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ARTICLE

Compounds isolated from *Salinispora arenicola* of the Gulf of California, México

Compuestos aislados de *Salinispora arenicola* del Golfo de California, México

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Resumen.- La actinobacteria marina AMS370, identificada mediante comparación de las secuencias del gen 16S del ARNr como *Salinispora arenicola*, fue aislada a partir de sedimento del Golfo de California, México. De su extracto semi-polar, se aislaron 8 compuestos conocidos: ácido-4-Hidroxi-fenil acético (1), 5-Metil-2-metilen-2,3-dihidro-1H-pirimidin-4-ona (2), 1H-Pirimidin-2,4-diona (3), ácido-3-amino-5-hidroxi-benzoico (4), 2-(4-Amino-imidazol-[4,5-d]piridazin-1-il)-5-hidroximetil-4-metil-tetrahidro-furan-3-ol (5), Acrilato de 3-(4-Hidroxi-genil)-metilo (6), 3-Bencil-6-isobutil-piperazin-2,5-diona (7) y 5,8-epidioxi-(22E,24R)-ergosta-6,22-dien-3-ol (Peróxido de Ergosterol) (8). La identificación química fue realizada mediante comparación de sus espectros de RMN con los espectros de los compuestos originales. Ésta sería la primera ocasión que el compuesto 4 se reporta como un producto natural a partir del género *Salinispora*. El extracto crudo y todas sus fracciones fueron ensayadas contra *Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Salmonella* sp. y *Candida albicans* para probar su actividad antibiótica y antifúngica en el caso de la última especie. Asimismo se evaluó su actividad citotóxica frente a las líneas celulares de cáncer de mama (MCF-7), cervicouterino (HeLa) y colorectal (HCT-116). Solamente el extracto crudo semipolar y las fracciones 5.1 y 5.2 resultaron activas contra *Klebsiella pneumoniae* y *Staphylococcus aureus.*

Palabras clave: Actinobacteria marina, actividad antibiótica, actividad citotóxica, Golfo de California

Abstract.- The marine actinobacterium AMS370, identified as *Salinispora arenicola* by 16S rRNA amplified gene comparison, was isolated from sediments of the Gulf of California, Mexico. From its semi-polar extract, 8 known compounds were isolated: 4-Hydroxy-phenyl acetic acid (1), 5-Methyl-2-methylene-2,3-dihydro-1H-pyrimidin-4-one (2), 1H-Pyrimidine-2,4-dione (3), 3-amino-5-hydroxy-benzoic acid (4), 2-(4-Amino-imidazol-[4,5-d]pyridazin-1-yl)-5-hydroxymethyl-4-methyl-tetrahydro-furan-3-ol (5), 3-(4-Hydroxy-phenyl)-acrylic acid methyl ester (6), 3-Benzyl-6-isobutyl-piperazine-2,5-dione (7) and 5,8-epidioxy-(22E,24R)-ergosta-6,22-dien-3-ol (Ergosterol peroxide) (8). The chemical identification was performed by comparison of their NMR spectra against the NMR spectra of the original compounds. This is the first time that compound 4 is reported as a natural product obtained from the *Salinispora* genus. The crude extract and all the fractions obtained were tested for antibiotic activity against *Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Salmonella* sp. and antifungic activity against *Candida albicans.* Also, breast cancer (MCF-7), cervical cancer (HeLa) and colorectal cancer cell lines (HCT-116) were tested to determine their cytotoxic activity. Only the semi-polar crude extract and its fractions 5.1 and 5.2 were active against *Klebsiella pneumoniae* and *Staphylococcus aureus.*

Key words: Marine actinobateria, antibacterial activity, cytotoxic activity, Gulf of California

INTRODUCTION

The order Actinomycetales harbor Gram positive bacteria that belong to the Actinobacteria phylum. This order represents the most important prokaryote organisms known due to the great diversity of bioactive compounds produced, among which we

can find antibiotics, antitumor and immunosuppressive agents. Their DNA base composition is characterized by a high percentage of guanine + cytosine (G+C) (Fenical & Jensen 2006, Bull & Stach 2007, Olano *et al*. 2009), higher than any other bacteria. Members of this class are known as actinomycetes or actinobacteria (Jensen & Lauro 2008) and include the *Streptomyces* genus, which is the main source of antibiotics discovered to date (Bérdy 2005). Currently, a large number of institutions around the world have developed research programs with the sole purpose of discovering new metabolites with pharmaceutical applications (Ellaiah *et al*. 2004) for its use in the treatment of human illnesses, as well as those with biotechnological applications (Jemimah *et al*. 2011). These studies have provided new knowledge of their taxonomy and description of new taxa, some of them without terrestrial counterparts (Olano *et al*. 2009) and many with salt requirements such as *Salinispora* (Feling *et al*. 2003, Maldonado *et al*. 2005, Becerril-Espinosa *et al*. 2013, Ng *et al*. 2014), *Serinicoccus* (Yi *et al.* 2004), *Salinibacterium* (Han *et al*. 2005) and *Sciscionella* (Tian *et al*. 2009).

