

ANTIBACTERIAL CONSTITUENTS FROM THE OCTOCORAL-ASSOCIATED BACTERIUM *Pseudoalteromonas* sp.

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ABSTRACT

Bioassay guided fractionation of the ethyl acetate extract obtained from the bacterium *Pseudoalteromonas* sp. isolated from the octocoral *Leptogorgia alba* led to the isolation of two known diketopiperazines identified as *cyclo*-(L-Ph-L-Pro) (**1**), and *cyclo*-(L-Leu-L-Pro) (**2**). Structures of **1-2** were defined based on their spectroscopic data, including NMR, HRESITOF-MS and optical rotations, and their comparison with the spectroscopic data reported in the literature. Both, compounds **1** and **2** showed strong inhibition on the growth of *Bacillus subtilis* and *Vibrio* sp. Analysis of the crude extract using APCI-MS revealed the presence of other compounds of the diketopiperazine family. www.relaquim.com

Keywords: *Pseudoalteromonas* sp., *Bacillus subtilis*, *Vibrio* sp., diketopiperazines, antibiotics.

RESUMEN

El asilamiento biodirigido del extracto de acetato de etilo obtenido de la bacteria *Pseudoalteromonas* sp. aislada del octocoral *Leptogorgia alba* llevó al aislamiento de dos diketopiperazinas conocidas, identificadas como *cyclo*-(L-Ph-L-Pro) (**1**), y *cyclo*-(L-Leu-L-Pro) (**2**). Las estructuras de los compuestos **1-2** fueron establecidas mediante análisis espectroscópico incluyendo RMN, ESITOF-MS y rotación óptica, y mediante su comparación con datos de la literatura. Los compuestos **1-2** mostraron una fuerte inhibición del crecimiento de *Bacillus subtilis* y *Vibrio* sp. El análisis del extracto crudo mediante espectrometría de masas APCI reveló la presencia de otros compuestos de la serie de las diketopiperazinas. www.relaquim.com

Palabras clave: *Pseudoalteromonas* sp., *Bacillus subtilis*, *Vibrio* sp., diketopiperacinas, antibióticos.

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INTRODUCTION

Antibiotic resistance of pathogenic bacteria is a major public health problem worldwide associated with increased rates of morbidity, mortality and increased health care costs for treatment of people infected by antibiotic resistant strains of bacteria (Gwynn *et al.*, 2010). Additionally, resistance to antibiotics has led to a significant economic impact because it has increased the cost of treating infections, plus it also has contributed to increases in morbidity and mortality (Högberg *et al.*, 2010). The number of new antibiotics introduced to the market has declined significantly over the past 20 years, despite the increasing problems with antimicrobial resistance among important bacterial pathogens (Moellering Jr. 2011; Gwynn *et al.*, 2010). Among the reasons leading to the reduction in the development of new antimicrobial agents are: 1) classical approaches of drug discovery have produced poor results due to the difficulty involved in obtaining new compounds; 2) new approaches, such as microbial genomics and high throughput screening, have not provided sufficient results yet, and 3) a lack of interest by major pharmaceutical companies in the development of new antimicrobials (Moellering Jr. 2011). For these reasons, there is an urgent need to seek new alternatives to current antibiotics.

Among the antibiotics available, a large group of low molecular weight compounds with antimicrobial activity were isolated from natural terrestrial sources during the past two decades (Butler and Cooper 2011; Hughes and Fenical 2010). The evolution of pathogenic bacteria resistant to antibiotics has stimulated the search for new antimicrobial agents from alternative sources, including sources from the ocean. In recent years, marine organisms have been investigated as potential producers of compounds with pharmaceutical application (Hughes and Fenical 2010). Thus, the

current study was designed to investigate the antibacterial activity of the bacterium *Pseudoalteromonas* sp. associated with the octocoral *Leptogorgia alba* collected from Otoque Island, Pacific coast of Panama. Herein we report the isolation, identification and antibacterial activity of compounds **1-2** produced by *Pseudoalteromonas* sp.

MATERIAL AND METHODS

General Experimental Procedures.

