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DE-REPLICATION FOR SMALL-SCALE INVESTIGATION OF METABOLITES FROM MARINE ACTINOMYCETES FOR THE SCREENING OF BIOACTIVE COMPOUNDS

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ABSTRACT

A total of 29 marine actinomycetes were isolated of which more than 75% showed potent antibacterial (MRSA; methicillin-resistant *Staphylococcus aureus*, VREF; vancomycin-resistant *Enterococcus faecium*) activity and 3% displayed anti fungal (ARCA; amphotericin-resistant *Candida albicans*, WTCA; wild type *Candida albicans*) activity against human drug-resistant pathogens. However all the strains (100%) exhibited cytotoxicity against brine shrimp larvae. All the crude extracts injected in the time-of-flight liquid chromatography mass spectrometry (HRESI-LC-MS) to analyse the metabolites profile with the respective UV profiles, molecular masses and the data were verified using natural product database, AntiBase and in-house database for screening of new compounds. Stauroporine, halomycin, rifamycin and their derivatives are commonly found in the microbial extracts. However, an actinomycete strain F-1568showed a unique profile with two derivatives of compounds have an UV profile similar to mycinamicin but had a different molecular mass, while another peak had an distinct UV profile but did not match any compounds in the in-house database revealing that F-1568 is a promising candidate for the discovery of new bioactive compounds.

Keywords: LC-MS profile, microbial metabolites, bioassay guided isolation, marine actinomycetes, bioactive compounds

INTRODUCTION

If there is no action today against multiple drug resistant human pathogens there would be no cure tomorrow due to its rapid emergence against current available multiple types or classes of antibiotics. The loss of effective current available antibiotics undermined the ability to fight against life threatening infections, the demand for new antibiotics are being increased. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of active compounds, nature stillremains the richest and the most versatile source for new antibiotics (Koehn and Carter, 2005; Baltz, 2006; Pelaez, 2006). Marine actinomycetes represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs (Bredholt *et al*., 2008). The interest in investigating natural products has led to the discovery of novel drug leads (Queiroz *et al*., 2012). Success in microbial natural products research is conditioned by proper strain selection, based on various criteria such as screening methods from large collection, chemotaxonomic data, bioactivity or metabolites profiling data. One main strategy in the isolation of new lead compounds consists of so-called bioactivity-guided isolation, in which biological assays are used to target the isolation of bioactive compounds (Queiroz *et al*., 2009). Metabolite profiling in crude microbial extracts is not an easy task since natural products display a wide structural diversity. Therefore, metabolite profiling using hyphenated techniques such as LC-UV, LC-MS and recently LC-NMR rapidly provides structural information, leading to a partial or a complete structure determination of the natural products of interest (Queiroz *et al*., 2009). Therefore, the present study investigated the bioassay-directed and LC-MS guided screening of new bioactive compounds from marine actinomycetes.

MATERIALS AND METHODS

Materials and avoidance of contamination

Liquid chromatography mass spectroscopy (LC-MS) were acquired using an Agilent1100 series separations module equipped with an Agilent1100 series LC-MSD mass detector in positive ion mode. Analytical grade solvents were utilized for extraction of metabolites. Riedel-de Haen, Chromasolv LC-MS grade solvents were used for LC-MS. All the ready-made media and media ingredients

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used in this study were purchased in Difco and Bacto. During sample preparation for LC-MS, glass containers were used whenever feasible, limiting exposure to plastics for minimizing contamination. In some cases, all pipetting measurements were carried out using polypropylene tips.

Samples, isolation and identification of marine actinomycetes

The collected marine sediment samples were dried under the sterile conditions using laminar air flow for 2-3 days. The dried sediments were directly (dumping) plated on M1A medium (40g starch, 4g yeast extract, 2g peptone, 18g agar and 1 liter of 0.45µm filtered natural seawater, pH 7.0) and incubated for 3-6 weeks at 27ºC. The medium also contained cycloheximide at 50 µg/ml to minimize the fungal contamination. A sponge *Melophlus* sp. was collected by hand using scuba at a depth of 10 m from Cicia, Lau group, Fiji Islands (17°47´33 S, 179°23´94 W). A small portion of the sponge was surface sterilized using 70% ethanol and then thoroughly washed by sterile natural seawater. The sterilized sponge tissues were cut into small pieces and grinded using pestle and mortar using sterile natural seawater. The resulting suspension was serially diluted in sterile natural seawater and plated on M1A medium. Media preparation and the culture conditions are the same as described above. Concurrently, an algae *Callophycus* sp. was collected using scuba diving at a depth of 5 m from Nananu-i-Ra, Fiji Islands. Immediately after the collection, a small portion of the algal tissue was surface sterilized and cut into small pieces and grinded by pestle and mortar using sterile natural seawater and the liquid suspension was plated on M1A medium using spread plate method.

