes. *ent*-Spathulenol (**9**) is the most frequently encountered aromadendrane-type sesquiterpenoid in Hepaticae.[1] Its enantiomer displays antibacterial action against *Staphyloccocus aureus*.[30] *ent*-4β,10α-Dihydroxyaromadendrane (**10**) has been isolated from *Plagiochila ovalifolia*. Its enantiomer has been found in the higher plant *Brasilia sickii*.[1] All aromadendranes found in liverworts possess the *ent*-configuration, while a number of analogues are known to have antipodal counterparts in higher plants.[31]

Porella species were divided into nine chemotypes: a drimane-aromadendrane-pinguisame type (I), a sacculatane type (II), a pinguisane type (III), a pinguisane-sacculatane type (IV), an africanane type (V), a santalane-africanane-cyclofarnesane type (VI), a guaiane type (VII), a germacrane-pinguisane-sacculatane type (VIII), and a germacrane-africanane-guaiane type (IX).[32] On the basis of the terpenoids present in *P. chilensis*, this species should be located in a new chemotype, X, containing pinguisanes, aromadendranes, and also fusicoccanes.

Effects on the QS Process and Biofilm Formation. Bacteria are able to interact with each other and coordinate gene expression according to population density employing chemical communication by the process of QS.[16] In Gram-negative bacteria, this process takes place via emission and reception of molecular diffusible signals, particularly *N*-acylhomoserin lactones (AHL), known as autoinducers of QS.[33] Control of the *P. aeruginosa* biofilm production and/or the QS process by natural compounds might be a good strategy in the search for new antimicrobials. The 10 terpenoids, isolated from *P. chilensis*, were evaluated for their ability to inhibit bacterial growth as well as biofilm and autoinducer formation on a strain of *P. aeruginosa*. The antibiotic ciprofloxacin, a known biofilm inhibitor, produced 73% growth inhibition and avoided 51% biofilm formation at 5 μg/mL, while the antibiotic azithromycin did not significantly alter bacterial growth but inhibited 76% of the QS process at the same dose.

Fusicoccanes. As shown in Figure 3a, fusicoccanes altered bacterial growth in different ways. While **1** produced a 15% increment at 50 μg/mL, compounds **3** and **4** significantly inhibit bacterial growth at the same dose (16 and 25%) in relation to the negative control. The experiment at 5 μg/mL indicated that the effects are dose-dependent (Figure 3a). At 50 μg/mL, fusicoccanes **1**, **2**, and **4** strongly enhance biofilm formation (95, 93, and 105%, respectively) compared to the negative control. Our results indicated that the biofilm formation depends on the increment of the autoinducer production (Figure 3a) rather than the bacterial growth. Compound **3** is the only fusicoccane that was able to inhibit bacterial growth and autoinducer formation. These effects led to an inhibition of the biofilm production.