Optical rotations were measured with a Jasco P-2000 polarimeter. NMR spectra were acquired on Jeol Eclipse 400 MHz spectrometer and referenced to residual solvent ^1H and ^{13}C signals (δ_{H} 7.26, δ_{C} 77.0 for CDCl_3). Low-resolution ESIMS spectra were acquired on Jeol LC-mate mass spectrometer, while high-accuracy mass measurements were obtained on an Agilent 6230 mass spectrometer. The purification of the compounds was carried out on Agilent 1100 HPLC system equipped with a quaternary pump, a diode array detector, and a normal phase silica gel column (Phenomenex Spherclone, 4.6 mm \times 100 mm, 5 μm) at a flow rate of 0.75 mL/min. Pre-fractionation of the extract was carried out using supelcleanTM LC-18 solid phase extraction (SPE) tubes. TLC (analytical) was performed on precoated silica gel 60 F254 plates (Merck). All solvents were HPLC grade and used without further purification.

Biological material collection and identification.

A healthy specimen of the octocoral *Leptogorgia alba* was collected by hand using SCUBA near Otoque Island in the Pacific Ocean off the coast of Panama in August 2009. A small piece of the coral was rinsed with sterile seawater and a small portion of the coral mucus was inoculated on Petri dishes with seawater-based nutrient media.

A total of 34 bacterial isolates were obtained from these collection plates. All strains were evaluated for their antimicrobial activity and one strain was found to inhibit the growing of *Bacillus subtilis* and *Vibrio* sp. Taxonomy of this active bacterium was carried out by sequencing of the 1317 bp of 16S rRNA gene. The gene sequence was submitted to The Ribosomal Database Project (RDP) (Michigan State University) and showed 100% similarity to 16S rRNA gene sequence of a strain identified in RDP like *Pseudoalteromonas* sp.

Fermentation and extraction

The bacterium *Pseudoalteromonas* sp. was inoculated in two Erlenmeyer flasks (500 mL), containing 250 mL of medium M1 (Agar, starch of potatoes, yeast extract and peptone) and 250 mL of medium M3 (Agar, yeast extract, peptone and glycerol). Erlenmeyer flasks were placed in an orbital shaker at 172 rpm at room temperature for 10 days. After this period, the culture broths (M1 and M3) were extracted separately with ethyl acetate (200 mL x 3). The organic extracts were washed with distilled water (200 mL x3) in order to remove traces of culture media from the organic extract. The extracts were then dried under reduced pressure to obtain 22 mg and 16.5 mg of crude extracts from M1 and M3 mediums, respectively.

Isolation of antibiotics.

Extract obtained from M1 medium (20 mg) was fractionated using C-18 solid phase extraction (SPE) cartridges eluted with a stepwise gradient of 20 %, 40 %, 60 %, 80 %, and 100 % of methanol in water to yield 5 fractions (F1-F5). Fraction F5 eluted with 100 % methanol, was purified by normal phase HPLC (Sphereclone silica 250 x 10 mm column, isocratic elution of 85% hexanes : 15% EtOAc, UV detector at 254 nm, flow of 0.75 mL/min) to afford 0.9

mg of *cyclo*-(L-Ph-L-Pro) (**1**) and 1.0 mg of *cyclo*-(L-Leu-L-Pro) (**2**).

Cyclo-(L-Ph-L-Pro) (1). Colorless oil; $[\alpha]_D^{25}$ -60.6 (c 0.66, EtOH); ^1H NMR (CDCl_3 , 400 MHz): δ : 7.20 – 7.36 (5H, m, Ar-H), 5.61 (1H, brs, NH), 4.25 (1 H, dd, J = 10.0, 3.0 Hz, H-9), 4.05 (1H, t, J = 8.0, Hz, H-6), 3.53-3.63 (2H, m, H-3), 2.76 (2H, dd, J = 14.0, 10.0 Hz, H-10), 2.29-2.35 (2H, m, H-5), 1.95-2.03 (1H, m, H-4b), 1.86-1.91 (1H, m, H-4a); ^{13}C (CDCl_3 , 100 MHz): δ : 169.2 (C-1), 164.7 (C-7), 135.7 (C-1'), 129.0 (C-2'), 128.8 (C-3'), 127.3 (C-4'), 58.9 (C-6), 55.9 (C-9), 45.2 (C-3), 36.5 (C-10), 28.1 (C-5), 22.3 (C-4); HR-ESI-TOFMS m/z 267.1104 $[\text{M} + \text{Na}]^+$, $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_2\text{Na}$ requires 267.1109).