The *Salinispora* genus is one of the most prolific and widely distributed in tropical and subtropical marine sediments both in the Pacific and the Atlantic Ocean (Mincer *et al*. 2002, Maldonado *et al*. 2005, Mincer *et al*. 2005, Jensen & Mafnas 2006). There are 3 species formally described, *S. arenicola*, *S. tropica* (Maldonado *et al*. 2005) and *S. pacifica* (Ahmed *et al*. 2013) from which a large number of compounds with cytotoxic activity against colorectal carcinoma cells (HCT-116) have been isolated, including arenicolide, salinisporamide A, staurosporine and arenimycin (Feling *et al*. 2003, Gerner & Meyskens 2004, Williams *et al*. 2007a, b; Fenical *et al*. 2009, Asolkar *et al*. 2010).

Salinisporamide A, is currently in clinical trials as one of the most potent anticancer agent isolated until today (Fenical *et al.* 2009). Metabolomics approach has been used to characterize the variation in secondary metabolites production when the cultures are subjected to different conditions. In the obligate marine *Salinispora* genus from The Great Barrier Reef (GBR) sponges were monitored by LC-MS analysis (Bose *et al*. 2014, 2015). Although marine Actinobacteria have been recognized as a major source of drugs, little is known about their diversity and ecological distribution in the Mexican territory, which has a coastal and ocean area comprising about 1,378,620 km^2 representing more than 50% of the land territory (De la Lanza 2004). This provides a great opportunity to learn more about its microbial diversity and potential applications in human disease treatments. Here, we carried out the isolation, purification and structural elucidation of the compounds found in the AMS370 strain obtained from sediments of the Gulf of California, as well as the molecular identification of the *Salinispora arenicola* strain.

Materials and methods

Sample collection

An extensive sampling along the Gulf of California was made in November 2007 and April 2008. 126 samples of sediment were collected at depths between 0 and 300 m, in 7 different areas located in the western part of the Gulf of California, from which, 1497 strains with actinomycete morphology were isolated (Becerril-Espinosa *et al.* 2013). The maximum strain recovery was achieved near Danzante Island and in the central part of the Gulf, specifically in Los Angeles Bay and Concepcion Bay. From all the obtained strains, AMS370 isolated from Los Angeles Bay, showed strictly marine characteristics, that is, it required salt to grow. Torres-Beltrán *et al.* (2012) concluded this after growing the bacteria in a culture medium containing deionized water and no growth was observed over a period of time. It also showed antibiotic activity (Torres-Beltrán *et al*. 2012) for which it was selected for this study.

Actinomycete isolation

The strain, cryopreserved in 30% glycerol at -70°C, was activated in Petri dishes in A1 medium (1.8% agar, 0.1% starch, 0.02% bactopeptone, and yeast extract 0.04% in sea water) supplemented with 10 mg l^{-1} of cycloheximide as an antifungal agent, and rifampicin (0.5 mg l^{-1}) as an antibiotic and incubated at 28°C under aerobic conditions for 3-4 weeks (Torres-Beltrán *et al.* 2012, Becerril-Espinosa *et al.* 2013).

The spores were inoculated into10 ml of liquid A1 medium for 7 days with continuous shaking at 215 rpm and 28°C. After the incubation period, the culture was transferred to 100 ml A1 liquid medium, and allowed time for optimal growth. Subsequently this volume was transferred to 1l of the same medium for a week to achieve maximum optical density.

Molecular identification

Pure bacterial strains were cultured in 25 ml of medium A1 and shaken at 215 rpm and 28°C for 7 days. Genomic DNA was extracted using Gontang *et al.* (2007) method. 16rRNA genes were PCR amplified using the primers FC27 (50-30AGAGTTTGATCCTGGCTCAG) and RC1492 (50-30TACGGCTACCTTGTTACGACTT) and initial naturalization at 95°C for 15 min followed by 72°C for 7 min. PCR products were purified with a Qiagen QIAquik PCR cleanup kit using protocols by the manufacturer (Qiagen Inc. Chatsworth, CA.). Sequence was obtained using the primers listed above at

SeqXcel, Inc. (http://www.seq_xcel.com/) using the BigDye terminator Cycle Sequencing Chemistry 3.1 technique and a Genetic Analyzer ABI Prism 3100 (Applied Biosystem). The forward and reverse 16SrRNA sequence obtained from this strain was assembled and analyzed using BLAST (Basic Local Alignment Search Tool) (Altschul *et al*. 1990) available on the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using CrustalX and imported into the Bioedit program (Hall & Brown 2001). The NCBI and GenBank database search using BLAST algorithm for highly similar sequences showed a 100% sequence identity to *Salinispora arenicola* 'A' [strain AMS300, Accession number (gb/HQ873946)].