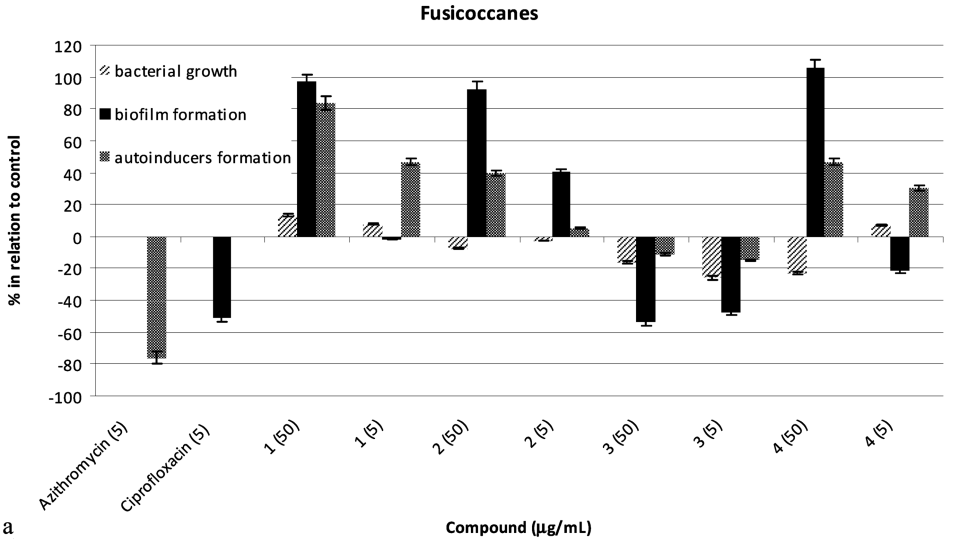
Pinguisanes. Bacterial growth was slightly enhanced by all pinguisanes at 50 and 5 μg/mL (Figure 3b). A good correlation between growth and biofilm and autoinducer production was observed for **5** and **6**. Compound **7** was able to inhibit 27 and 20% of biofilm production at 50 and 5 μg/mL, respectively. It is noteworthy that compound **8** produced increments in bacterial growth and autoinducer formation that did not result in an enhancement of the biofilm percentage.

Aromadendranes. Compounds **9** and **10** significantly inhibited not only bacterial growth but also biofilm and autoinducer formation even at 5 μg/mL (Figure 3c). The inhibition of autoinducers produced by **10** (80%) was related to the effect on bacterial growth (24%), suggesting that the QS process was altered as well.

Only fusicoccane **3** is as potent as ciprofloxacin in biofilm formation (Figure 3a) inhibition, while the aromadendranes **9** and **10** are slightly weaker than the antibiotic at the same concentration (Figure 3c). Regarding autoinducer formation, none of the tested compounds interfere as strongly as azithromycin (76%) at 5 μg/mL, aromadendrane **9** being the most active (33%) at the same dose (Figure 3c).

Fusicoccanes and pinguisanes share some structural features with a few natural products known to inhibit autoinducer production in *P. aeruginosa*. Some halogenated furanones[34,35] as well as penicillic acid and patulin[36] carry furanone rings, which apparently play a role in the inhibition. From our results, it is difficult to assess the structural requirements for the activity. In the series of fusicoccanes (all contain a cyclopentenone ring) and pinguisanes (**6**−**8** carry a furane ring), small differences in the molecular structure alter the QS process, inhibiting or stimulating it. These differential effects (inhibition or stimulation) were also previously reported for a series of structurally related *N*-acylhomoserin lactone analogues.[37] It is important to note that aromadendranes **9** and **10**, inhibiting both the biofilm formation and QS process, are not structurally related to a previously reported active compound. Since the strongest QS inhibitors (two AHLs synthetic analogues[38]) cause 50% inhibition at 2 and 6 μM, the effects of the terpenoids of *P. chilensis* might be considered mild (**9** produces 33% inhibition at 16.4 μM).

Among natural products that affect biofilm production in *P. aeruginosa*, the strongest are four germacranolides (sesquiterpene lactones) that inhibit more than 70% at around 0.6 μM.[16] None of the compounds isolated from *P. chilensis* are as potent. In fact, the