Cyclo-(L-Leu-L-Pro) (2): Colorless oil, $[\alpha]_D^{25}$ -15.9 (c 0.66, EtOH); ^1H NMR (CDCl_3 , 400 MHz): δ : 6.07 (1H, brs, NH), 4.10 (1 H, t, J = 7.8, H-6), 4.00 (1H, dd, J = 8.3, 4.0 Hz, H-9), 3.45-3.61 (2H, m, H-3), 2.29-2.35 (2H, m, H-5), 1.98-2.16 (2H, m, H-4), 1.84-1.92 (1H, m, H-11), 1.72 (1H, m, H-10b), 1.51 (1H, m, H-10a), 0.97 (3H, d, J = 7.0, CH_3), 0.93 (3H, d, J = 7.0, CH_3); ^{13}C (CDCl_3 , 100 MHz): δ : 170.1 (C-1), 166.2 (C-7), 59.1 (C-6), 53.5 (C-9), 45.6 (C-3), 38.7 (C-10), 28.2 (C-5), 24.8 (C-11), 23.4 (C-4), 22.8 (C-12), 21.3 (C-12'); HR-ESI-TOFMS m/z 211.1441 $[\text{M} + \text{H}]^+$, $\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_2$ requires 211.1447).

Screening for bacterial antagonistic activity

Pseudoalteromonas sp. was screened for antibacterial activity according to the method described by Castillo and collaborators (Castillo *et al.*, 2001) with some modifications. The method consisted in spreading a suspension of 1×10^5 CFU of the target bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella* sp., *Pseudomonas aeruginosa* and *Vibrio* sp.) on Petri dishes with M1 agar. After 20 minutes a small cluster of *Pseudoalteromonas* sp. was placed on the agar plate, followed by overnight incubation at 37 °C. After this period, the plates were observed for the formation of inhibition