Various characteristics such as colony appearance, growth nature, morphological characteristics such as substrate and aerial mycelia, identification and preservation of marine actinomycetes were performed as described by Bergey and Holt (1994) and Subramani and Narayanasamy (2009).

Extraction of metabolites and antimicrobial assay

All the respective 29 actinomycete strains were inoculated with 3% of seed culture in each 500 ml flasks containing 100 ml A1 broth (40g starch, 4g yeast extract, 2 g peptone and 1 liter of 0.45 µm filtered natural seawater, pH 7.0) and were incubated on a rotary shaker with the speed of 200 rpm at 27°C. The cultures were harvested after 7-9 days, equal volume of ethyl acetate were added to each culture and the mixtures were sonicated for 30 min. After that, the mixture was vigorous shaken using separating funnel, and then allowed to settle for 5-10 min. The solvent phase was separated and the procedure was repeated twice on residual aqueous phase to extract the maximum amount of metabolites. Further the solvent fractions were combined and concentrated in a roto evaporator at 35ºC. The dried crude extracts were maintained at a concentration of 25 mg/ml.

Susceptibility discs (6 mm in diameter) were impregnated with 250 µg of the each crude metabolites dissolved in MeOH and placed on nutrient agar [vancomycin-resistant *E. faecium* (VREF, ATCC12952)], Luria-Bertani agar [methicillin-resistant *Stapylococcus aureus* (MRSA, ATCC 10537) and wild type *S. aureus* (WTSA)]and potato dextrose agar [amphotericin resistant *Candida albicans* (ARCA, ATCC 90873) and wild type *C. albicans* (WTCA, ATCC 32354)] plates inoculated with the respective test bacterial/fungal pathogens, and zones of inhibition was recorded after 24 h of incubation at 37°C. The same concentration (250 µg/ disc) of positive controls were maintained for VREF (rifampicin), MRSA and WTSA (vancomycin) and ARCA and WTCA (nystatin). In all cases, for the negative controls containing only the respective amount of solvent, no zone of inhibition was observed.

Brine shrimp (*Artemiasalina*) lethality assay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of the crude extracts. Brine shrimp (*A. salina*) eggs were hatched in a beaker (150 ml), filled with filtered seawater under constant aeration for 48 h at 27 ± 2°C. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. 100 µl of seawater containing ten to fourteen nauplii were drawn through a pipette and placed in each 96-well plate. In each experiment, 100 µl of crude metabolites was added in respective well and maintained at room temperature for 24 h. Experiments were conducted in triplicates with different concentrations (250-62.5ppm) of crude metabolites to calculate the lethality dosage. Filtered seawater was used as negative control. The procedure was followed as previously described by Sam (1993).

HRESI-LC-MS of crude extracts

To avoid contamination and dust particles in the MS profile, the crude extracts were filtered using 0.4 µm syringe filter before injecting in HRESI-LC-MS. The HRESI-LC-MS of the crude extracts (10 µl; 0.1 mg/ml) were injected using ALS (Automated Liquid Sampler, G1313A) and analysed on a Waters C18 symmetry column (3.5 µm 2.1×3.0 cm) on Agilent 1100 series LC-MS-TOF mass spectrometer (Agilent Technologies, USA)using electro-spray ionization in positive ion mode(ESI^+; m/z range 100-3200). A linear water-acetonitrile gradient of 20% acetonitrile-water to 100%acetonitrile in 9.5 min was applied at a constant flow of 0.35 ml/min; then 100% acetonitrile was maintained for 1before returning to the starting conditions in 3 min and equilibrating for 2.5 min using a binary pump (G1312A). Acetic acid was added to all solvents at a concentration of 10 mM. UV spectra were collected by a DAD (G1315B) every second from 190 to 400 nm with are solution of 4 nm. The MS-TOF (G1969A) parameters as follows: fragmentor voltage 135 V, gas temp. 320°C,

flow rate of drying gas 8 l/min, nebulizer pressure 25 psig and the capillary voltage 3500 V.

