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Production and purification of a bioactive substance against multi-drug resistant human pathogens from the marine-sponge-derived *Salinispora* sp.

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PEER REVIEW

Peer reviewer
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Comments
This is a good research paper in which the authors obtained rifamycin W (compound 1) from the EtOAc extract of the culture of *Salinispora* sp. FS-0034 and found that it had potent antibacterial activity. The results are meaningful and valuable and lay a solid foundation for further studies.
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ABSTRACT

Objective: To isolate, purify, characterize, elucidate structure and evaluate bioactive compounds from the sponge-derived *Salinispora* sp. FS-0034.
Methods: The symbiotic actinomycete strain FS-0034 with an interesting bioactivity profile was isolated from the Fijian marine sponge *Theonella* sp. Based on colony morphology and obligatory requirement of seawater for growth, and mycelia morphological characteristics the isolate FS-0034 was identified as a *Salinispora* sp. The bioactive compound was identified by using various spectral analysis of ultraviolet, high resolution electrospray ionization mass spectroscopy, ^{1}H nuclear magnetic resonance, correlated spectroscopy and heteronuclear multiple bond coherence spectral data. A minimum inhibitory concentration assay were performed to evaluate the biological properties of the pure compound against multi-drug resistant pathogens.
Results: Bioassay guided fractionation of the ethyl acetate extract of the culture of *Salinispora* sp. FS-0034 by different chromatographic methods yielded the isolation of an antibacterial compound, which was identified as rifamycin W (compound 1). Rifamycin W was reported for its potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*, wild type *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* and displayed minimum inhibitory concentrations of 15.62, 7.80 and 250.00 μg/mL, respectively.
Conclusions: The present study reported the rifamycin W from sponge-associated *Salinispora* sp. and it exhibited appreciable antibacterial activity against multi-drug resistant human pathogens which indicated that sponge-associated Actinobacteria are significant sources of bioactive metabolites.

KEYWORDS
Marine actinomycetes, Sponge-derived, *Salinispora*, Multi-drug resistant, Antibacterial, Rifamycin

1. Introduction

Infectious diseases have always been serious health problems with high morbidity and mortality in humans. Even though pharmaceutical companies have produced a number of new antibiotics in the past decade, resistance to these drugs has increased and has now become a global concern[1]. The global emergence of multi-drug resistant bacteria is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure[2]. *Staphylococcus aureus* (*S. aureus*) is one of the most important human pathogens associated with hospital and community-acquired infections. Over the decades, the number and proportion of drug resistant pathogens, including methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococci*, cephalosporin-resistant *Klebsiella pneumonia*, fluoroquinolone-resistant *Pseudomonas*

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aeruginosa, multi-drug resistant Gram-negative bacteria and extensively drug resistant tuberculosis infections in different countries have increased due to the rise of epidemics in humans[3-5]. Consequently, new and innovative antimicrobial agents are urgently needed to combat these life threatening infections.

New trends in drug discovery from natural sources emphasize investigation on the marine ecosystem to explore numerous complex and novel chemical entities[6]. It is noteworthy that marine sources have also demonstrated tremendous abilities as producers of anticancer compounds and bioactive secondary metabolites which act against infectious diseases and inflammation[7]. Among the marine organisms, microbial-derived natural products are playing a significant role in the drug discovery process[8]. Marine Actinobacteria represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs[9,10]. Recently, sponge-derived actinomycetes have been attracting increasingly interests as potential sources of unique and unusual bioactive secondary metabolites[11]. The isolation of secondary metabolite-producing bacteria from sponges and of microbial secondary metabolism gene clusters from the metagenome of sponges has led to the general understanding that these metabolites are, in many cases, the products of microbial symbionts and are not derived from the microbial diet of sponges[12]. In continuation of our studies on sponge-derived actinomycetes we now report on the bioactive compound against multi-drug resistant pathogens produced by *Salinispora* sp. isolated from the marine sponge *Theonella* sp. The goal of the present study is to isolate a bioactive compound showing antibacterial activities from the strain of *Salinispora* sp. FS-0034, which was isolated from the Fijian marine invertebrate.