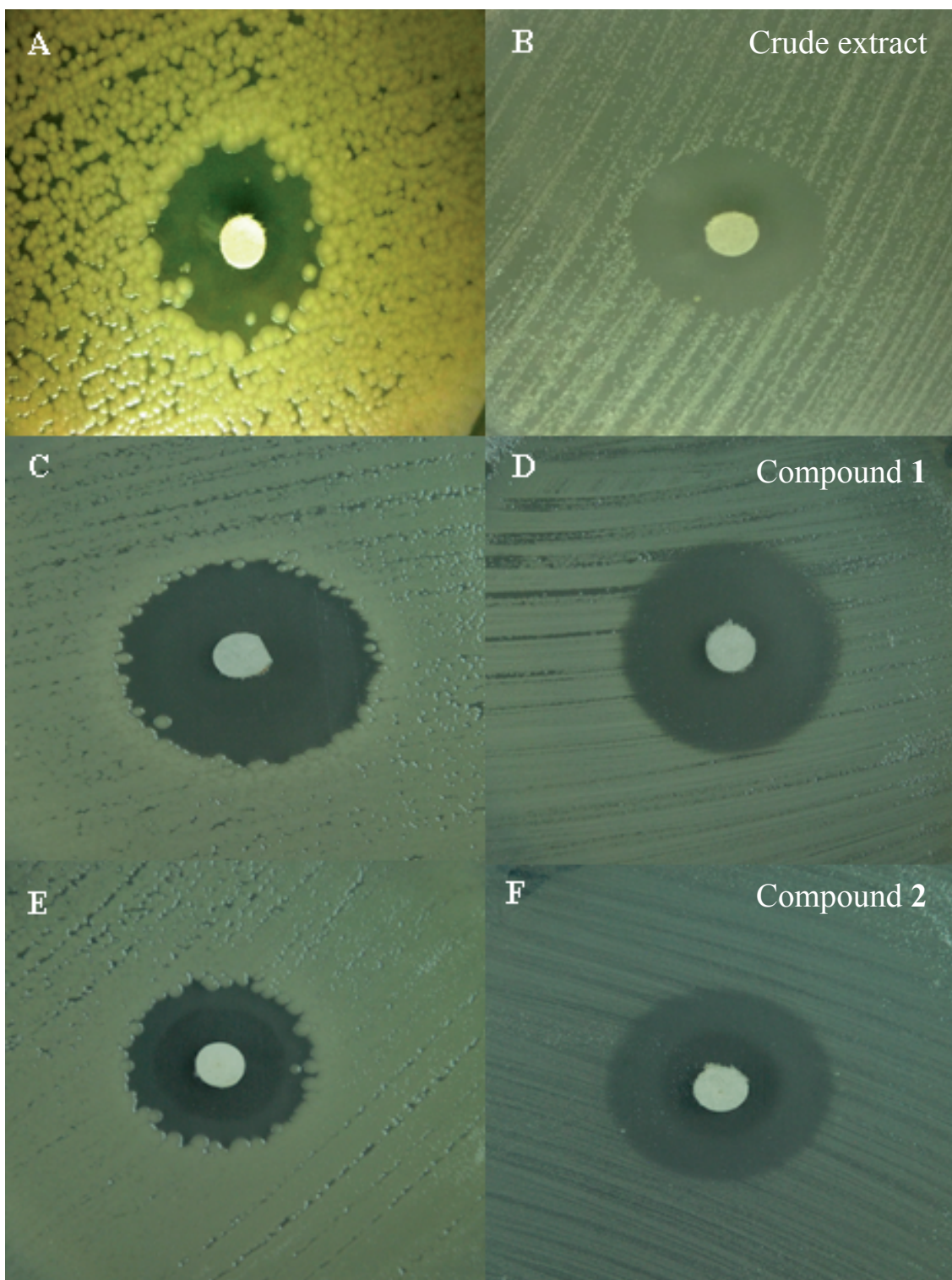


Figure 1. Antibacterial activity of crude extract and compounds **1** and **2** from *Pseudoalteromonas* sp. against *Bacillus subtilis* (A, C and E) and *Vibrio* sp. (B, D, F).

zones around the *Pseudoalteromonas* sp. clusters.

Antimicrobial activity

The antimicrobial activity of extracts and pure compounds obtained from *Pseudoalteromonas* sp. was evaluated using the disc diffusion susceptibility test according to the National Committee for Clinical Laboratory Standards (Espinel-Ingroff and Canton 2007). Briefly, the disc diffusion test was carried out by spreading 500 μ L of a suspension of 1×10^5 CFU/mL of the target bacteria (*B. subtilis*, *S. aureus*, *P. aeruginosa*, *Klebsiella* sp. and *Vibrio* sp.) on agar plates with a sterile cotton swab. After 10 minutes 6 mm diameter paper discs containing the extracts or compounds (100 μ g/disc) obtained from *Pseudoalteromonas* sp. were applied and incubated at 37°C for 24 h. After this period the inhibition zones were measured. Discs containing 100 μ g of chloramphenicol were used as positive controls, whereas discs containing 10 μ L of ethyl acetate were used as negatives controls for each experiment. Each test was performed in duplicate.

RESULTS AND DISCUSSION

The bacterium *Pseudoalteromonas* sp. was isolated from the mucus of the octocoral *L. alba* collected near Otoque Island in the Pacific of Panama. Taxonomic identifica-

tion of the bacterium was done based on its 16S rRNA sequence.

Pseudoalteromonas sp. was evaluated for its antagonistic activity against several target bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio* sp. and *Klebsiella* sp.) and it was found to be active against *Vibrio* sp. and *B. subtilis* with inhibition zones of 21 mm and 16 mm, respectively.

In order to isolate the active compounds, the organic extract of *Pseudoalteromonas* sp. obtained from M1 medium was fractionated by C-18 SPE, followed by normal phase HPLC purification to yield the diketopiperazines **1-2**. Compounds **1** and **2** were identified as *cyclo*-(*L*-Ph-*L*-Pro) and *cyclo*-(*L*-Leu-*L*-Pro), respectively, by spectroscopic analysis including HRESITOF-MS, NMR, and optical rotations. The structures were confirmed by comparison of their spectroscopic data with those reported in the literature (Li *et al.*, 2006; Capon *et al.*, 2007; Furtado *et al.*, 2005; Fdhila *et al.*, 2003; Fdhila *et al.*, 2006).