Analysis MS for identification of new molecules

The acquired mass profiles were analysed in the Agilent Mass Hunter Qualitative Analysis B.04.00 for obtaining the molecular masses. The accurate molecular mass values have been verified with mass adducts[M + H]$^+$, [M + Na]$^+$, [2M + H]$^+$, [2M + Na]$^+$,[M - OH]$^+$,and the confirmed molecular masses along with the UV profiles were taken to search in the natural product database, AntiBase (Laatsch, 2010) and in-house database.

RESULTS AND DISCUSSION

Biological screening followed by activity guide dfractionation is the standard procedure for searching active metabolites. However, the number of available targets is limited (Queiroz *et al*., 2012). Moreover, bioassays are not always predictive for clinical efficacy. Further, the bioassay-guided fractionation strategy frequently leads to the isolation of known metabolites (Queiroz *et al*., 2012). Chemical screening of crude extracts therefore constitutes an efficient complementary approach, allowing localisation and targeted isolation of new types of constituents with potential activities (Queiroz *et al*., 2009, 2012). In our approach (Fig. 1), the LC-MS is used as a first step for the chemical profiling of crude microbial extracts, and compounds are tentatively identified based on molecular weight and fragment information with manual search in natural product database (AntiBase; Laatsch, 2010) as well as on matching with the in-house UV spectral libraries (Paul Jensen Laboratory, Scripps Institution of Oceanography). High resolution LC-MS has the major advantage that it can provide accurate mass, which can be used as a query in nearly all natural product databases. The rapid identification of known compounds present in a mixture (de-replication) by LC-NMR has advanced substantially in the past few years because of developments in nanoprobes and cryoprobes (Lang *et al*., 2008; Molinari, 2009). However, LC-MS is still more sensitive, providing reliable results within the nanogram range (Nielsen and Smedsgaard, 2003; Feng and Siegel, 2007; Molinari, 2009). Along with its many clear advantages, LC-MS screening with ESI or APCI ionization faces five major problems: (i) Sensitivity is highly compound-dependent, with some compounds unable to ionize at all in positive and/or negative polarity. This can lead to incorrect assignment of the molecular mass from coeluting impurities and resultinion suppression. (ii) Determining the pattern of adducts such as [M + H]$^+$, [M + Na]$^+$, [M + NH_4]$^+$, [M −H]$^-$, [M + HCOO]$^-$, and others for correct assignment of the molecular ion can be difficult (Huang*et al*., 1999; Nielsen and Smedsgaard, 2003; Nielsen *et al*., 2005; Lang *et al*., 2008). (iii) Adduct patterns vary substantially from one LC-MS system to another and can even change during a sequence because of sodium extraction from solvent glass bottles (Gorlach and Richmond, 1999; Nielsen and Smedsgaard, 2003; Schug and Mcnair, 2003; Fredenhagen *et al*., 2005; Overy *et al*., 2008). (iv) Some compounds predominantly form di- and tri-meric ions, e. g., [2M + H]$^+$, [2M + Na]$^+$,[3M + H]$^+$, and [2M - H]$^-$, which can further complicate assignment. Finally, (v) some compounds fragment very easily, losing HCOOH, CH_3COOH, one or more H_2O equivalents, and/or CO_2 (Levsen *et al*., 2007). Any of these problems can result in an erroneous assignment of molecular mass (Nielsen and Smedsgaard, 2003; Nielsen *et al*., 2005).

