Identification bioactive compounds from marine microorganism and exploration of structure–activity relationships (SARs)

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ABSTRACT: Marine natural products (MNPs) have become new strong leads for antimicrobial drug discovery and an effective alternative to control drug resistant infections. Herein we report the bioassay guided fractionation of marine extracts from sponges Lendenfeldia, Ircinia and Dysidea that led us to identify novel compounds with antimicrobial properties. Tertiary amines or quaternary amine salts: anilines 1, benzylamines 2, tertiary amines 3 and 4, and quaternary amine salt 5, along with three known compounds (6-8) were isolated from a crude extract and MeOH eluent marine extracts. The absolute configurations of the new compounds were assigned based on tandem mass spectrometry (MS) analysis. Several of the compounds exhibited potent in-vitro antibacterial activity, especially against Methicillin-resistant Staphylococcus aureus (MRSA) (MICs from 15.6 to 62.5 micro g/mL). Herein, we also, report structure activity relationships of a diverse range of commercial structurally similar compounds. The structure activity relationships (SARs) results clearly demonstrate that modification of the amines through linear chain length, and inclusion of aromatic rings, modifies the observed antimicrobial activity towards different biological activity. Several commercially available compounds, which are structurally related to the molecules we discovered showed broad spectrum antimicrobial activity...
against different test pathogens with an MIC50 range of 50 to 0.01 microM. The results of cross-referencing antimicrobial activity and cytotoxicity establish that these compounds are promising potential lead molecules, with a favourable therapeutic index for antimicrobial drug development. Additionally, the SAR studies show that simplified analogues of the isolated compounds with increased bioactivity

**INTRODUCTION**

Almost 70 percent of earth's surface is covered by ocean, representing a huge reserve of natural biological and chemical diversity on our planet. Marine ecosystems have long been a rich source of bioactive natural products in the search for interesting molecules and novel therapeutic agents. Many interesting and structurally diverse secondary metabolites have been isolated from marine sources over the last 70 years. In addition, the preclinical pharmacology of seventy-five compounds isolated from marine organisms have been reported to have biological activities. Yet the first ‘drugs from the sea’ were only approved in the early 2000s: the cone snail peptide ziconotide (ω-conotoxin MVIIA) in 2004 to alleviate chronic pain, and sea squirt metabolite trabectedin in 2007 for treatment of soft-tissue sarcoma. Marine natural products (MNPs) have displayed exceptional potency and potential as anticancer therapeutics. Interest in MNPs has continued to grow, spurred in part by the spread of antimicrobial resistant pathogens and the need for new drugs to combat them.

The most prolific marine organisms are sponges, and the oldest metazoans on earth belong the phylum *Porifera*. The *Demospongiae* are the most abundant class of *Porifera*, representing 83% of described species, and has the largest number of bioactive compounds. The genus
Lendenfeldia are known as a source of sulfated sterols.\textsuperscript{18} The Lendenfeldia species metabolites have an anti-HIV, anti-tumor\textsuperscript{19}, anti-inflammatory, antifouling\textsuperscript{20} activities but they lack antimicrobial activity.\textsuperscript{18} Secondary metabolites of the genus Ircinia and Dysidea are prime candidates for further study to unveil their biological metabolites with antibacterial activity.\textsuperscript{14, 21-23}

Human pathogens are associated with a variety of moderate to severe infections and the recent rise of multi-drug resistant pathogens makes treatment more difficult. The last two decades have seen the emergence of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) strains resistant even to ‘drugs of last resort’ such as vancomycin,\textsuperscript{24} and \textit{Mycobacterium tuberculosis} resistant to all first-line agents,\textsuperscript{25-27} highlighting the urgent need to find new effective antibiotics with distinct mechanisms of action. Natural products continue to offer a productive source of structural diversity and bioactivity, and are an important source for new drugs.\textsuperscript{4-5, 7-8}

In the search for new antimicrobial agents, we screened a set of marine extracts\textsuperscript{28} to determine activity against antibiotic resistant microorganisms using a high-throughput screening (HTS) assay. Fractionation and purification of active components by high-performance liquid chromatography (HPLC), Nuclear magnetic resonance (NMR) and structural elucidation using high resolution and tandem mass spectrometry (MS) led us to a series of potential scaffolds for new, bioactive amine natural products (Figures 1, S20, S21 and S24).
**RESULTS AND DISCUSSION**

**Identification of active Marine Extracts.** To identify marine samples with activity against MRSA, 1434 compounds from the AIMS Bioresources Library\(^2\) (provided by the Queensland Compound Library,\(^3\) now called Compounds Australia\(^4\)) were screened in a resazurin cell viability assay. Of the samples tested, 29 inhibited the growth of MRSA by greater than 50% compared to non-treated controls. Minimum inhibitory concentrations (MICs) were determined for the 23 most promising samples, representing extracts and fractions from the phyla Porifera (90%), Echinodermata (5%) and Chordata (5%) (Table 1). The five most active samples showed MICs at 31.3 µg mL\(^{-1}\) (all Porifera samples), while another four samples returned MICs of 62.5 µg mL\(^{-1}\) (also all Porifera). Cytotoxicity screens against HepG2, HEK 293, A549 and THP-1 cell lines were performed to define the cytotoxicity profile of the most active samples. Pleasingly, all the samples most active against MRSA were also nontoxic to the cell lines tested (Tables 1).