Preparation of extracts

Crude extracts were obtained after the fermentation period ended; the broth was separated from the cell mass by filtration in order to process both samples separately. Broth was extracted with ethyl acetate (EtOAc) and concentrated by reduced pressure distillation (semi-polar extract). Amberlite XAD7HP (20 g l^{-1}) was added to the cell mass and stirred for 3h, then the resin was extracted with methanol (MeOH) (40 g l^{-1}) and stirred for 2 h, then filtered and concentrated (polar extract). Both extracts were subjected to analysis of cytotoxic, antibiotic and antifungal activity.

Separation of the semi-polar fraction (EtOAC)

The crude extract of the semi-polar fraction (783.3 mg) was fractionated by reverse phase chromatography using a 2.5 cm inner diameter column with silica gel 100C-18 (Fluka, 60758) to a height of 10.0 cm (Blunt *et al*. 1987) and H_2O 100%, mixtures of H_2O-MeOH (3:1, 3:2, 2:3 and 1:4), MeOH (Anhydrous, Sigma-Aldrich) followed by EtOAc (Anhydrous, Sigma-Aldrich) and CH_2Cl_2 (Anhydrous, Sigma-Aldrich) to 100% each were used as mobile phase, giving a total of 8 fractions. The course of the chromatography was monitored by thin layer chromatography analysis (TLC) and protonic nuclear magnetic resonance (^{1}HNMR) as well as by antibiotic activity assays.

TLC were performed over silica gel 60F_{254} aluminum base preparative plates (20 x 20 cm) (Merck, 105554), and developed with fluorescence detection in an Ultra Violet (UV) lamp LourmatVilberta at a wavelength of 254 nm, oleum [H_2SO_4:H_2O:AcOH (1:4:20)] and phosphomolybdic acid reagent [10 g of $H_3[P(Mo_3O_{10})_4]$, 4 g of $Ce(SO_4)_2 \cdot 4H_2O$, 2 ml of H_2SO_4 and 470 ml H_2O] spray solution followed by heating.

According to the ^{1}HNMR spectra and/or bioactivity, the selected fractions to undergo further analysis were fractions 2, 4, 5, and 6. These fractions were processed as follows:

Fraction 2 (H_2O-MeOH 3:1)

This fraction (119.5 mg) was purified by gel filtration chromatography on Sephadex LH-20 (intϕ= 3.5 cm; HGEL= 22.0 cm) eluted with 100% MeOH and yielded 60 subfractions.

Fraction 4 (H_2O-MeOH 2:3)

Sephadex LH-20 (intϕ= 3.5 cm; HGEL= 22.0 cm) was used to purify fraction 4 (71.6 mg). This chromatography was eluted with a mixture of CH_2Cl_2-Hex-MeOH (3:1:1) to yield 2 subfractions [4.1 (14 mg) and 4.2 (13.1 mg)].

Subfraction 4.1 was re-chromatographed using identical conditions that in the previous step eluting with 100% methanol.

Subfraction 4.2 was purified by HPLC [Supelco Analytical If Ascentis (25 cm x 10 mm)], gradient Hex-EtOAc 10:90 to 100% EtOAc, and flow rate: 3 ml min^{-1}.

Fraction 5 (H_2O-MeOH 1:4)

For fraction 5 (46.5 mg), biological activity guided the purification process. The sample was chromatographed on a Sephadex LH-20 column (finϕ= 3.5 cm; HGEL= 22.0 cm) eluted with a mixture of Hex-CH_2Cl_2-MeOH (3:1:1) to yield 10 fractions, 2 of them were purified as follow:

Subfractions 5.1 and 5.3 were subjected to purification by HPLC. For this technique an Agilent1200 chromatograph Quaternary LC Series system was used, equipped with 2 detection systems: UV diode array detector (DAD, G1315D) and a refractive index detector (RIDG1362A). JAIGELSil-AMS-043-10 (25 cm x 20 mm, 10 μm) and Ascentis (25 cm x 10 mm columns, 5 μm) were used.

Fraction 6 (MeOH 100%)

Fraction 6 (46.5 mg) was purified by Sephadex LH-20 gel filtration chromatography (finϕ= 3.5 cm; HGEL= 22.0 cm) eluted with a mixture of Hex-CH_2Cl_2-MeOH (3:1:1).

Infrared spectroscopy (IR)

Infrared spectra were performed as film on a Perkin-Elmer FTIR spectrophotometer, model 1600.

Mass spectrometry (MS)

For low resolution mass spectra (MS) and high resolution mass spectra (HRMS) a Vg-Micromass model Zab 2F spectrometer (Scientific Instrument Services: SIS), with ionization source electron impact (ionization energy EI, 70 eV and temperature source 220°C) and a Micromass model LCT Premier XE spectrometer, with electrospray (ESI) ionization source of positive and negative mode were used.