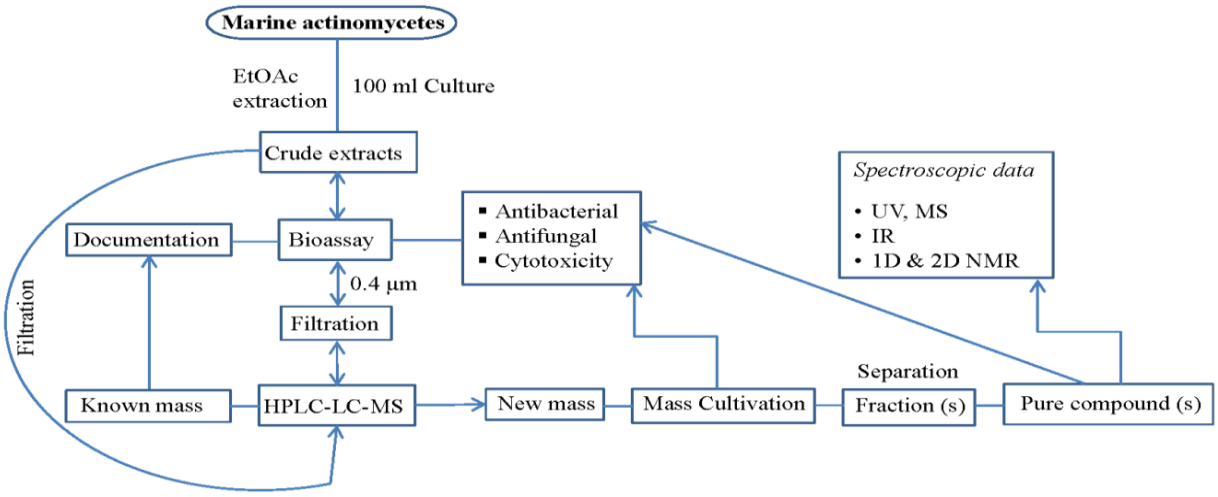

Fig. 1. Work flow for searching and identification of new bioactive compounds

In the present study, a total of 29 actinomycetes (6 from marine sediments; 5 from algae *Callophyus* sp.; 18 from marine sponge *Meophlus* sp.) were isolated, among them, 23 were *Salinispora* spp. 5 were non-*Salinispora* spp. and 1 was *Streptomyces* sp. Crude metabolites of all the strains were subjected to bioassay

against drug resistant human pathogens. Totally 22 strains (75.86%) showed antibacterial activity against MRSA, WTSA and VREF and 1 (3.4%) strain displayed antifungal activity against ARCA and WTCA however 5 strains did not exhibit both antibacterial and antifungal activity. Nevertheless all strains' crude extracts displayed cytotoxicity against brine shrimps (Tables 1, 2 and Fig. 2). The current results along with on-going research (data not shown) in our group revealed that actinomycetes, particularly *Salinispora* spp., produce appreciable antibacterial and anticancer metabolites than antifungal metabolites. Feling *et al*. (2003) reported that *Salinispora* strains are a prolific source of structurally diverse secondary metabolites, including salinosporamide A, a potent proteasome inhibitor that has entered phase I human clinical trials as an anticancer agent.

Table 1. Screening of marine actinomycetes for antimicrobial and cytotoxicity properties.

Source/ Strain	Seawater requirement	†Biological activity of actinomycetes (250 µg/disc)					Cytotoxicity (LD_{50} in ppm)
		Antibacterial			Antifungal		
		MRSA	WTSA	VREF	ARCA	WTCA	
Marine sediments							
F-1021	+	30	26	-	-	-	13
F-1077	+	-	-	-	-	-	47
F-1085	+	-	-	-	-	-	47
F-1195	-	-	-	-	14	14	13
F-1241	+	-	-	-	-	-	38
F-1250	+	32	32	11	-	-	8
Marine Algae							
F-1563	+*	-	-	-	-	-	562
F-1564	+*	10	9	-	-	-	616
F-1568	+*	9	10	-	-	-	501
F-1569	+*	-	-	-	-	-	610
F-1570	+*	-	-	-	-	-	562
Marine sponge							
SS-0028	+	20	18	10	-	-	112
SS-0029	+	16	17	11	-	-	180
SS-0030	+	29	27	10	-	-	12
SS-0031	+	18	16	10	-	-	141
SS-0032	+	20	16	10	-	-	47
SS-0033	+	30	30	14	-	-	13
SS-0034	+	29	29	11	-	-	47
SS-0035	+	30	30	13	-	-	17
SS-0036	+	17	18	-	-	-	13
SS-0037	+	20	18	10	-	-	123
SS-0038	+	18	16	9	-	-	138
SS-0039	+	20	21	-	-	-	47
SS-0040	+	30	30	12	-	-	12
SS-0041	+	17	18	10	-	-	117
SS-0042	+	23	22	-	-	-	44
SS-0043	+	16	16	9	-	-	190
SS-0044	+	30	29	10	-	-	12
SS-0045	+	28	29	-	-	-	47
Control		19	19	22	25	25	AA
Total activity (%)		76	76	52	3	3	100