Antibacterial activity of compounds **1-2** against *Bacillus subtilis* and *Vibrio* sp. was evaluated using the disc diffusion susceptibility test in agar plates. Compound **1** showed inhibition zones of 25 mm against *Bacillus subtilis* and 14.5 mm against *Vibrio* sp., while compound **2** showed inhibition zones of 23 mm against *Bacillus subtilis* and 24 mm against *Vibrio* sp. (Figure 1). The fact that diketopiperazines **1-2** inhibit the growing of *B. subtilis*, a Gram positive

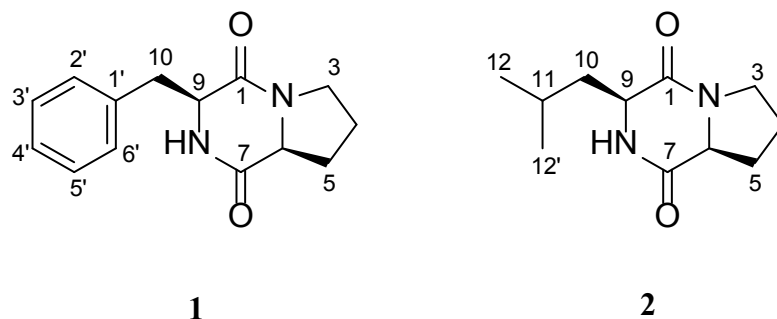


Figure 2. Compounds isolated from *Pseudoalteromonas* sp.