2. Materials and methods

2.1. General experimental procedures

The ultraviolet (UV) spectrum was acquired in spectroscopy grade methanol using a PerkinElmer Lambda 35 spectrophotometer. Nuclear magnetic resonance (NMR) experiments were performed on Varian Inova spectrometer 600 MHz. The chemical shifts were expressed in δ (ppm) and referred to the residual solvent (δ_H 2.50 ppm for dimethylsulfoxide-d_6). High resolution electrospray ionization mass spectra (HRESIMS) were acquired by using an Agilent 1 100 series separations module equipped with an Agilent G1969A MSD (mass spectroscopy detector) in positive ion mode. Purification was done on a Zorbax ODS 5 μm 9.4 mm×250 mm column. Analytical and semi-preparative high performance liquid chromatography (HPLC) was performed using a Waters 515 pump connected to a 2487 UV-visible detector. Thin-layer chromatography (TLC) analyses were carried out by using glass plate pre-coated silica gel 60 reversed phase (RP)-18 F_{254}S (Merck, Darmstadt, Germany). Analytical grade solvents were utilized for TLC analysis. Burdick and Jackson high purity solvents were used for HPLC while Riedel-de Haen, Chromasolv liquid chromatography-mass spectrometry (LC-MS) grade solvents were used for LC-MS.

2.2. Sample collection and processing

The sponge *Theonella* sp. was collected by hand using selfcontained underwater breathing apparatus at a depth of 10 m from Cicia, Lau group, Fiji Islands (17°47′33′′ S, 179°23′94′′ W) in September 2008 during a three week biodiversity expedition in the central Lau Group. The sponge material was transferred into a sterilized bag immediately after harvesting and was transported cooled to the nearby laboratory. The isolation of bacteria was subsequently carried out. The sponge was identified by Dr. Paco Cardenas, Uppsala University, Sweden. A voucher specimen (G-0634) has been preserved at the Marine Reference Collection, The University of the South Pacific, Fiji Islands and at Georgia Institute of Technology, USA.

The sponge sample was rinsed three times with sterile seawater to eliminate nonspecific microbial propagules that stick to the sponge surface from the seawater, and the surface of the sponge tissue was subjected to surface-sterilization by using 70% ethanol under aseptic conditions. The surface sterilized sponge tissue was then cut into small pieces of approximately 0.1 cm^3, homogenized and diluted with autoclaved membrane-filtered seawater. The resulting homogenate was diluted with sterile seawater at three dilutions (1:10, 1:100, and 1:1 000). Hundred microliters of each dilution was plated onto A1 agar (10 g of starch, 4 g of yeast extract, 2 g of peptone, 18 g of agar and 1 L of filtered natural seawater) medium. The isolation medium was amended with cycloheximide (100 μg/mL) and polymixin B (5 μg/mL) after autoclaving to avoid unwanted Gram negative bacteria and fungi contamination. The plates were incubated at 28 °C for 3-4 weeks until the morphology of actinomycetes could be distinguished.

2.3. Isolation of Salinispora strains

Actinomycetes were evaluated on each plate by eye and with the aid of an Olympus SZ51 binocular zoom stereomicroscope (8-40×). Actinomycetes were recognized by the presence of filamentous hyphae, a characteristic that was just within the range of detection at the highest magnification used, and/or by the formation of tough, leathery colonies that adhered to the agar surface. Colonies were assigned to the genera *Salinispora* if, for larger colonies, they produced orange pigment, black spores that darkened the colony surface, and lacked areal hyphae. Smaller colonies, viewed microscopically, could be ascribed to the *Salinispora* group if they possessed finely branched, scattered hyphae that formed a moderately developed substrate mycelium as

described by Jensen, *et al*[13]. *Salinispora*-like colonies were successively transferred onto new media until pure cultures were obtained. All pure strains were grown in liquid culture and cryopreserved at −80 °C in 10% glycerol.

2.4. Seawater requirement test

The *Salinispora* strains were reported as the first obligatory marine actinomycetes which needed seawater for their growth[13,14]. Thus, all of the isolated *Salinispora*-like actinomycetes were tested for the requirement of seawater for growth. Fresh *Salinispora*-like actinomycetes' colonies were streaked on A1 agar plate prepared with seawater and a plate prepared with filtered distilled water. Growth was monitored on both plates visually and with the aid of a stereomicroscope for up to 4 weeks. If no growth was observed on the plate prepared with filtered distilled water, that strain was determined to require seawater for growth.