*Non-salinispora strains but requires seawater for better growth; †Zone of inhibition in mm; AA: All are alive

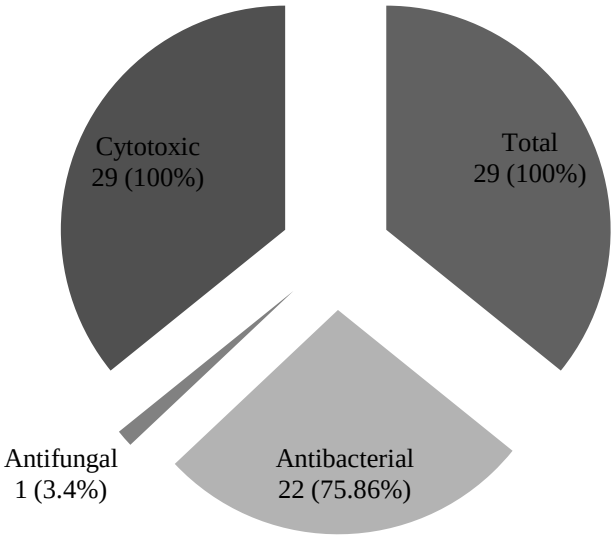

Fig. 2. Antimicrobial and cytotoxicity potential of marine actinomycetes against human drug resistant pathogens.

The crude metabolites of the 29 strains in this study were analysed on the positive mode HRESILC-MS for metabolite profiling (Table 2). Accurate mass measurements in the de-replication of unknown compounds reduce the number of predicted elemental molecules. This ensures that database searches are conducted with the fewest possible candidates (Nielsen *et al*., 2004). Therefore only accurate masses were selected and searched in the natural product database. To characterize the general mass distribution of microbial natural products, the accurate mass was verified for all compounds in AntiBase2010. AntiBase, which is used extensively in our laboratory, contains data for secondary metabolites from micro organisms and higher fungi. AntiBase contains up to 95-98% of the compounds published in journals and patents. Additionally, the identification of the compounds in the crude extracts has been confirmed using molecular weight, UV profile and biological property (Table 2).

Among the 23 *Salinispora* and 5 non-*Salinispora* extracts analysed, most of the compounds are stauroporine, halomycin, rifamycin and their analogs (Table 2, Fig. 3). Notably *Salinispora* extracts did not show any chemical diversity, and rifamycin analogs were identified as common metabolite among the strains. The lack of chemical diversity may be attributed to the lack of diversity in the strains. The detection of rifamycin analogs in most of the strains indicates the presence of the rifamycin gene cluster. However, Jensen *et al*. (2007) reported that >300*Salinispora* metabolites of LC-MS analyses revealed a high degree of structural diversity. Moreover, *Salinispora* produces species specific metabolites reported that *S. arenicola* is the only species that possesses the *rifK* gene suggesting that the genecluster responsible for rifamycin biosynthesis(Jensen *et al*., 2007). Supportive of these data and metabolite profiling of this study revealed that most of the strains may be belongs to *S. arenicola*. Further, LC-MS profile of the crude extracts of all *Salinispora* strains, the common masses m/z 269, 296, 326, 396, 316 and 711 were also observed but we were not able to find any mass adducts(e. g., $[M + H]^+$, $[M + Na]^+$, $[2M + H]^+$, $[2M + Na]^+$and$[M - OH]^+$) for the respective molecular masses in the LC-MS profile.

A total of 5 antimycinanalogs and ossamycin were identified in the crude extract of *Streptomyces* sp. F-1195 (Table 2). Further, the non-*Salinispora* strain F-1568 showed interesting metabolite profile, two analogs having similar UV profile as mycinamicin but had different molecular mass, and a peak that had a unique UV profile, which did not match any compounds in the in-house database. Thus strain F-1568 deserved to be taken for further study. However, the crude extracts of some strains did not ionize well and therefore we were not able to pick the correct masses or it might have been overlooked at low concentration and/or in complex extracts (Table 2).