Nuclear magnetic resonance (NMR)

NMR spectra were performed on Bruker Avance 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C), Bruker AMX 500 (500 MHz for $^{1}HNMR$ and 125.7 MHz for $^{13}CNMR$) and Bruker Avance III 600, equipped with a 5 mm cryoprobe TCI reverse detection (600 MHz for $^{1}HNMR$ and 150.0 MHz for $^{13}CNMR$). All the 2D experiments ^{1}H-^{1}H-COSY and ^{13}C-^{1}H-HMBC were run with the same equipment.

For data acquisition, deuterochloroform ($CDCl_3$) was used as solvent and chloroform as internal reference standard unless otherwise indicated (7.25 ppm δ H; 77.0 ppm δ C). Chemical shift values (δ) are expressed in parts per million (ppm) in relation to the solvent used as internal reference, and coupling constants (*J*) in Hertz (Hz). The spectroscopic information was processed using SpecManager program (version 6.0), ACD /Labs: Copyright 1997-2002.

Assessment of antibiotic activity

The following panel of strains from the Spanish Collection [CECT(Valencia, Spain)] or American Type Culture [ATCC(USA)] were used for this bioassay: *Bacillus cereus* (ATCC 21772, Gram+), *Escherichia coli* (ATCC 9637, Gram-), *Enterococcus faecalis* (ATCC 29212, Gram+), *Klebsiella pneumoniae* (ATCC 23357, Gram-), *Proteus mirabilis* (CECT 170, Gram-), *Staphylococcus aureus* (ATCC 6538, Gram+), *Salmonella* sp. (CECT 456, Gram-) and the yeast *Candida albicans* (ATCC MYA-2876). These strains were chosen for their chemical and physical characteristics commonly found in aerobic microorganisms.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the broth dilution method (Torres-Beltrán *et al*. 2012). Two ml of tryptic soy broth medium (TSB, Fluka) and an aliquot of crude or test compound dissolved in dimethylsulfoxide (DMSO) were added to a test tube to achieve a final concentration of 50-100 μg ml^{-1}. The tubes were inoculated with 0.1 ml of an indicator organism suspension. The suspension cell density was maintained in the 1 to 5 x 10^5 viable units ml^{-1} range. After a 20-24 h period at 28°C in a shaker, the turbidity degree was determined visually and the viable cells was counted on TSA plates for those tubes that had transparency. A control growth tube was included in the assay, it lacked product but had the same proportion of DMSO. For *C. albicans* yeast YPD medium [2% (w/v) Bactopeptone, 1% (w/v) yeast extract and 2% (w/v) glucose, +/- 1.5% agar] was used.

Cytotoxic activity

The cytotoxicity activity test was performed with 3 cancer cell lines: breast cancer MCF-7 (ATCC HTB-22 ™), cervical cancer HeLa (ATCC CCL-2 ™), and colorectal cancer HCT-116 (ATCC CCL-247 ™), obtaining the average inhibitory concentration (IC_{50}) values in μg ml^{-1} after a 24 h exposition period; then, the absorbance was read at 495 nm. DMSO was used as negative control and etoposide as positive control, both in the same concentrations as the rest of the compounds used in the experiment. In this assay, crude extracts and pure compounds isolated from the active strains were evaluated.

Cell line culture

Cells lines used for this assay were stored at -70°C in Bovine Fetal Serum (FBS) 10% DMSO. The cells were thawed at a temperature of 4°C and centrifuged at 4000 rpm for 2 min, then the pellet was resuspended in 1 ml Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, antibiotic-antimycotic 1% in glucose 0.5%. The cellular pellet was resuspended in a Petri dish and slowly homogenized. Immediately after homogenization, 10 ml supplemented medium were added and incubated at 37°C in a 5% CO_2 atmosphere until a confluent monolayer growth was observed.

Cytotoxic assay

From the confluent monolayer, the cells were detached from the bottle by trypsinization. Initially they were washed twice with the monolayer of cells, one with 1% fetal bovine serum (FCS) and the other with 1% ethylenediaminetetraacetic acid (EDTA), then subsequently 1 ml of 1% trypsin was added. The mixture was allowed to act for about 1 minute in order to detach the cells that were attached to the support. Cells were deposited in 96-well plates at a density of 5 x 10^3 cells, and a total volume of 200 μl in each well. Plates were incubated for 24 h at 37°C and a 5% CO_2 atmosphere. Once the incubation time had elapsed, 20 μl of each test sample (10 mg ml^{-1}) was placed in 200 μl of supplemented media in a separate plate. Finally, the cell medium was removed and replaced with 200 μl of each sample (sample mixture with supplemented medium), and incubated for 24 h. Each extract or compound as well as the controls and targets were run in triplicate. The positive control contained 10% DMSO while the negative control RPMI in sterile water. After a 24 h incubation period, the samples were observed with an inverted 10 or 20x magnification microscope. Subsequently, 20 μl of Cell Titer solution was added and incubated for 1 to 2.5 h at 37°C, the optical density of the wells was determined with an ELISA reader at 495 nm.