2.5. Bioassay and selection of potential strain

All the isolated *Salinispora* strains were subjected for small scale fermentation by inoculating each strain in 100 mL A1B medium (1 g of starch, 0.4 g of yeast extract, 0.2 g of peptone) incubating at 28 °C in temperature controlled incubator at 200 r/min for 7-14 d. After incubation, the whole cultures (100 mL) were extracted twice with equal volumes of ethyl acetate (EtOAc) using a separating funnel. The EtOAc crude extract was dried *in vacuo* using rotary evaporator under 35 °C. Each crude extract of the strains was tested for bioassay against methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA), wild type *S. aureus* (WTSA), vancomycin-resistant *Enterococcus faecium* (*E. faecium*) (VREF), amphotericin resistant *Candida albicans* (*C. albicans*) and wild type *C. albicans* at 250 μg/disc concentration. The highest bioactivity displayed strain was selected for mass production in order to produce enough metabolites for purification of bioactive compounds.

2.6. Extraction and isolation

Salinispora sp. FS-0034 was cultured in 24 Fernbach flasks (2.8 L) each containing 1 L of a natural seawater-based A1B medium. The whole culture broth along with cells was extracted twice with EtOAc by sonication for 20-30 min. The EtOAc phase was concentrated *in vacuo* to give a crude extract (2.77 g). The crude EtOAc extract was subjected to normal phase vacuum liquid chromatography with mixtures of direct-current main and MeOH in gradually increasing the polarity. A total of eleven fractions were obtained which were subjected to bioassay. All the fractions showed activity against MRSA, WTSA and VREF. However, fraction three (FS-0034F3) was selected for further purification based on higher bioactivity and quantity. FS-0034F3 was subjected to RP C_{18} column chromatography using mixtures of MeOH/H_2O. A total of sixteen fractions were collected and pooled to three fractions (FS-0034F3F1 to FS-0034F3F3) based on the profiles on TLC. Fraction FS-0034F3F1 was further selected for purification by RP C_{18} column chromatography based on the bioactivity. Totally, ten fractions were collected; bioactive fractions (FS-0034F3F1F6 and FS-0034F3F1F7) were further purified by RP (Zorbax ODS 5 μm 9.4 mm×250 mm column) semi-preparative Waters 515 HPLC system yielding a pure bright red powder (compound 1; 2.1 mg).

Rifamycin W (compound 1): Bright red powder, UV (MeOH) λ_{max} 230, 275, and 325 nm; molecular formula $C_{35}H_{45}NO_{11}$; HRESIMS (m/z): 656.3068 $[M+H]^+$ (calcd. for $C_{35}H_{46}NO_{11}$, 656.3065); ^1H NMR, correlated spectroscopy (COSY) and heteronuclear multiple-bond correlation (HMBC) spectroscopic data, see Table 1.

Table 1

NMR chemical shifts (600 MHz, dimethylsulfoxide-d_6) for rifamycin W.

Position	δ_H (multi, J in Hz)	COSY	HMBC[b]
NH	9.50		
H-3	7.47 s		127.6 (C10), 140.6 (C2), 182.6 (C1)
H-13	2.02 s		132.9 (C12), 169.9 (C11), 131.2 (C29)
H-14	2.14 s		116.9 (C7), 159.9 (C6), 161.5 (C8)
H-17	6.20 ovl	H18	
H-18	6.31 m	H17	
H-19	6.00 dd (15.8, 6.0)	H20	
H-20	2.22 m	H19, H21, H31	
H-21	3.99 m	H22, H20	
H-22	1.96 m	H32	
H-23	3.56 m	H22, H24	
H-24	1.51 m	H23, H33	
H-25	3.67 m	H26	
H-26	1.19 m	H34, H25	
H-27	3.18 m	H28	
H-28	3.31[a]	H27, H34a	
H-29	6.196 ovl	H28	
H-30	1.94 s		196.0 (C15), 137.6 (C17), 139.5 (C16)
H-31	0.84 d (6.86)	H20	36.4 (C20), 71.2 (C21),
H-32	0.87 d (7.04)	H22	29.5 (C22), 76.9 (C23)
H-33	0.62 d (6.80)	H24	76.9 (C23), 67.9 (C25), 37.5 (C24)
H-34	0.17 d (6.82)	H26	67.9 (C25), 65.4 (C27), 41.9 (C28), 39.7 (C26)
H-34a	2.39 m		

ovl: Overlap; [a]: Signal hidden by solvent peak in ^1H NMR (obtained from COSY); [b]: Tentative assignments.