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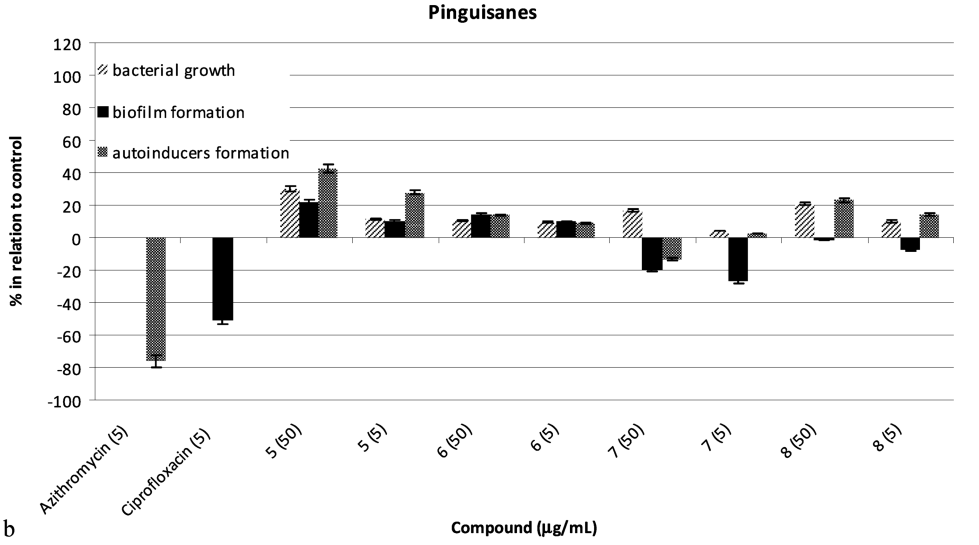

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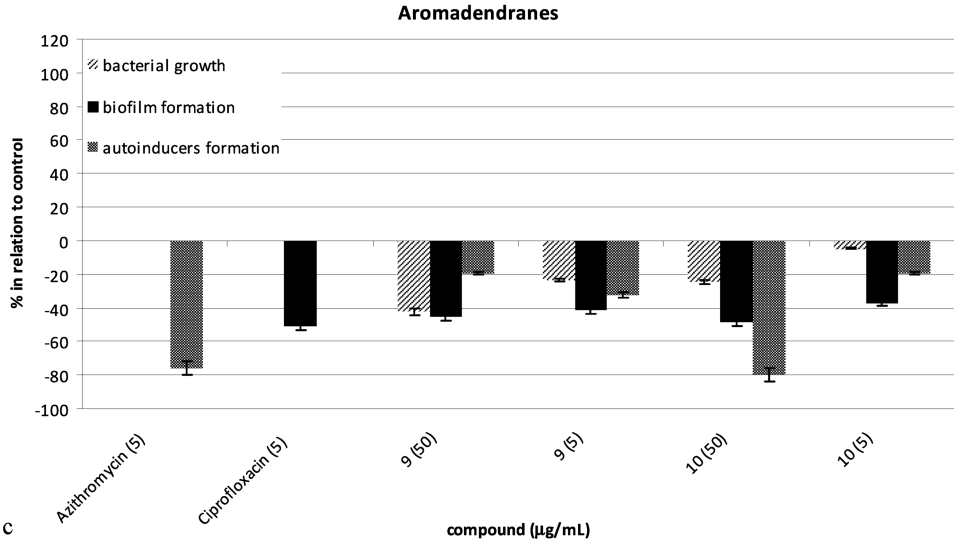

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Figure 3. Effects of compounds **1**−**10** and positive controls on bacterial growth and biofilm and autoinducer formation (expressed in percentage, in relation to control). Values > 0 or < 0 indicate that bacterial growth and biofilm and autoinducer production are higher or lower than control, respectively.

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fusicoccane **3** and the aromadendranes **9** and **10** inhibited 47, 41, and 37% biofilm formation at 16.4 μM, respectively.

Our results suggest that **3**, **9**, and **10** could be good candidates for further research on antipathogenic natural substances.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a Horiba SEPA-300 polarimeter. The UV spectra were obtained on a Shimadzu UV-160A spectrophotometer. The CD spectra were recorded on a JASCO J-725 spectrometer. The IR spectra were recorded on a Perkin-Elmer GX1. NMR spectra were recorded on a Varian Unity spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C with TMS as internal standard in $CDCl_3$. Low- and high-resolution mass spectra were recorded on a JEOL JMS AX 500 spectrometer (HRCIMS). For MPLC and HPLC a Gilson chromatograph with refractive index detector was used. MPLC column: (A) Merck LiChroprep Si 60 (40−60 μm). HPLC columns: (B) Chemo Pack Develosil 60 (5 μm, 10 mm i.d. × 250 mm), (C) Phenomenex Luna C18 (5 μm, 10 mm i.d. × 250 mm), and (D) Phenomenex Luna C8 (5 μm, 10 mm i.d. × 250 mm).

