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Isolation and characterisation of Rifamycin W and Phenylethylamides from a Fijian Marine Actinomycete *Salinispora arenicola*

Kavita Ragini and William Aalbersberg

Institute of Applied Sciences, Faculty of Science, Technology and Environment, The University of the South Pacific, Laucala Campus, Suva, Fiji Islands

Abstract

An ansamycin, rifamycin W, and three phenylethylamides, N-(2'-phenylethyl)isobutyramide, 2-methyl-N-(2'-phenylethyl)butyramide, and N-(2'-phenylethyl)isovaleramide were isolated from the fermentation broth of a marine actinomycete strain identified as Salinispora arenicola. *The structures of these compounds were confirmed by detailed interpretation of NMR spectroscopic and high resolution ESILC-MS data. Moderate antibacterial activity was observed for rifamycin W only.*
***Keywords**: Rifamycin, Phenylethylamides,* Salinispora arenicola

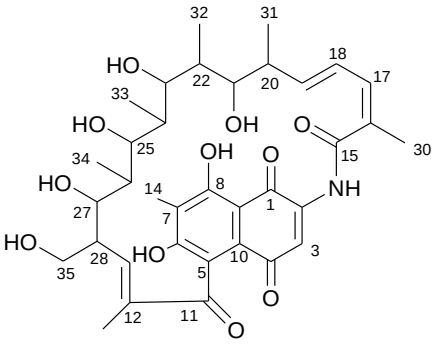
1. Introduction

Actinomycetes are the most significant source of microbial natural products ever discovered, accounting for more than half of all the known antibiotics (Moore *et al*. 1999). Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances (Jensen *et al*. 1991). Examples include antibiotic (erythromycin and tetracycline), anticancer (mitomycin and daunomycin), immunosuppressant (rapamycin and FK506) and veterinary agents (thiostrepton and monensin) (Moore *et al*., 1999; Fenical and Jensen, 2008). The obligate marine actinomycetes of the genus *Salinispora* possess antibiotic and anticancer activities from a high percentage of the organic extracts of cultured strains, which suggests that these bacteria are an excellent resource for drug discovery (Fenical and Jensen, 2006). More than 80% of the strains inhibit human tumour cell growth and 35% show antibacterial properties toward drug-resistant human pathogens (Fenical and Jensen, 2006). *S. arenicola* strains produce compounds in the well-studied rifamycin (antibiotic) and staurosporine (protein kinase inhibitor) classes, as well as the new bicyclic compound saliniketal which shares some biosynthetic features with rifamycin (Jensen *et al*. 2007).

As part of an investigation into actinomycete diversity in marine sediments around the Fiji Islands, we isolated rifamycin W together with the three amides: *N*-(2'-phenylethyl)isobutyramide, 2-methyl-*N*-(2'-phenylethyl)butyramide, and *N*-(2'-phenylethyl)isovaleramide from *Salinispora arenicola*. Rifamycin W was originally isolated by White *et al*. in 1974 from a mutant strain of *N. mediterranei* as a pure crystalline yellow solid while the amides were first isolated from cultures of limnic bacterial strains within the genus *Bacillus* by Maskey *et al*. in 2002. However, this is the first report of these known metabolites from an obligate marine bacterium, *S. arenicola* and also the first production of rifamycin W from a *S. arenicola* strain. Herein we report the isolation, structural elucidation and bioactivity of these metabolites.