2.7. Antibacterial assay

The minimum inhibitory concentrations (MICs) of compound 1 against different test organisms were determined by the broth dilution method[15]. The isolated compound 1 was dissolved in MeOH at 10 mg/mL and diluted further to give required concentrations (μg/mL) 500.00, 250.00, 125.00, 62.50, 31.25, 15.62, 7.80, 3.90, 1.90, 0.97, 0.48, 0.24, 0.12, 0.06, 0.03 and 0.01. The diluted solutions (100 μL) were added to separate wells on a 96 wells plate. An inoculum of 100 μL from 24 h old culture of each test human pathogens, MRSA (ATCC 10537), WTSA and VREF (ATCC 12952) were inoculated separately in each well. The antibacterial agents vancomycin (MRSA and WTSA) and rifampicin (Voltage Reference VREF) were used as positive controls and the solvent MeOH was included

in the bioassay as negative control. The cultures were incubated for 24 h at 37 °C. Replicates were performed for each treatment. The MIC was defined as the lowest concentration of the pure compound/antibiotics showing no visible growth after the incubation time.

3. Results

A total of eighteen *Salinispora* strains were isolated from the sponge tissues of *Theonella* sp. The characteristics of obligatory growth in seawater based medium and colony morphology confirmed the isolates as *Salinispora* spp. Further all the *Salinispora* strains were subjected for bioassay against drug-resistant pathogens. All the eighteen strains displayed activity against MRSA and WTSA with fourteen strains active against VREF. The strain FS-0034 exhibited highest antibacterial activity against multi-drug resistant human pathogens (Table 2).

Table 2

Screening of marine-sponge-derived *Salinispora* sp. against multi-drug resistant human pathogens (zone of inhibition in mm; 250 μg/disc).

Strains	Seawater requirement	Antibacterial			Antifungal	
		MRSA	WTSA	VREF	ARCA	WTCA
FS-0028	+	20	18	10	-	-
FS-0029	+	16	17	11	-	-
FS-0030	+	29	27	10	-	-
FS-0031	+	18	16	10	-	-
FS-0032	+	20	16	10	-	-
FS-0033	+	28	29	11	-	-
FS-0034	+	30	30	14	-	-
FS-0035	+	30	30	13	-	-
FS-0036	+	17	18	-	-	-
FS-0037	+	20	18	10	-	-
FS-0038	+	18	16	9	-	-
FS-0039	+	20	21	-	-	-
FS-0040	+	30	30	12	-	-
FS-0041	+	17	18	10	-	-
FS-0042	+	23	22	-	-	-
FS-0043	+	16	16	9	-	-
FS-0044	+	30	29	10	-	-
FS-0045	+	28	29	-	-	-
Control		19	19	22	25	25

MRSA: Methicillin-resistant *S. aureus*; WTSA: Wild type *S. aureus*; VREF: Vancomycin-resistant *Enterococcus faecium*; ARCA: Amphotericin resistant *C. albicans*; WTCA: Wild type *C. albicans*.

The *Salinispora* sp. FS-0034 was mass cultivated (24 L) in seawater based A1 medium for 14 d at 28 °C in shaking incubator at 200 r/min. After 14 days of growth in A1 broth, the total culture medium was extracted with EtOAc and concentrated *in vacuo* to give a crude extract (2.77 g). The crude extract (active against MRSA, WTSA and VREF) was fractionated by using different chromatography (Figure 1) yielded a metabolite as pure red powder (compound 1; 2.1 mg). The pure compound was further subjected to RP analytical HPLC (Zorbax ODS 5.00 μm 4.60 mm×250.00 mm) to analyze the purity and further submitted for NMR and other spectroscopic studies for structure elucidation.