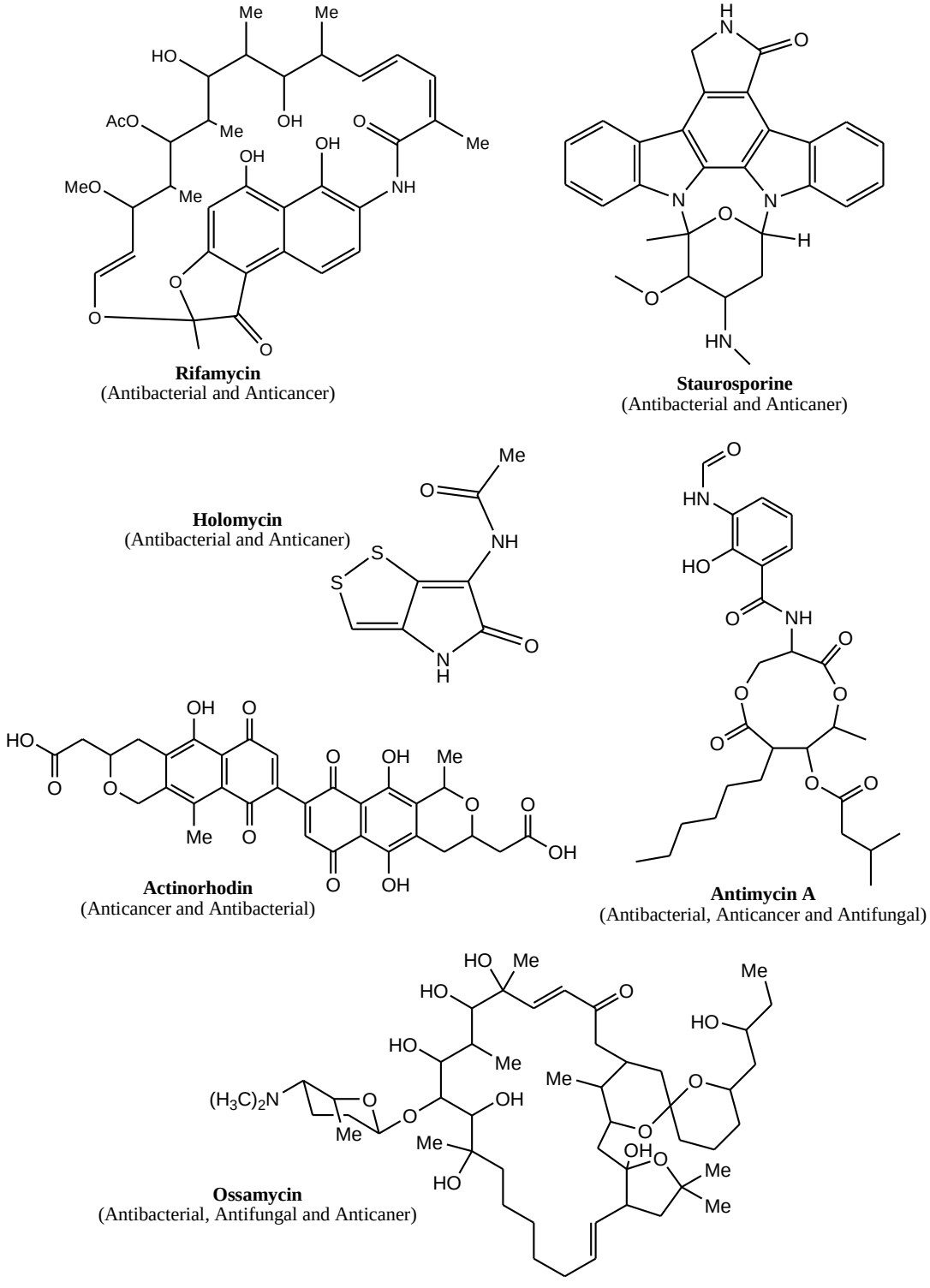

Fig. 3. Structures of some unique compounds identified from marine actinomycetes.

Table 2. Bioassay and LC-MS guided screening of bioactive compounds from crude metabolites of marine actinomycetes.