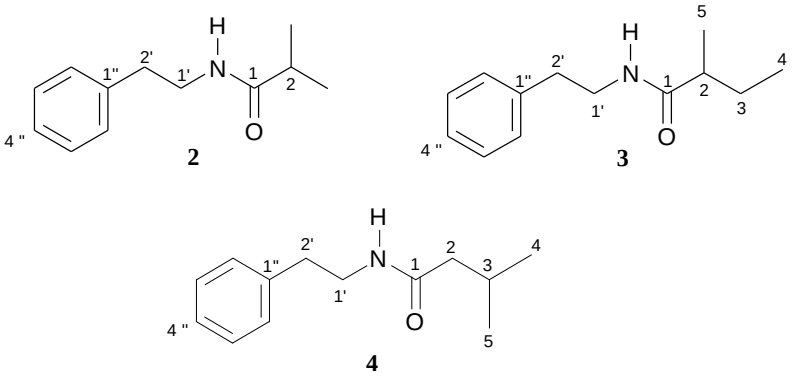
2. Results and Discussion

The actinomycete strain F-0017 was isolated and morphological examination indicated a *Salinispora* species. Analysis of the 16S rRNA gene sequence from gDNA of this strain yielded a 747 base pair sequence, which was compared to available sequences in GenBank using the BLAST algorithm. The strain was identified as *S. arenicola* with maximum sequence similarity of 99%. LC-MS chemotyping revealed that this strain produces four known compounds not previously observed from *S. arenicola* species. The culture medium was extracted with ethyl acetate, and the crude extract subjected to bioassay guided fractionation. Further purification of active fractions by reverse phase HPLC yielded four compounds (**1**-**4**).



1

Compound **1** possessed a molecular formula of $C_{35}H_{45}NO_{11}$, as determined by HRESIMS (*m/z* $[M+H]^+$ 656.3065; calcd for $C_{35}H_{46}NO_{11}$ *m/z* 656.3065; Δ = 0.0 ppm). An AntiBase search for the molecular formula mass revealed a match to the known rifamycin W. Examination of the ^1H-^1H



COSY spectrum of **1** revealed the following connectivities of the carbons: 21-CH–20-CH–19-CH=18-CH–17-CH=16-C–30-CH_3–, 32-CH_3–22-CH–, 23-CH–24-CH–33CH_3–, 25-CH–26-CH–34-CH_3– and 35-CH_2–28-CH–29-CH=. These fragments were connected to form a partial structure in Figure 1 based on the analysis of the ^{1}H-^{13}C HMBC couplings. An amide linkage was determined between C-2 and the C-15 carbonyl group by HMBC. Further analysis of the HMBC spectra showed that the aromatic proton (3-CH) coupled with C-5, C-6, C-7, C-8, C-9 and C-10 signals as shown in Figure 1. The methyl proton (14-CH_3) shows correlation with C-1, C-2 and C10. This, confirms the presence of a naphthoquinone chromophore as indicated in **1**. The structure of **1** was concluded to be rifamycin W which was supported by the detailed spectroscopic data (^{1}H NMR, COSY, HSQC and HMBC) obtained from Scripps Institution of Oceanography, University of California San Diego. Moreover, spectral data of **1** is in good agreement with the literature values of rifamycin W (Martinelli *et al*. 1974).

Compounds **2**-**4** showed strong similarities to each other over most parts of the ^{1}H-NMR spectra. Compound **2** possessed a molecular formula of $C_{12}H_{17}NO$ as determined by HRESIMS while compounds **3** and **4** were isomers with the molecular formula of $C_{13}H_{19}NO$. Structural difference is evident by the position of the methyl substituent on parent structure, forming two different compounds. In compound **3** the methyl group is attached to 2-C and based on the NMR spectrum there are 9 signals, while in compound **4** the methyl group is attached to 3-C with 7 signals in the NMR spectrum. Further analysis of HRESIMS and ^{1}H-NMR spectra of **2** with **3** and **4** revealed the difference of a mass unit of 14 amu (CH_2). Compounds **2**, **3** and **4** were established to be *N*-(2'-phenylethyl)isobutyramide, 2-methyl-*N*-(2'-phenylethyl)butyramide, and *N*-(2'-phenylethyl)isovaleramide, respectively, by the direct comparison of the ^{1}H NMR data with reported values as obtained from AntiBase (Maskey *et al*. 2002).

All the amides were found to be inactive against all the test bacteria and fungi. Rifamycin W had a minimum inhibitory concentration (MIC) of 12.5 µg/mL against methicillin resistant *Staphylococcus aureus*, 6.25 µg/mL against wild type *Staphylococcus aureus* and above 250 µg/mL against vancomycin resistant *Enterococcus faecium*. Rifamycin W was inactive against amphotericin B resistant and wild type *Candida albicans*.