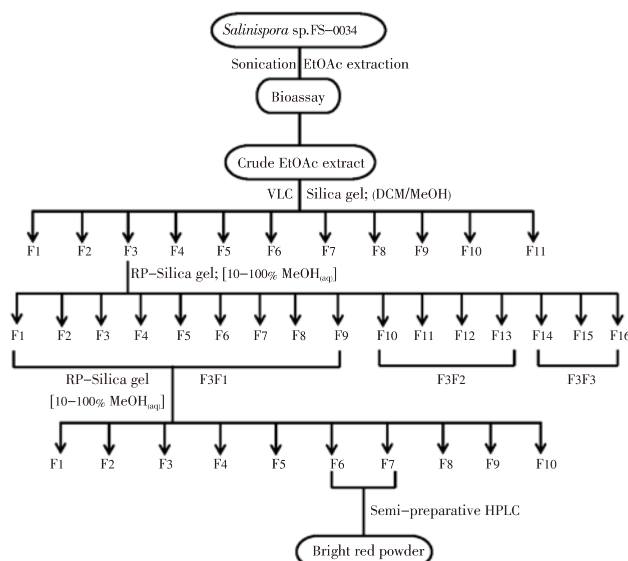

Figure 1. Schematic diagram of procedure for isolation of compound 1.

Compound 1 was obtained as bright red powder. Its molecular formula $C_{35}H_{45}NO_{11}$ was established by a pseudomolecular ion at $[M+H]^+$ m/z 656.3068 from high resolution electrospray ionization mass spectroscopy [$(M+H)^+$; calcd for $C_{35}H_{46}NO_{11}$ m/z 656.3065) (Figure 2). Compound 1 also showed characteristic UV absorption maxima at 230, 275 and 325 nm suggesting a naphthoquinone nucleus chromophore. Further, AntiBase (2012) search for the corresponding molecular mass together with the given UV maxima and the single aromatic proton from ^{1}H NMR spectrum revealed a match to the known rifamycin W. In addition, the ^{1}H NMR, COSY and HMBC experiments assisted to establish the structure of compound 1. The ^{1}H NMR spectrum of compound 1 showed two downfield singlets at δ 7.47 ppm and δ 9.50 ppm, the latter representing the amine proton while the former representing the single aromatic proton. The up-field region of the ^{1}H NMR spectrum revealed a total of seven methyl signals from which four signals were present as doublets and three as singlets. Furthermore, the methyls at H_3-31, H_3-32, H_3-33 and H_3-34 showed COSY correlations to the corresponding methines at H-20 (δ 2.22 ppm), H-22 (δ 1.96 ppm), H-24 (δ 1.51 ppm) and H-26 (δ 1.19 ppm), respectively. Moreover, the methine proton H-20 showed further COSY to the olefinic proton H-19 (δ 6.00 ppm) and methine H-21 (δ 3.99 ppm). The olefins H-17 and H-18 were overlapped in the ^{1}H NMR spectrum while olefin H-29 showed COSY to H-28 as H-28 showed COSY to H-34a. The COSY spectrum also displayed other methine-methyl and methine-methine correlations as given in Figure 3. In addition, the carbon values were tentatively assigned by comparison to the literature[16]. Supportively, HMBC correlations (Figure 4) confirm the correct assignments of the methyls and the aromatic proton, further affirming the proposed structure for compound 1 to be rifamycin W.

Rifamycin W (compound 1) showed appreciable antibacterial activity against MRSA, WTSA and VREF exhibiting MICs of 15.62, 7.80 and 250.00 μg/mL, respectively.