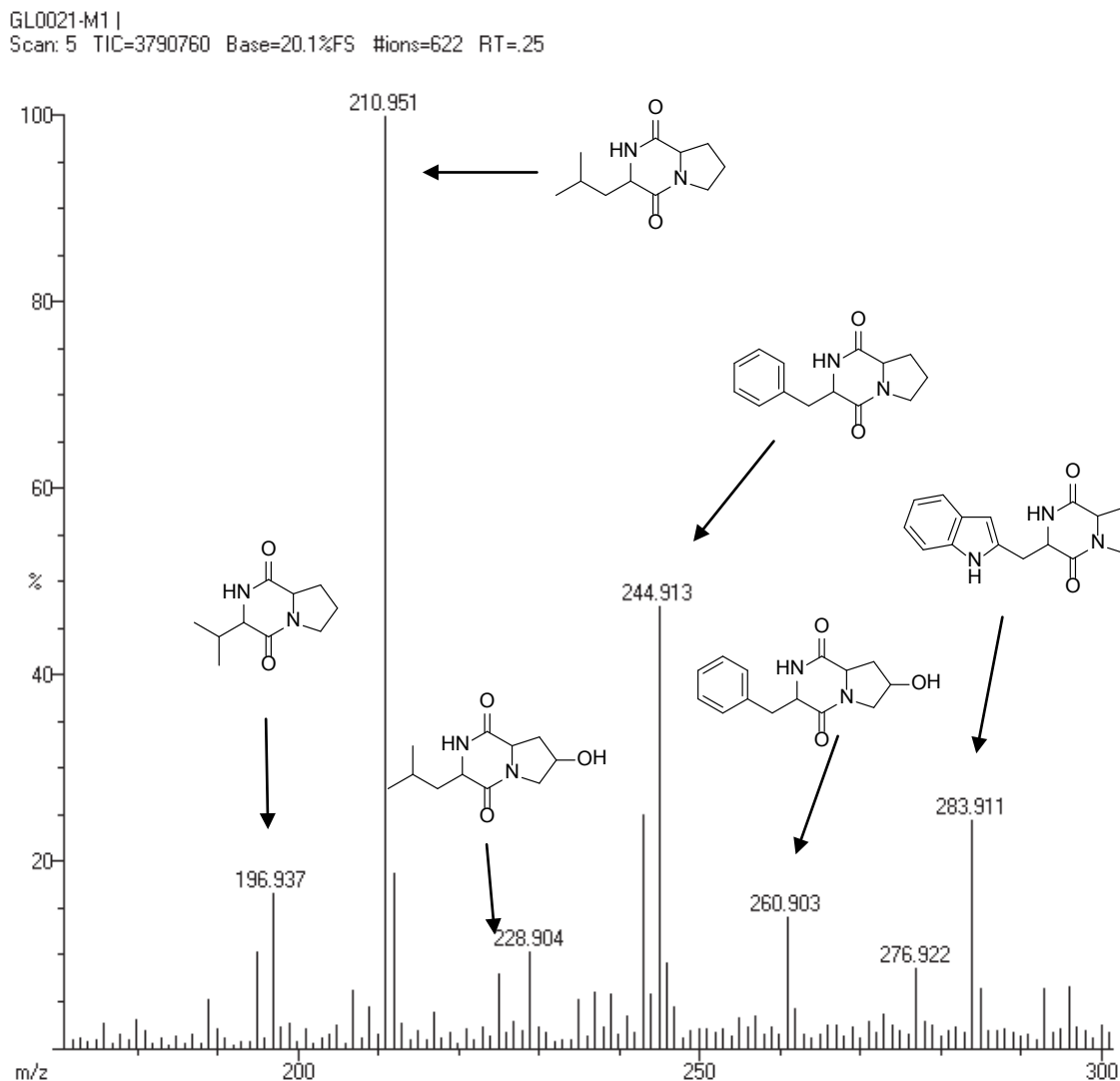


Figure 3. Positive mode APCI full scan mass spectrum of crude extract obtained from fermentation of *Pseudoalteromonas* sp. in M1 liquid medium.

bacterium, and *Vibrio* sp., a Gram negative bacterium, indicates their broad spectrum activity.

Marine diketopiperazines have been isolated mainly from microorganisms and there are a few reports on their isolation from marine invertebrates (Huang *et al.*, 2010). The fact that this family of compounds is widely produced by microorganisms, strongly suggests that the “marine invertebrate-derived diketopiperazines” might also have a microbial biosynthetic origin. To our knowledge, this is the first

report on diketopiperazines from a bacterium associated with an octocoral.

It is well documented that corals hold a complex microbial community in their mucus (Sunagawa *et al.*, 2010; Shnit-Orland and Kushmaro, 2009; Ritchie, 2006). However, the ecological role of these microbes in the coral holobiont remains largely unknown. It has been proposed that bacterial communities in the coral mucus provide defense against coral pathogens by production of antibiotics (Ritchie, 2006; Shnit-Orland and Kushmaro, 2009).