Results

The AMS370 strain was collected in Los Angeles Bay, B.C. México (Becerril-Espinosa *et al.* 2013); the strain was identified as *Salinispora arenicola* by 16S rRNA amplified gene comparison with GenBank database sequences of the 100% identities to *Salinispora arenicola* 'A' [strain AMS300, Accession number (gb/ HQ873946)] sequence.

From the AMS370 strain culture, two crude extracts were obtained, the first one, semi-polar fraction, consisted of the EtOAc extraction of the liquid phase of the culture and the second one, the polar fraction, representing the extraction of the residue with methanol.

The semipolar crude extract showed some antibiotic activity (Table 1) being inactive against *Candida albicans* as well as to all the cancer cell lines assayed. Positive activity was found against Gram- bacteria *Klebsiella pneumoniae* and the Gram+ bacteria *Staphylococcus aureus* with a MIC ≤ 100 μg ml^{-1} for both microorganisms

The EtOAc extract was separated by chromatographic methods yielding 8 fractions (Fig. 1) that were monitored by ^{1}HNMR spectra and bioactivity. From the results obtained by the monitoring process, only fractions 2, 4, 5 and 6 were selected for further analysis.

Table 1. Assessment of antibiotic activity semipolar extract of the strain AMS370 / Evaluación de la actividad antibiótica del extracto semipolar de la cepa AMS370

Strain name	Minimum Inhibitory Concentration (MIC) μg ml^{-1}
	Semipolar fraction (ETOAc)
Bacillus cereus	*
Escherichia coli	*
Enterococcus faecalis	*
Klebsiella pneumoniae	≤100
Proteus mirabilis	*
Staphylococcus aureus	≤100
Salmonella sp.	*
Candida albicans	*

*= countless or MIC ≥100 μg ml^{-1}

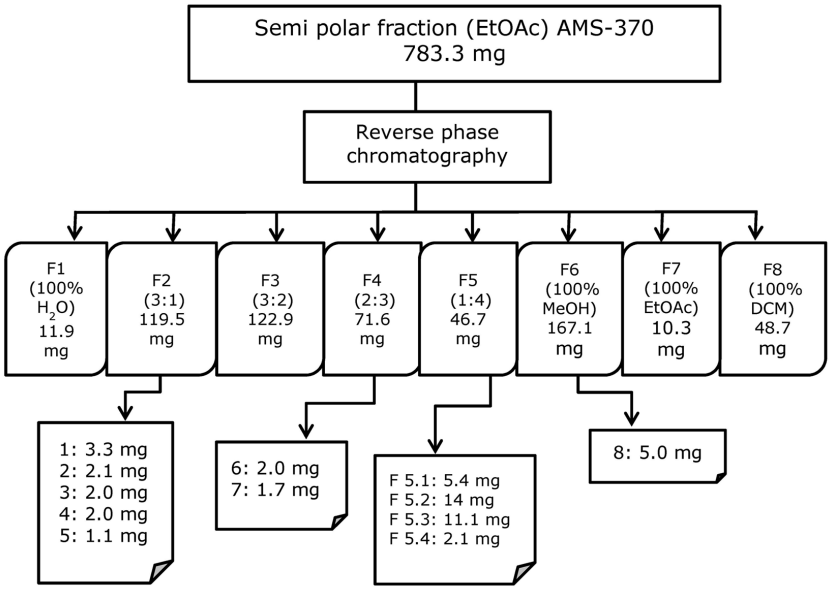


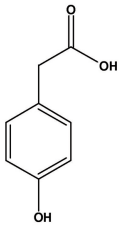
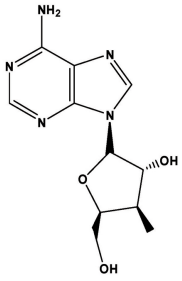
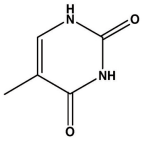
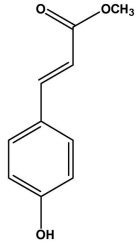
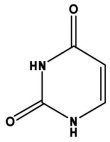
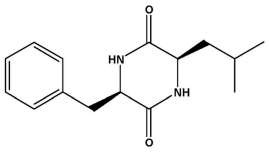
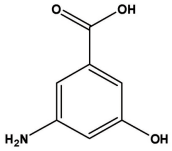
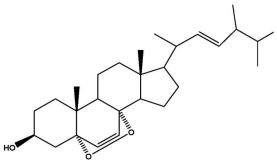
Figure 1. Chromatographic separation of the semipolar fraction (EtOAc) of the strain AMS370 from the Gulf of California, Mexico / Separación cromatográfica de la fracción semipolar (AcOEt) de la cepa AMS370 del Golfo de California, México