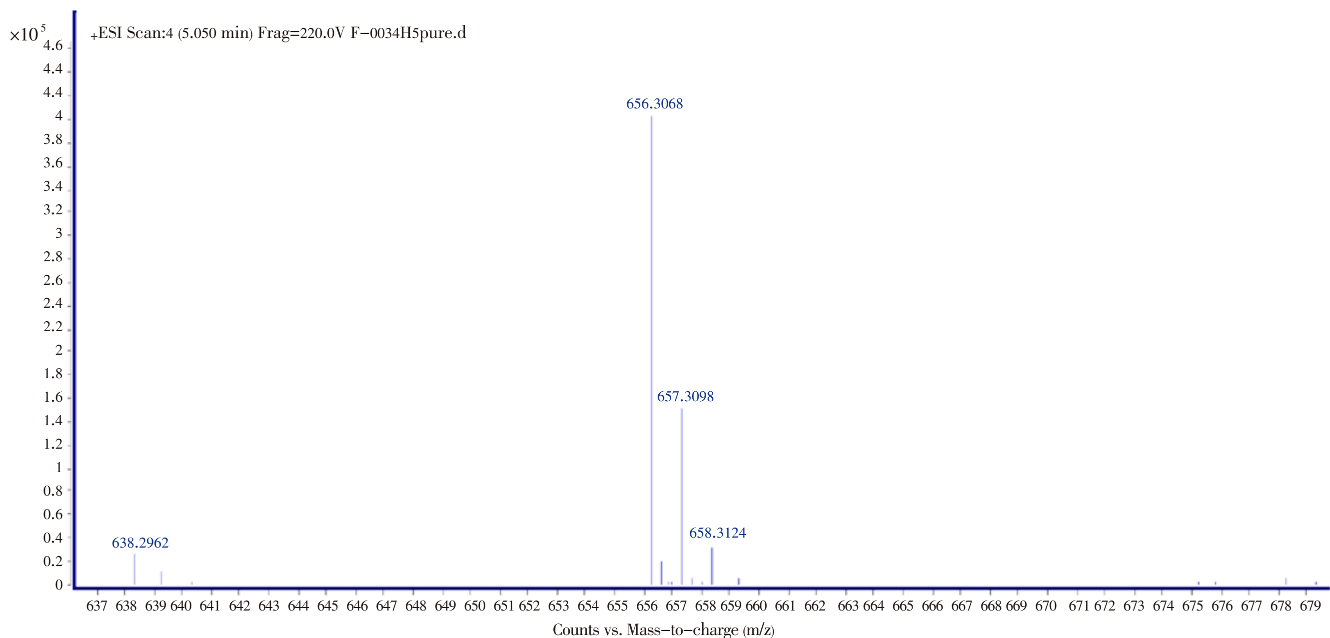

Figure 2. High resolution electron spray ionization mass spectroscopy of compound 1.

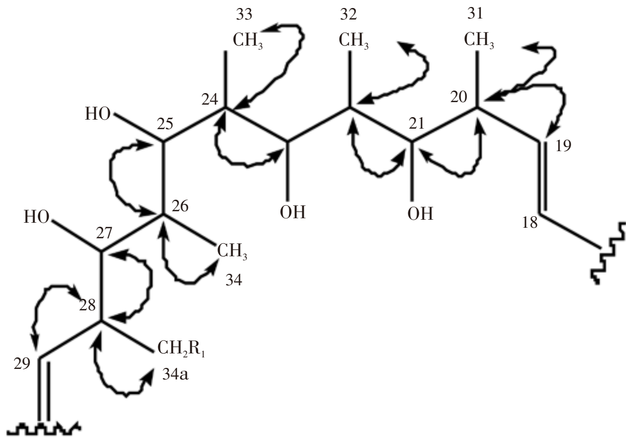

Figure 3. COSY correlations of compound 1.

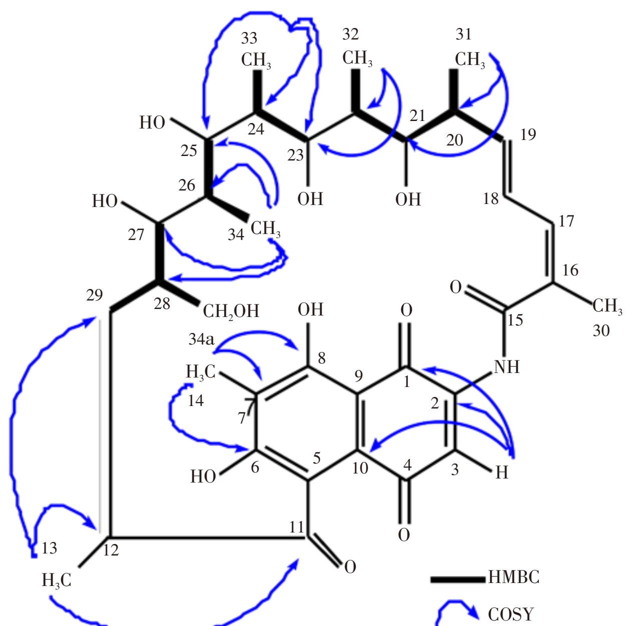

Figure 4. COSY and HMBC correlations of compound 1.

