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Sesqui- and triterpenoids from the liverwort *Lepidozia chordulifera* inhibitors of bacterial biofilm and elastase activity of human pathogenic bacteria

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ABSTRACT

Five dammarane-type triterpenoids, five pentacyclic triterpenoids (three of them carrying a carboxylic acid group), and two aromadendrane-type sesquiterpenoids were isolated from an Argentinian collection of the liverwort *Lepidozia chordulifera*. Compounds were characterized by comparison of their spectral data with those previously reported and tested in their ability to control bacterial growth, biofilm formation, bacterial Quorum Sensing process (QS), and elastase activity of *Pseudomonas aeruginosa*, as well as bacterial growth and biofilm formation of *Staphylococcus aureus*. The key role played by biofilm and elastase activity in bacterial virulence make them a potential target for the development of antibacterial agents. The aromadendrane-type sesquiterpenoid viridiflorol was the most potent biofilm formation inhibitor, producing 60% inhibition in *P. aeruginosa* and 40% in *S. aureus* at 50 μg/ml. Ursolic and betulinic acids (two of the pentacyclic triterpenoids isolated) were able to reduce 96 and 92% the elastase activity of *P. aeruginosa* at 50 μg/ml, respectively. Among the analyzed triterpenoids, those that carry a dammarane skeleton were the most potent inhibitors of the *P. aeruginosa* biofilm formation and were active against both *P. aeruginosa* and *S. aureus*. Subsequently, a computer-assisted study of the triterpenoid compounds was carried out for a better understanding of the structure-activity relationships.

Introduction

The genus *Lepidozia* belonging to the Porellaceae family of the Class Hepaticae (liverworts) is mainly distributed in tropical and subtropical regions of the southern hemisphere and its species are rich sources of various types of secondary metabolites including isobicyclogermacranes, lepidozanes, cadinanes, eudesmanes, maalianes, vitranes, and bibenzyls (Asakawa et al., 2012). *Lepidozia chordulifera* is a light green to green-brown plant that grows over rocks, trees, and soil forming loose tufts (Ardiles et al., 2008). The only previous chemical investigation of a Chilean collection of *L. chordulifera* revealed that triterpenoids represented the major constituents of the dichloromethane extract in which 21, 28-epoxy-18β,21β-dihydroxybaccharan-3-one, taraxerol, shoreic, betulinic, betulonic, and ursolic acids were present (Zapp et al., 2008).

Bacterial biofilms are complex communities of bacteria embedded in a self-produced matrix and attached to inert or living surfaces (Costerton et al., 1999). These microorganisms are more resistant to antibiotics and to the immunologic system than planktonic cells (Stewart and Costerton, 2001). Processes of biofilm formation, virulence factors production, and resistance to antimicrobials are regulated by the Quorum Sensing (QS) process, a bacterial communication system mediated by diffusible chemical signals called autoinducers (van Delden and Iglewski, 1998). Sesquiterpene lactones, fusicoccane-type diterpenoids, annonaceous acetogenins, and acylphloroglucinols from plants are able to alter biofilm formation of *Pseudomonas aeruginosa* (Cartagena et al., 2007; Gilabert et al., 2011) and *Staphylococcus aureus* (Arena et al., 2011; Socolsky et al., 2010), as well as the production of some virulence factors of *P. aeruginosa* such as elastase (Amaya et al., 2012). *Pseudomonas* elastase, also known as pseudolysin or LasB, is a metalloprotease that has long been recognized as a key

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virulence factor produced by *P. aeruginosa* (Stewart and Costerton, 2001). The secreted protease degrades a broad range of host tissue proteins and key biomolecules involved in innate immunity such as inmunoglobulins, complement factors, and cytokines (Liu, 1974). In addition, LasB acts within the bacterial cell as a key regulator in the generation of the secreted polysaccharides that constitute the bacterial biofilm (Cathcart et al., 2009). Inhibition of one of the main virulence factors (elastase) of bacteria turn them susceptible to the attack of the host immune system. Thus, LasB inhibition by natural products could be an important strategy for controlling *Pseudomonas* virulence in bacterial infections (Sokol et al., 2000).

Herein we report the ability of ten *L. chordulifera* terpenoids (**1**–**10**) to control bacterial growth, biofilm formation, QS process, and elastase activity of *P. aeruginosa*, as well as, bacterial growth and biofilm formation of *S. aureus*.

Experimental

General

NMR spectra were recorded on a Bruker spectrometer operating at 300 MHz for ^{1}H and 75 for ^{13}C with TMS as internal standard in $CDCl_3$. For preparative HPLC a Gilson chromatograph with refractive index detector was used. HPLC columns: (A) Chemo Pack Develosil 60 (5 μm, 10 mm i.d. × 250 mm), (B) Phenomenex Luna C18 (5 μm, 10 mm i.d. × 250 mm), and (C) Phenomenex Luna C8 (5 μm, 10 mm i.d. × 250 mm).

Plant material

L. chrodulifera, growing over rocks and trees was collected in February 2009 in Lago Steffen, Rio Negro province, Argentina. A voucher specimen (LIL N° 3414) is deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina.