Purification of fraction 2 (H_2O-MeOH (3:1), 119.5 mg) yielded 60 subfractions. This process resulted in the isolation of 5 known compounds, 1 (3.3 mg), 2 (2.1 mg), 3 (2.0 mg), 4 (2.0 mg) and 5 (1.1 mg) (Table 2). By NMR data comparison, compound 1 was identified as 4-Hydroxy-phenyl acetic acid (Zhuravleva *et al*. 2011). Compound 2 resulted identical to 5-Methyl-2-methylene-2,3-dihydro-1*H*-pyrimidin-4-one, (thymine) previously reported by Ying *et al.* (2011). Compound 3 (2.0 mg) was identified as 1*H*-Pyrimidine-2, 4-dione (uracile) (Liu *et al*. 2009). Compound 4 (2.0 mg) was shown to be identical to the synthetic compound 3-Amino-5-hydroxy-benzoic acid (Wang *et al*. 2011) and obtained for the first time as a natural product in this study from the *Salinispora* genus. Compound 5 was identified as 2-(4-Amino-imidazol-[4,5-d]pyridazin-1-yl)-5-hydroxymethyl-4-methyl-tetrahydro-furan-3-ol (adenosine) (Patching *et al*. 2005) (Table 2).

Compound 4 was obtained as an amorphous yellow solid with a molecular ion at *m/z* 153.05 $[M]^+$, which satisfies the empirical formula $C_7H_7NO_3$ (HRMS 153.0427, calculated 153.0426 for $C_7H_7NO_3$), with 5 degrees of unsaturation (Table 2). Its infrared spectrum showed absorptions for hydroxyl, carbonyl and amine groups at 3345, 1690 and 1597 cm^{-1}, respectively. Its ^{13}CNMR spectrum confirmed signals for 7 carbon atoms: one carbonyl at δ 170.6 ppm, 3 sp^2 methines at δ 109.4, 107.5 and 107.4 ppm and 3 olefin quaternary carbons at δ 159.3, 150.2 and 133.7 ppm.

In the ^{1}HNMRspectrum, 3 aromatic protons were distinguished at δ 6.87 (dd, *J*= 1.6, 2.1Hz), δ 6.78 dd, *J*= 1.3-3, 2.1Hz) and δ 6.37 (t, *J*= 2.1Hz) ppm (Table 3, Fig. 2).

Table 2. Compounds isolated from the strain AMS370 of Gulf of California, Mexico / Compuestos aislados de la cepa AMS370 del Golfo de California, México

Compound	Structure	Compound	Structure
1 **4-Hydroxy-phenylacetic acid** (Zhuravleva *et al.* 2011)		**5** **2-(4-Amino-imidazol-[4,5-d]pyridazin-1-yl)-5-hydroxymethyl-4-methyl-tetrahydro-furan-3-ol** (Patching *et al.* 2005)	
2 **5-Methyl-2-methylene-2,3-dihydro-1*H*-pyrimidin-4-one** (Ying *et al.* 2011)		**6** **3-(4-Hydroxy-phenyl)-acrylic acid methyl ester** (Lee *et al*. 2013)	
3 **1*H*-Pyrimidine-2,4-dione** (Liu *et al.* 2009)		**7** **3-Benzyl-6-isobutyl-piperazine-2,5-dione** (Tullberg *et al.* 2006)	
4 **3-Amino-5-hydroxy-benzoic acid** (Wang *et al.* 2011)		**8** **5α,8α-Epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol** (Ying *et al.* 2011)	

^{13}C-^{1}HHMBC correlations of the C-7 carbonyl carbon with H-2 and H-6, as well as correlations between C-6 with proton H-2 and H-4 were observed.

Fraction 4 (H_2O-MeOH 2:3, 71.6 mg) yielded subfractions 4.1 and 4.2. TLC and ^{1}HNMR analysis of subfraction 4.1 allowed to identify compound 6 (2.0 mg) as 3-(4-Hydroxy-phenyl)-acrylic acid methyl ester (Lee *et al*. 2013) (Table 2). Subfraction 4.2 yielded compound **7** (tr= 42 min 100% EtOAc), identified as3-Benzyl-6-isobutyl-piperazine-2,5-dione (Tullberg *et al*. 2006) (Table 2).

In the case of fraction 5 (H_2O-MeOH 1:4, 46.5 mg), several sub-fractions were obtained according to TLC and ^{1}HNMR spectroscopy. They were classified as follows: 5.1 (5.4 mg), 5.2 (14 mg), 5.3 (11.1 mg), 5.4 (2.1 mg) and 5.5 (12.2 mg).