4. Discussion

Novel Actinobacteria with biopharmaceutical potential have been increasingly isolated from marine habitats[10,14,17]. The *Salinispora* group, as a relatively newly discovered group of Actinobacteria have been found in diverse habitats such as marine sediments and marine sponges[14,17,18]. The marine actinomycete genus *Salinispora* is composed of three closely related species (*Salinispora arenicola*, *Salinispora pacifica* and *Salinispora tropica*) that have been identified so far[19]. These bacteria have proven to be a rich source of secondary metabolites, which are produced in species-specific patterns that include antibacterial, potent cytostatic, anticancer, antimalarial and proteasome inhibitor activities[9,10]. In the present study, eighteen strains were isolated and confirmed as *Salinispora* strains due to its stringent requirement of seawater for growth and other morphological characteristics[13,14]. All isolated strains in the present study showed significant antibacterial activity against multi-drug resistant pathogens. Among them, a highest bioactivity showing *Salinispora* sp. FS-0034 was subjected to production and purification of bioactive compounds yielding the strong MRSA and VREF active rifamycin W.

Rifamycins, a group of antibiotics of the ansamycin family[20], are clinically important antibacterial agents active against Gram-positive bacteria. Several semi-synthetic rifamycin variants (e.g., rifampin) have been used clinically for the treatment of tuberculosis and other bacterial infections, but resistance threatens their

effectiveness[21]. Rifamycins are known to be produced by the soil actinobacterial species *Amycolatopsis mediterranei* and *Nocardia mediterranei* and rifamycin synthesis has been most intensively studied in these species[22,23]. The gene cluster for the biosynthesis of rifamycin B, a compound forming the basis for all other rifamycins, has been characterized only in *Amycolatopsis mediterranei*[22,23]. However it has recently been reported that members of marine genus *Salinispora* are known to produce compounds of the rifamycin class, including rifamycin B and rifamycin SV[18,24]. In the present study we reported rifamycin W from the *Salinispora* sp. FS-0034. Rifamycin W was previously isolated from the fermentation medium of the mutant strain *Nocardia mediterranei*[16]. Therefore, the marine obligate *Salinispora* group of actinobacteria represents a potential new source of rifamycins outside the soil genera *Amycolatopsis* and *Nocardia*. Further, the present study has added strength to the biological properties of rifamycin W as it was showed strong antibacterial activity against multi-drug resistant human pathogens.

Herein we report the isolation of rifamycin W from a sponge-associated *Salinispora* sp. and describe its strong antibacterial activity against multi-drug resistant human pathogens including MRSA, WTSA and VREF. The consistent production of specific classes of secondary metabolites by *Salinispora* species will raise the expectancy in this group to discover novel drug leads.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The increasing emergence of multi-drug resistant human pathogens is seriously limiting the effectiveness of currently available drugs and significantly causing treatment failure. The ocean is an important source of bioactive substances and numerous antibiotics derived from the marine play a significant role in the treatment of many diseases. The search for new and innovative antimicrobial agents has become an urgent issue facing humanity.

Research frontiers

In the present study, the authors isolated a bioactive compound, rifamycin W, from sponge-associated *Salinispora* sp. using various spectrum analysis methods and examined its appreciable antibacterial activity against multi-drug resistant human pathogens by MIC assays.

Related reports

This study also provided the specific information about the rifamycin W, including the molecular formula and the molecular structure using various spectral analysis of UV, HRESIMS, ^{1}H NMR, COSY and HMBC spectral data.

Innovations and breakthroughs

Recently members of marine genus *Salinispora* are reported to produce compounds of the rifamycin class, including rifamycin B and rifamycin SV. In the present study, the authors isolated rifamycin W from the *Salinispora* sp. with strong antibacterial activity, showing that the marine obligate *Salinispora* group of actinobacteria can represent a potential new source of rifamycins.

Applications

The research work illustrated the strong antibacterial activity of rifamycin W (compound 1) against multi-drug resistant human pathogens, indicating that secondary metabolites from *Salinispora* species have potential to be

developed as novel antibacterial agents.

Peer review

This is a good research paper in which the authors obtained rifamycin W (compound 1) from the EtOAc extract of the culture of *Salinispora* sp. FS-0034 and found that it had potent antibacterial activity. The results are meaningful and valuable and lay a solid foundation for further studies.

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