Rifamycins are a family of closely related ansamycin antibiotics, the first examples of a novel class of secondary metabolites characterized by the possession of an aliphatic ansa chain bridging an aromatic chromophore (Nakata *et al*. 1990). Rifamycins are clinically important antibacterial agents active against Gram-positive bacteria (Kim *et al*. 2006). Several semi synthetic rifamycin variants (eg. rifampicin, which is a broad spectrum antibiotic) have been used clinically for the treatment of tuberculosis and other bacterial infections. Kim *et al*. (2006) identified specific gene clusters responsible for the production of rifamycin B and rifamycin SV in a marine-sponge-derived Salinispora M403 (which had previously been isolated from the soil actinobacterial species *Amycolatopsis mediterranei*). The *Salinispora* KS gene amino acid sequence displayed 90% similarity to the amino acid sequence of the RifB gene of *A. mediterranei*.

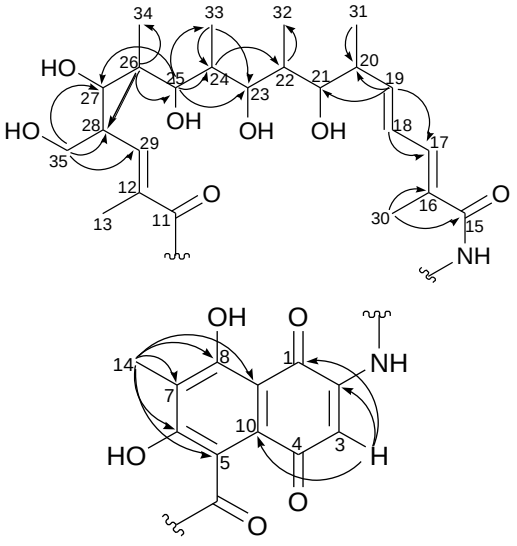


Figure 1. Partial structures of **1** elucidated by ^{1}H-^{13}C HMBC long range experiments.

It is likely that a similar gene sequence is present in the *S. arenicola* studied that was responsible for the production of rifamycin W, which had previously only been isolated from a marine bacterium of the genus *Norcadia*.

3. Experimental

3.1. General Experimental Procedures

^{1}H, ^{13}C and 2D NMR spectroscopic data were obtained on a Bruker 600 MHz (with cryoprobe) NMR spectrometer. HRLC-MS spectra were obtained on an Agilent 6200 TOF LC-MS system with a reverse-phase C_{18} column (Symmetry, 30 x 2.1 mm, 3.5 µm) at a flowrate of 0.4 mL/min in a positive ESI mode. Reversed-phase separations were performed using a semipreparative C_{18} Alltech Econosil (10 x 250 mm, 10 µm) column with a 50:50 MeOH/H_2O isocratic solvent system, flow rate of 4 mL/min on a Waters 515 system with UV detection at 254 nm. Semipreparative HPLC was also performed on a C_{18} Phenomenex Luna RP column (250 x 10 mm, 5 µm) with a 30:70 CH_3CN/H_2O isocratic system, with UV detection at 254 nm.

3.2. Bacterial Isolation and Identification

The actinomycete strain F-0017 was obtained from a marine sediment sample collected on the shore of Suva reef in Nasese, Fiji (18° 09' 21.05" S, 178° 25' 22.02" E) on the 11th of October 2005, and stored in freezer at -20°C until processed on the 20th of February 2006. The sediment sample was treated using the method of heat shock and the supernatant was inoculated onto agar-based M1 isolation medium as previously described (Mincer *et al*. 2002). Strain F-0017 was obtained in pure culture by repeated, single colony transfer on M1 agar media and DNA was extracted using Marmur's (1961) modified protocol for genomic DNA from actinomycete bacteria. The PCR amplification of the gDNA was accomplished using the forward primer FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) at SIO and the strain was identified as *S. arenicola* based on 16S rDNA analysis.

3.3. Fermentation and Extraction

Strain F-0017 was cultured at 27 ºC for 12 days while shaking with the aid of a magnetic stirrer in 9 x 1 L volumes of the liquid medium (A1B, composed of 10 g starch, 4 g yeast extract, 2 g peptone, per 1 L filtered seawater). At the end of the fermentation period after 12 days when the broth was nice and thick and dark orange whole broth cultures were extracted with a separating funnel with 9L of EtOAc. The ethyl acetate extract was reduced to dryness under vacuum (water bath temperature 35°C) to give a 974.7 mg of crude extract.