Table 3. Nuclear Magnetic Resonance data of compound 4 (3-amino-5-hydroxy benzoic acid) (CD_3OD, 400 MHz, δ ppm, (*J*) Hz) / Datos de Resonancia Magnética Nuclear del compuesto 4 (ácido-3-amino-5-hidroxibenzóico) (CD_3OD, 400 MHz, δ ppm, (*J*) Hz)

#	δ_H	δ_C	^{1}H-^{1}H COSY	^{13}C-^{1}H HMBC
1	---	133.7		
2	6.87 (1H, dd, *J* =1.5, 2.1)	109.4	H-4	H-6, H-4
3	---	150.2		H-4
4	6.37 (1H, t, *J*=2.1, 4.2)	107.5	H-6	H-6
5	---	159.3		H-4, H-6
6	6.78 (1H, dd, *J*=1.3, 2.1)	107.4		H-2, H-4
7		170.6		H-2, H-6

Subfractions 5.1 and 5.3 were subjected to HPLC. No purification process enabled us to obtain a pure compound, however, both sub-fractions obtained by HPLC were active with a MIC ≥ 50 mg ml^{-1}, but the sample amounts (mg) were insufficient to continue with the isolation process.

The purification of fraction 6 (100% MeOH, 46.5 mg) resulted in the isolation of compound 8 (5 mg), after comparison of its NMR data with those reported for the original compound it was identified as 5,8-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3-ol (ergosterol peroxide) (Ying *et al*. 2011) (Table 2).

Antibiotic activity

The crude extract showed only antibacterial activity, as well as the semi-polar extract. All the fractions selected from the semi-polar extract (2, 4, 6, and their subfractions) were inactive, only fraction 5 showed active against bacterial strains *S. aureus* and *K. pneumoniae*, with a MIC ≤ 100 μg ml^{-1} (Table 4), similar to that obtained from the crude extract. Furthermore, after purification, sub-fractions 5.1 to 5.3, showed the same or an increment of such activity (MIC ≥ 50 μgml^{-1} for 5.1 and 5.2 and ≥ 100 μgml^{-1} for 5.3 subfractions). No activity was found against the rest of the tested bacteria.

All the 8 isolated compounds were tested against the same cells lines that the original extract tested, resulting inactive with MIC ≥ 100 μgml^{-1}.

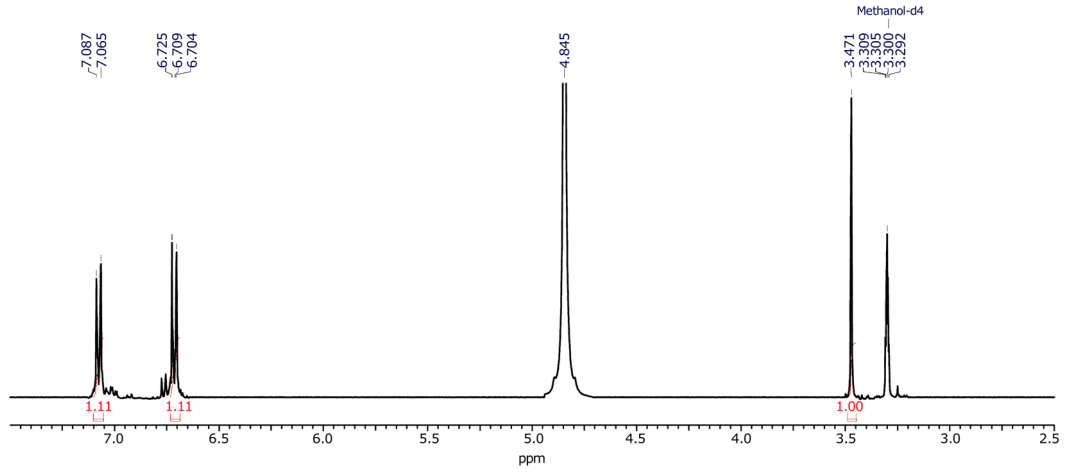


Figure 2. ^{1}HNMR spectra of the compound 4, (3-amino-5-hydroxy benzoic acid) [CD_3OD, 400 MHz, δ ppm, (*J*) Hz] / Espectro de Resonancia Magnética Nuclear protónica del compuesto 4 (ácido-3-amino-5-hidroxibenzóico) [CD_3OD, 400 MHz, δ ppm, (*J*) Hz]

Table 4. Evaluation of the minimum inhibitory concentration of F5 sub-fractions against the *S. aureus* y *K. pneumoniae* strains / Evaluación de la concentración inhibitoria mínima de las sub-fracciones F5, frente a las cepas indicadoras *S. aureus* y *K. pneumoniae*

F5 Sub-fractions	*S. aureus*	*K. pneumoniae*
5.1	≥50 μgml^{-1}	≥50 μgml^{-1}
5.2	≥50 μgml^{-1}	≥50 μgml^{-1}
5.3	≥100 μgml^{-1}	≥100 μgml^{-1}
5.4	*	*
5.5	*	*

*= count less

Discussion

The distribution of genus *Salinispora* in the Gulf of California has been reported to extend from Los Angeles Bay, Baja California to Loreto Bay, Baja California Sur. It is important to state that the AMS370 strain as well as 88% of the strains sequenced by Becerril-Espinosa *et al*. (2013) belongs to the *S. arenicola* species.