Extraction and isolation

The air-dried plant material (240 g) was extracted at room temperature for 7 days with diethyl ether (Et_2O), in a shaker, to give 5.9 g of residue after solvent removal (yield 2.46%). The extract was subjected to Sephadex LH20 CC (MeOH–CH_2Cl_2, 1:1) to get rid of chlorophylls, and then to silica gel CC with *n*-hexane with increasing amounts of EtOAc (0–100%), and finally MeOH as eluents, to give five fractions (I–V).

Fr I (370 mg) was submitted to HPLC (Column A, *n*-hexane-EtOAc, 49:1) to give compound **1** (17 mg). Fr II (103 mg) was a mixture further separated by HPLC (Column A, *n*-hexane-EtOAc, 26:1) to yield compounds **2** (34 mg), **11** (10 mg), and **12** (15 mg). Fr III (80 mg) was processed by RP-HPLC (Column B, MeOH–H_2O, 9:1) to give compound **8** (7 mg). RP-HPLC (Column B, MeOH–H_2O, 17:1) of Fr IV (150 mg) yielded compounds **9** (3 mg), **10** (4 mg) and a mixture that was re-processed by RP-HPLC using column C (MeOH–H_2O, 17:1 + 1% Acetic acid) to obtain compounds **3** (11 mg), **4** (3 mg), and **5** (6 mg). Fr V (150 mg) was submitted to Sephadex LH20 CC (MeOH–CH_2Cl_2, 1:1) and then to RP-HPLC (Column B, MeOH–H_2O, 22:3 + 1% acetic acid) to give **6** (14 mg) and a mixture submitted to RP-HPLC with column C (MeOH–H_2O, 21:4 + 1% Acetic acid) to obtain **7** (3 mg) and a new portion of **6** (3 mg). Purity and chemical structures were assessed by HPLC (single peaks in the chromatogram) and spectroscopic data which were compared with those previously reported.

Tests

Compounds **2**–**11** were tested on their ability to alter bacterial growth, biofilm formation, Quorum Sensing process, and elastase activity of *P. aeruginosa* as well as the bacterial growth and biofilm formation of *S. aureus*. Compound **1** (taraxerone) underwent partial decomposition therefore it could not be tested, while compound **12** (*ent*-spathulenol) had been previously evaluated (Gilabert et al., 2011).

Microorganisms

Pseudomonas aeruginosa ATCC 27853 (Gram negative bacilli) and *Staphylococcus aureus* ATTC 6538 P (Gram positive cocci) strains were selected for the bioassays.

Bacterial growth

An overnight culture of *P. aeruginosa* ATCC 27853 was diluted to reach an OD 0.125 ± 0.02 at 560 nm in Luria–Bertani (LB) medium. An overnight culture of *S. aureus* ATTC 6538 P was diluted to reach an OD 0.13 ± 0.03 at 560 nm in Mueller Hinton medium. The diluted cultures (190 μl) were placed in each of the 96 wells of a microtitre polystyrene plate. Solutions containing 1 and 0.1 mg/ml of compounds **2**–**11** in DMSO/distilled water (1:1) were prepared separately and 10 μl of each were pipetted to the plastic microtitre plate wells individually (8 replicates). Control growth wells (8 replicates) contained the diluted culture (190 μl) and 10 μl of a solution of DMSO/water (1:1) in which the final concentration of DMSO is 2.5%. Ciprofloxacin, a known biofilm inhibitor, was incorporated to the bioassay at 5 μg/ml in the same experimental conditions employed to evaluate the compounds (8 replicates). At this concentration, ciprofloxacin inhibited the biofilm formation but did not modify significantly the bacterial growth (Sandasi et al., 2011). After 24 h incubation at 37 °C, bacterial growth was detected as turbidity (560 nm) using a microtitre plate reader (Power Wave XS2, Biotek, VT, USA). Control absorbance values were 1.59 for *P. aeruginosa* and 1.35 for *S. aureus*.

Biofilm formation assay

For biofilm quantification, a micro method based on a protocol previously reported was employed (O'Toole and Kolter, 1998). Biofilms formed after 24 h incubation of bacterial cultures prepared as described in the previous paragraph, were stained with 20 μl of an aqueous solution of crystal violet (0.1%, w/v) for 20 min. After washing with water, the liquid was discarded from the wells and the material that remained fixed to the polystyrene (containing biofilm) was washed with PBS (thrice). Crystal violet bound to biofilm was removed from each well employing 200 μl absolute ethanol during 30 min at 37 °C with shaking. Absorbance (560 nm) of ethanol solutions of crystal violet was determined using a microtitre plate reader (Power Wave XS2, Biotek, VT, USA). Wells containing ciprofloxacin at 5 μg/ml were employed as a positive control of biofilm formation. Control growth wells treated as described above were employed as a control for biofilm formation. Absorbance values were 2.09 for *P. aeruginosa* and 0.35 for *S. aureus*.

Screening for Quorum sensing inhibition

An overnight culture of the reporter strain *P. aeruginosa* qsc 119 (Whiteley et al., 1999), 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 cell-free culture supernatant obtained from *P. aeruginosa* ATCC 27853 cultured in LB media containing 50 and 5 μg/ml of compounds **2**–**11**, during 24 h. Azithromycin, known to interfere with the QS process (Tateda et al., 2001) was used at 5 μg/mL as QS inhibition positive control under the same experimental conditions as for compounds **2**–**11**. Quorum sensing control wells (eight replicates) contained