No.	Strain	Biological activity	λ_{max} (nm)	$[M + H]^+$	Peak identification (compound)
1.	F-1021	Antibacterial, Anticancer	235, 275	811.3997	Halomycin C
			240	795.4055	Halomycin B
			240	696.2998	Rifamycin S
			244	696.3008	Rifamycinanalogs
2.	F-1077	Antibacterial, Anticancer	-	-	-
3.	F-1085	Antibacterial, Anticancer	245	649.4894	Actinorhodin
4.	F-1195	Antibacterial, Antifungal, Anticancer	234, 365, 384	912.6391	Ossamycin
			237	479.2014	Antimycin
			239	507.2324	Antimycin A4B
			240	521.2485	Antimycin A3B
			238	535.2645	Antimycin A2B
			239	549.2787	Antimycin A1B
5.	F-1241	Antibacterial, Anticancer	-	-	-
6.	F-1250	Antibacterial, Anticancer	371, 292, 235	483.2025	Gentamycin
			240, 270	811.3998	Halomycin C
			248	795.4055	Halomycin B
			241	696.2880	Rifamycin S
7.	F-1563	Antibacterial, Anticancer	-	-	-
8.	F-1564	Antibacterial, Anticancer	-	-	-
9.	F-1568	Antibacterial, Anticancer	245, 290, 340	225.1123	**
			220, 290, 380	358.1021	**
10.	F-1569	Antibacterial, Anticancer	-	-	-
11.	F-1570	Antibacterial, Anticancer	-	-	-
12.	SS-0028	Antibacterial, Anticancer	220, 275	682.2864	Rifamycinanalogs
13.	SS-0029	Antibacterial, Anticancer	-	-	-
14.	SS-0030	Antibacterial, Anticancer	205, 220, 278	622.2664	Rifamycinanalogs
			217, 280	638.2616	Rifamycinanalogs
			218, 280	682.2852	Rifamycinanalogs
15.	SS-0031	Antibacterial, Anticancer	195, 246, 292, 336, 373	467.2099	Staurosporine
			205, 220, 280	622.2657	Rifamycinanalogs
			218, 278	682.2878	Rifamycinanalogs
16.	SS-0032	Antibacterial, Anticancer	197, 292, 372	467.2086	Staurosporine
17.	SS-0033	Antibacterial, Anticancer	197, 292, 372	467.2093	Staurosporine
			218, 275	622.2661	Rifamycinanalogs
			219, 280	696.3029	Rifamycin S
18.	SS-0034	Antibacterial, Anticancer	197, 292	467.2085	Staurosporine
			204, 220. 280	622.2663	Rifamycinanalogs
19.	SS-0035	Antibacterial, Anticancer	195, 247, 292, 372	467.2093	Staurosporine
20.	SS-0036	Antibacterial, Anticancer	-	-	-
21.	SS-0037	Antibacterial, Anticancer	205, 220, 280	622.2647	Rifamycinanalogs
			220, 278	638.2600	Rifamycinanalogs
22.	SS-0038	Antibacterial, Anticancer	220, 280	696.2737	Rifamycin S
23.	SS-0039	Antibacterial, Anticancer	215, 275	622.2658	Rifamycinanalogs
			215, 272	696.2661	Rifamycin S
24.	SS-0040	Antibacterial, Anticancer	196, 245, 292, 335, 372	467.2101	Staurosporine
			215, 275	622.2669	Rifamycinanalogs
			215, 275	638.2635	Rifamycinanalogs
			218, 275	682.2870	Rifamycinanalogs

25.	SS-0041	Antibacterial, Anticancer	220, 278	638.2585	Rifamycinanalogs
26.	SS-0042	Antibacterial, Anticancer	-	-	-
27.	SS-0043	Antibacterial, Anticancer	205, 220, 275	622.2650	Rifamycinanalogs
			218, 275	638.2579	Rifamycinanalogs
28.	SS-0044	Antibacterial, Anticancer	215, 275	638.2626	Rifamycinanalogs
			205, 275	622.2662	Rifamycinanalogs
29.	SS-0045	Antibacterial, Anticancer	195, 292	467.2073	Rifamycinanalogs
			220, 275	622.2640	Rifamycinanalogs
			220, 275	682.2850	Rifamycinanalogs
			220, 275	696.2995	Rifamycinanalogs

** Probably new compound

CONCLUSION

The present study highlights the combination of metabolite profiling and bioassay describes the rapid de-replication of known and new secondary metabolites from the microbial crude extracts. The bioassay and LC-MS guided screening among 29 microbial crude metabolites lead to the identification of the possible new compounds producing strain, F-1568. However further downstream process study is warranted for purification of the new molecules.

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