The fractions 2, 4, 5 and 6 obtained from the semi-polar phase of the original extract were purified. From fraction 2 compounds 1, 2, 3, 4 and 5 were obtained and identified as 4-Hydroxy-phenyl acetic acid (Zhuravleva *et al.* 2011), 5-Methyl-2-methylene-2,3-dihydro-1*H*-pyrimidin-4-one (Ying *et al*. 2011), 1*H*-Pyrimidine-2, 4-dione (Liu *et al*. 2009), 3-Amino-5-hydroxy-benzoic acid (Wang *et al*. 2011) and 2-(4-Amino-imidazol-[4,5-d]pyridazin-1-yl)-5-hydroxymethyl-4-methyl-tetrahydro-furan-3-ol (Patching *et al.* 2005), respectively. Even though compound 4 (3-Amino-5-hydroxy-benzoic acid) has been obtained previously as an intermediate product in the Ferrimycin A synthesis (Bickel *et al.* 1966), this is the first time it is found as a natural product in the *Salinispora* genus.

Despite the inactivity revealed for compound 1 in this work, Aissa *et al*. (2012) found antimicrobial activity against the Gram+ bacteria *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus xylosus* and Gram- *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae* and *Pseudomona aeruginosa* as well as an antioxidant capacity equivalent to 0.87 ± 0.0015 TEAC mM Trolox l^{-1} (ABTS assay) for the same compound.

Yan *et al*. (2012) reported a low cytotoxic activity of the compounds 2 and 3 against SF-268, NCl-H460, K-562, SMMC-7721 y SGC-7901 cancer cell lines (IC_{50} >100 μg ml^{-1}).

Compound 5 was reported to possess activity as antithrombotic, neurotransmission inhibitor and vasodilator; also, several analogs of this compound showed anticancer, antifungal and antiviral activity. It has been reported that the 5'-hydroxyl group in the molecule can enter nucleoside metabolic pathways (Moukha-chafiq & Reynolds 2013).

Compound 6 has been reported with weak antibiotic activity with inhibition diameters between 8.0-11.5 mm at 10 mg ml^{-1} (disc diffusion method) against *E. coli, P. aeroginosa, B. subtilis* and *S. aureus* (Venkateswarlu *et al.* 2006).

The structure elucidation of compound 4 was based on the main characteristics of the ^{13}CNMR data obtained. The existence of one carbonyl group and 6 sp^2 carbons, and the need to meet the 5 degrees of unsaturation indicated by its empirical formula ($C_7H_7NO_3$), suggests that 4 must possess a single ring that should be a tri substituted aromatic ring.

^{1}HNMR and two dimensional experiments ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC confirmed compound 4 structures (Table 2). Long-range COSY correlations between H-2 (*J*= 1.5Hz) and H-6, and between H-4 and H-6 (*J*= 2.1Hz) indicated that protons H-2, H-4 and H-6 are all *meta* positioned.

^{1}H-^{13}C HMBC correlations of carbonyl C-7 with H-2 and H-6 established the position of the carboxylic group on C-1. C-3 showed δ150.2 ppm and C-5 at δ159.3 ppm indicating that the amine must be at C-3 and the hydroxyl group at C-5. These data allowed confirmation of the chemical structure of compound 4 as 3-amino-5-hydroxy-benzoic acid. In this paper was described, for the first time, the isolation of this acid as a natural product in the *Salinispora* genus, which was previously obtained by synthetic pathways (Wang *et al*. 2011) and was claimed for the first time as a natural product, inferring the result by the reduction in the ^{13}C content of the carboxyl group from 85 to 52 atom % excess over natural abundance during the fermentation experiment with unlabeled material produced by *S. verticillatus* (Kibby & Rickards 1981). However, never until this investigation, was isolated as a natural product *per se*.

Ergosterol peroxide (8) is other interesting compound obtained in this work. It has been reported and isolated from a number of organisms, including bacteria. According to published results (Ying *et al*. 2011), 8 presents mild cytotoxic activity against the SNU-1 cell line (human gastric tumor), SNU-C4, SNU-354 and Sarcoma-180 (Kyong *et al*. 2001). In addition, activities such as anti-inflammatory (Lishuai *et al*. 2013), immunosuppressive (Fujimoto *et al*. 1994) and anti-atherosclerosis (Kim *et al*. 2005) have been reported.

However we did not find any cytotoxic, anti-fungicidal or antibacterial activity for this compound.

In conclusion we can say that AMS370 strain was identified as *Salinispora arenicola* 'A' on the basis of its 16S rRNA sequence analysis. Chromatographic purification and structural determination of 8 known compounds, one of them, 3-amino-5-hydroxybenzoic acid, reported for the first time as a natural product in *Salinispora* genus, were made. Fraction 5 was responsible for the bioactivity of the crude extract, with antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* strains (MIC_{50} ≤100 μg ml^{-1}). Actinobacteria from marine sediments such as the ones found in the Gulf of California with the capability to biosynthesize bioactive compounds may have a great impact in human health since they are a potential source of compounds with very promising antibacterial activity.

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