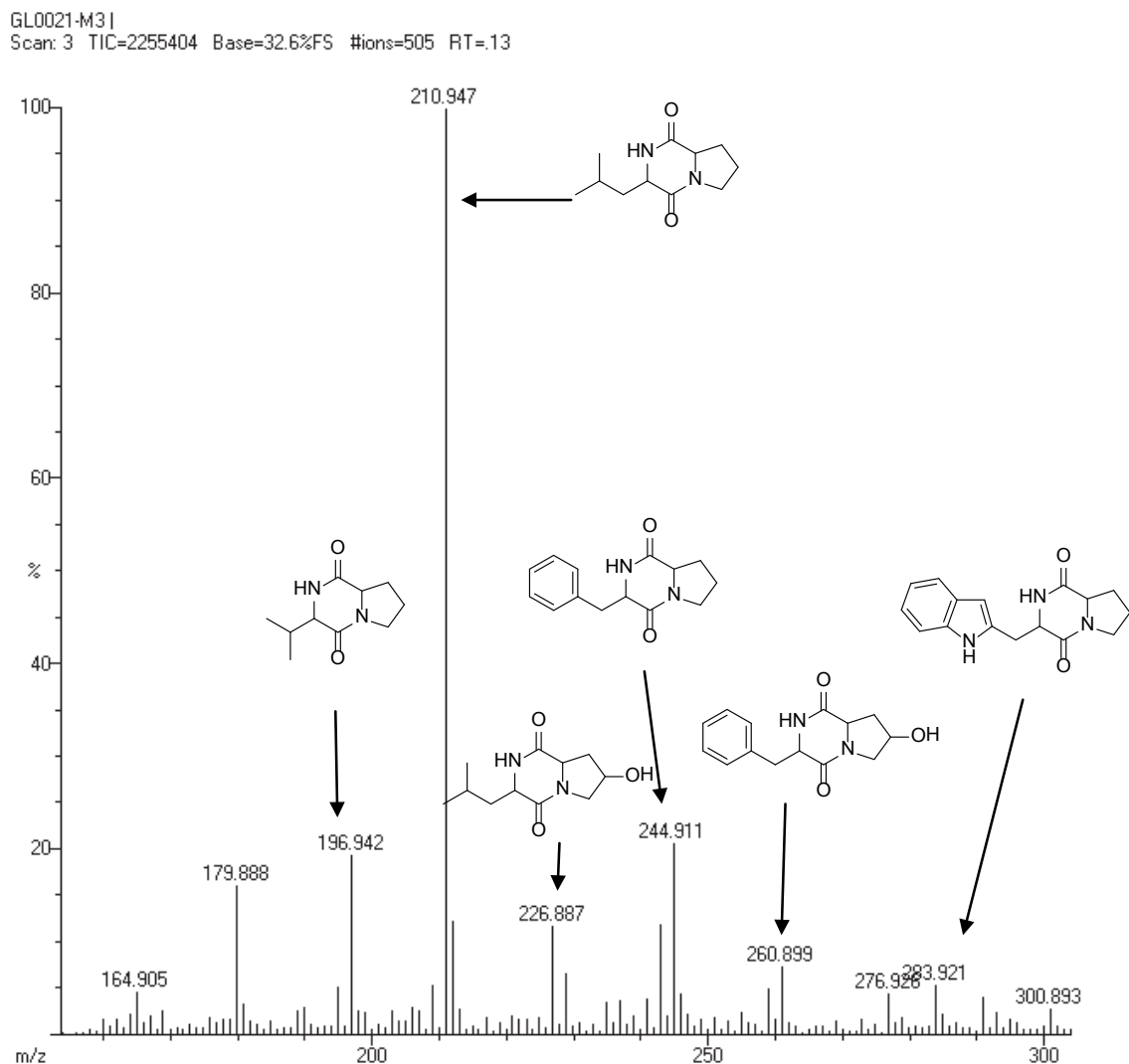


Figure 4. Positive mode APCI full scan mass spectrum of crude extract obtained from fermentation of *Pseudoalteromonas* sp. in M3 liquid medium.

Among pathogens that affect the coral health, bacteria of the genus *Vibrio* have been found to produce several coral diseases (Vidal-Dupiol *et al.*, 2011). The fact that compounds **1-2** were isolated from an octocoral-associated bacterium, together with the fact that compounds **1-2** possess antibiotic activity against a marine strain of *Vibrio*, support the hypothesis that corals are chemically defended by their associated bacteria.

Due to the small amount of crude extract obtained, we only isolated compounds

1 and **2**. However, to learn more about the metabolites produced by this bacterium, we analyzed the crude extract obtained from medium M1 by LC-MS. Using this technique we detected the presence of other diketopiperazines including *cyclo*-(Val-Pro), *cyclo*-(Leu-4-OH-Pro), *cyclo*-(Phe-4-OH-Pro) and *cyclo*-(Trp-Pro) (Figure 3).

To explore the effect of different culture media on the production of secondary metabolites by *Pseudoalteromonas* sp., we cultured the bacterium using two different media M1 and M3. Analysis of the extracts

by APCI-MS indicated that *Pseudoalteromonas* sp. produced a chemical profile quite similar using both M1 and M3 medium. (Figures 3-4).

characterization of the additional diketopiperazine's detected in the *Pseudoalteromonas* sp. extract is required to confirm if they also possess antibacterial activity.

CONCLUSIONS

The bacterium *Pseudoalteromonas* sp. associated with the octocoral *L. alba* showed antagonistic activity against *Vibrio* sp. and *B. subtilis*. Bioassay guided fractionation of the crude extract resulted in the isolation of two diketopiperazines **1-2**. Compounds **1-2** were responsible for the antibacterial activity presented by *Pseudoalteromonas* sp., and they are not selective for Gram positive or Gram negative bacteria. Analysis of the crude extract by liquid chromatography coupled to a mass spectrometer revealed the presence of more compounds of the diketopiperazine series. Isolation and

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