3.4. Bioactivity testing

Extracts (250 µg per disc) were tested for biological activity against the methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC: 10537), wild type *Staphylococcus aureus* (WTSA), vancomycin-resistant *Enterococcus faecium* (VREF; ATCC: 12952), wild type *Candida albicans* (WTCA; ATCC: 32354), and amphotericin B-resistant *Candida albicans* (ARCA; ATCC: 90873) by using standard disc diffusion method. MICs (WTSA, MRSA, and VREF) of the purified compounds (**1**−**4**) were also determined. Antibiotics were used as standards for comparison: (200µg) vancomycin for MRSA and WTSA, (50µg) rifamycin for VREF and (100µg) nystatin for ARCA and WTCA.

3.5. Isolation of Rifamycin W and Phenylethylamides

The crude extract 974.7 mg was fractionated by RP-FCC (Baberbond^{TM} Octadecyl $C_{18,}$ 40 µm) using a step-wise gradient of 20−100% $MeOH_{(aq)}$ to give 14 fractions. Bioassay guided fractionation led to repetitive RP-HPLC of active fraction five using a Alltech Econosil column which led to isolation of compound **2** (*N*-(2'-phenylethyl)isobutyramide, 5.7 mg). Bioactive fraction six was rechromatographed on NP-FCC (Silica Gel 60, 0.04−0.063 mm, 230−400 mesh) using a step-wise gradient of 5−100% MeOH/DCM, to give nine sub-fractions. Sub-fraction one from NP-FCC was also subjected to repetitive RP-HPLC with Alltech Econosil column to yield compound **3** (2-methyl-*N*-(2'-phenylethyl)butyramide, 0.7 mg) and compound **4** (*N*-(2'-phenylethyl)isovaleramide, 4.2 mg). Sub-fraction six was subjected to RP-HPLC (isocratic solvent system of 50:50 MeOH-H_2O, flow rate of 4 mL/min) and the bioactive peak obtained as a semi-solid was further purified by RP-HPLC using C_{18} Phenomenex Luna column (isocratic solvent system of 30:70 CH_3CN:H_2O, flow rate of 4 mL/min), to obtain compound **1** rifamycin W (0.7 mg).

3.6. Rifamycin W (1)

Purple solid; ^{1}H NMR (600 MHz, CD_3OD), see Table 1; HRESIMS *m/z* 656.3065 $[M+H]^+$ (calcd for $C_{35}H_{46}NO_{11}$ 656.3065).

3.7. N-(2'-phenylethyl)isobutyramide (2)

White crystals; ^{1}H NMR (600 MHz, CD_3OD) δ 7.24 (m), ~ 4.9 (s), 3.33 (q), 2.81 (t), 2.40 (m); HRESIMS *m/z* 192.2146 $[M+H]^+$ (calcd for $C_{12}H_{18}NO$ 192.1383).

3.8. 2-methyl-N-(2'-phenylethyl)butyramide (3)

White crystals; ^{1}H NMR (600 MHz, CD_3OD) δ 7.26 (m), 4.85 (s), 3.43 (q), 2.82 (t), 2.15 (m), 1.61 (m), 1.39 (m), 1.06 (d), 0.86 (t); HRESIMS *m/z* 206.1000 $[M+H]^+$ (calcd for $C_{13}H_{20}NO$ 206.1539).

3.9. N-(2'-phenylethyl)isovaleramide (4)

White crystals; ^{1}H NMR (600 MHz, CD_3OD) δ 7.22 (m), 4.79 (s), 3.41 (q), 3.25, 2.79 (t), 2.01 (d), 0.89 (d); HRESIMS *m/z* 206.3035 $[M+H]^+$ (calcd for $C_{13}H_{20}NO$ 206.1539).

4. Conclusions

Four known compounds (**1**−**4**) were successfully isolated from a Fijian marine actinomycete *S. arenicola* and the structures elucidated by mass and NMR spectra. Rifamycin W exhibited moderate activity against resistant strain MRSA, which was anticipated because naphthalene ansamycins are strongly active against Gram-positive and mycobacteria. The phenylethylamides have no activity against any of the test microorganisms. This report is the first recorded production of rifamycin W and the phenylethylamides by a marine sediment derived actinomycete.

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Correspondence to: K. Ragini
Email: ragini_k@usp.ac.fj
Tel.: +679 3232805; Fax: +679 3231534