Plant Material. *P. chilensis*, growing over rocks and trees, was collected in February 2005 in Lago Steffen, Rio Negro Province, Argentina. One of the authors (M.M.S.) was responsible for the taxonomic determination of plant material. A voucher specimen (LIL No. 3411) is deposited at the Herbarium, Fundación Miguel Lillo, Tucumán, Argentina.

Extraction and Isolation. The air-dried plant material (573 g) was extracted at room temperature for 7 days with Et_2O to give 4.82 g of residue after solvent removal in a rotary evaporator (yield 0.84%). The extract was subjected to Sephadex LH20 CC (MeOH−CH_2Cl_2, 1:1) and then silica gel CC (70−230 mesh) with *n*-hexane, increasing amounts of EtOAc (0−100%), and finally MeOH, as eluents, to give five fractions.

Compound **6** (250 mg) was obtained as a crystalline material (*n*-hexane was used for crystallization) from 600 mg of fr I. After separation of **6** by filtration, the supernatant was submitted to MPLC on column A, with hexane and increasing amounts of EtOAc (0−100%) to give compound **5** (17 mg) and an additional amount of **6** (12 mg).

Fr II (98 mg) was submitted to HPLC (column B, *n*-hexane−EtOAc, 23:2) to give compounds **8** (6.5 mg) and **9** (6 mg).

Fr III (296 mg) was processed by MPLC on column A, with *n*-hexane and increasing amounts of EtOAc (5−100%), to give compounds **3** (5 mg) and **7** (39 mg).

CC rechromatography of fr IV (200 mg) on silica gel using *n*-hexane and increasing amounts of EtOAc (10−100%) gave two fractions. Compound **3** (39 mg) was obtained from fr 1. Fr 2 was processed by HPLC (column D, MeOH−H_2O, 17:3) to give compounds **1** (1.6 mg), **2** (4.3 mg), **4** (7 mg), and a new portion of **3** (7 mg).

Fr V (201 mg) was submitted to Sephadex LH20 CC and then HPLC (column C, MeOH−H_2O, 3:1) to give compound **10** (9 mg).

Compound ***1***: amorphous, white solid; $[\alpha]^{27}_D$ −10 (*c* 0.4, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 221 (4.0) nm; CD (EtOH) $\Delta\varepsilon_{326}$ +4.14, $\Delta\varepsilon_{234}$ −8.96, $\Delta\varepsilon_{209}$ +3.96 (*c* 2.88 × 10^{-4} M); FTIR ν_{max} ($CHCl_3$) 3416, 2920, 1690, 1633, 1452, 1378 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HREIMS *m*/*z* 304.2395 $[M]^+$ (calcd for $C_{20}H_{36}O_2$, 304.2402); EIMS *m*/*z* 304 $[M]^+$ (11), 286 $[M - H_2O]^+$ (69), 271 $[M - H_2O - Me]^+$ (8), 261 $[M - C_3H_7]^+$ (8), 243 $[M - H_2O - C_3H_7]^+$ (62), 176 $[M - 128]^+$ (97), 135 $[M - 169]^+$ (100).

Compound ***2***: colorless oil; $[\alpha]^{29}_D$ −4 (*c* 0.6, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 241 (4.0) nm; CD (EtOH) $\Delta\varepsilon_{317}$ −5.11, $\Delta\varepsilon_{245}$ +19.29, $\Delta\varepsilon_{210}$ −8.69 (*c* 4.11 × 10^{-4} M); FTIR ν_{max} ($CHCl_3$) 3407, 2920, 1685, 1624, 1462, 1372 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HREIMS *m*/*z* 304.2401 $[M]^+$ (calcd for $C_{20}H_{36}O_2$, 304.2402); EIMS *m*/*z* 304 $[M]^+$ (47), 286 $[M - H_2O]^+$ (27), 271 $[M - H_2O - Me]^+$ (5), 261 $[M - C_3H_7]^+$ (80), 243 $[M - H_2O - C_3H_7]^+$ (100), 225 $[M - 79]^+$ (95), 201 $[M - 103]^+$ (37).

Compound ***4***. Not previously reported CD data: (EtOH) $\Delta\varepsilon_{320}$ +5.69, $\Delta\varepsilon_{243}$ −19.94, $\Delta\varepsilon_{216}$ +8.11 (*c* 5.76 × 10^{-4} M).

Bacterial Growth. Bacterial growth was measured by optical density at 600 nm in a tunable microplate (PowerWave XS2, Biotek, VT, USA). Control absorbance value was 1.34.[39]

Biofilm Formation Assay. For determination of biofilm concentration a micromethod based on a protocol previously reported[40] was employed. Overnight cultures of *P. aeruginosa* ATCC 27853 were diluted to 10% (v/v) in Luria−Bertani (LB) medium. The diluted culture (190 μL) was poured in each of the 96 wells of a microtiter plate. Solutions containing 1 and 0.1 mg/mL of compounds **1**−**10** in DMSO−distilled H_2O (1:1) were prepared separately, and 10 μL of each was added to the microtiter plate wells individually (8 replicates). Negative control wells (8 replicates) contained the diluted culture (190 μL) with 10 μL of DMSO−H_2O (1:1). Plates were incubated at 37 °C for 24 h, and growth was determined spectrophotometrically at 600 nm. After absorbance measurement, the liquid was discarded from the wells and the material that remained fixed to the plastic (containing biofilm) was washed with PBS (three times). Only the biofilm remained after washing. It was stained with an aqueous solution of crystal violet (0.1% w/v) for 20 min, which was further eliminated by washing with H_2O. Crystal violet bound to biofilm was removed from each well employing 200 μL of absolute EtOH. Absorbance (540 nm) was determined using a microtiter plate reader (Power Wave XS2, Biotek, VT, USA). Negative control absorbance value was 2.75. Ciprofloxacin, a known biofilm inhibitor,[41] was incorporated in the bioassay at 5 mg/mL as a positive control in the same experimental conditions employed to evaluate compounds **1**−**10**.

Screening for Quorum Sensing Effect. An overnight culture of the reporter strain *P. aeruginosa* qsc 119,[42] grown at 37 °C in LB, was diluted 10 times in the same medium. A 100 μL portion of this suspension was mixed, in each microplate well, with 100 μL of cell-free culture supernatant obtained from *P. aeruginosa* ATCC 27853 cultured in LB media containing 50 and 5 μg/mL of compounds **1**−**10**, during 24 h. Azithromycin, known to interfere with the QS process,[43] was used at 5 μg/mL as QS inhibition positive control under the same conditions as compounds **1**−**10**. Negative control wells (8 replicates) contained cell-free culture supernatant (100 μL) obtained from *P. aeruginosa* ATCC 27853 cultured in LB media with 10 μL of DMSO−water (1:1).

P. aeruginosa qsc 119 is a mutant that cannot produce its own AHL (QS signal molecules), but responds to exogenous active signal molecules by the production of β-galactosidase. As a consequence, β-galactosidase activity is under QS control and in direct relationship with the autoinducer (AHL) activity. β-Galactosidase activity was measured spectrophotometrically by the Miller test.[40] Negative control activity was 25.2 expressed as Miller units.

Statistical Analysis. Data are presented as mean ± SD. The statistical significance of differences between mean values was evaluated by Student's test. A value of $p \leq 0.05$ was considered significant.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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