**Table 1. Summary of the Nine Marine Samples Selected for Further Study.**

| Entry | AIMS Sample Code | QCL Sample Number | MIC (µg mL\(^{-1}\)) | Cytotoxicity (% cell survival) |
|-------|------------------|--------------------|----------------------|-------------------------------|
|       |                  |                    | MRSA                 | Hep G2 | A549 | HEK  |
| 1     | 19033            | SN00733110         | 31.25 ±0.9           | 91 ±1.2 | 91 ±0.3 | 98 ±1.6 |
| 2     | 20608            | SN00760947         | 31.25 ±1.3           | 97 ±1.0 | 101 ±2.9 | 102 ±0.7 |
Isolation and Characterization of Bioactive Compounds. Following the primary screening of the AIMS library and selection of positive hits, HPLC was used to separate and isolate active compounds, guided by bioassays against MRSA. Extracts were fractionated by analytical HPLC (see Experimental section and Supporting Information for further details), and fractions evaluated for bioactivity. Preparative scale HPLC was carried out on each bulk sample to isolate the active component (Table S1, Figures S1–S10), NMR and tandem mass spectrometry (MS/MS) methods used to deduce structures for novel compounds (Table 2 and Supporting Information). Insufficient quantities were obtained for positive ion high resolution mass spectrometry (HRMS) analyses.

Table 2. Key HRMS Data for Bioactive Samples, and Proposed Structures as Shown in SI.

| Sample | Molecular ion (m/z) | Molecular formula          | Proposed structures | Spectra               | MS/MS     | NMR analyses |
|--------|---------------------|---------------------------|---------------------|-----------------------|-----------|--------------|
| 19033  | 326.37813 [M + H]⁺  | [C₁₂H₁₄N]⁺                | 3                   | FiguresS11            | Table S2  | -            |
|        |                     | Calc. = 326.37802 (Δm = 0.11 ppm) RDBE = 0 |                     | FiguresS12            |           |              |
| 20608  | 332.33115 [M + H]⁺  | [C₁₃H₁₆N]⁺                | 1, 2                | FiguresS13            | Table S3  | -            |
|        |                     | Calc. = 332.33118 (Δm = 0.03 ppm) RDBE = 4² |                     | FiguresS14            |           |              |
| 26051  | 368.42508 [M + H]⁺  | [C₁₄H₁₈N]⁺                | 4, 5                | FiguresS15            | Table S4  | -            |
|        |                     | Calc. = 368.42495 (Δm = 0.13 ppm) RDBE = 0 [C₁₈H₂₄O₂N₃] |                     | FiguresS16            |           |              |
| 25663  | 306.0635 [M + Na]⁺  | [C₁₈H₂₄O₃N₃]⁺             | 6                   | FiguresS17            | -         | Table S5     |
|        |                     | Calc. = 306.0637 (Δm = 0.8 ppm) RDBE = 16 |                     | FiguresS20            |           |              |
The same active species was observed for all five fractions SN00760947, SN00760956, SN00732222, SN00734298 and SN00760958.

‡ RDBE = ring or double bond equivalents

Active components were isolated and characterised for five of the six extracts shown in Table 1: anilines 1 and benzylamines 2 from the Lendenfeldia sp. samples (AIMS Sample Code 20608, Table 1 entries 2-4); tertiary aliphatic amine 3 from the Dysidea herbacea extract (AIMS Sample Code 19033, Table 1 entry 1); aliphatic tertiary amine 4 and quaternary amine salt 5 from Ircinia gigantea (AIMS Sample Code 26051, Table 1 entry 5); ascididemin 6 and 2-bromoascididemin (2-Bromoleptoclinidinone) 7 from the Flavobranchia samples (AIMS Sample Code 25663, Table 1 entries 7); and halisulfate 8 from Ircinia (AIMS Sample Code 26104, Table 1 entry 8).

Aniline/amines 1-5 are all known compounds, 34-36 however they have not previously been identified as natural products, nor has their antibiotic activity been assessed.

Compounds 6, 7 37-38 and 8 39-42 have previously been reported, and in the current study these structures were confirmed by comparison of data with NMR and literature values. In 1994, the first biological activity of compounds 6 was reported as an antitumoral and antineoplastic activity 43. Antimycobacterial activity of compounds 6 with wide range of MIC50 against different microorganism’s reported 43, 38,44. Compounds 7 shows a similar activity profile against these organisms 38, 45-54. The inhibitory effects of compounds 6 are not limited to terrestrial microorganisms as it has also shown anti-predatory properties against both puffer and damselfish 43, 55.
Compound 8 has previously been identified and isolated from the marine sponges *Theonella swinhoe, Halicondriidea,* and *Coscinoderma matthewsi* and its inhibitory effects on different enzymes reported before 56-58. The bioactivity of 8 was first reported in 1998, when it was identified as a thrombin inhibitor 56. Antimicrobial activity against *S. aureus, C. albicans,* and *B. subtilis* has also been demonstrated 59. This compound also inhibited PMA-induced inflammation, phospholipase A2, ATPase activity (with IC$_{50}$ = 8 $\mu$M), RNA binding (IC$_{50}$ = 8 $\mu$M), serine protease activities (IC$_{50}$ = 14 $\mu$M) 59 and calcineurin (IC$_{50}$ = 69 $\mu$M) 59-61.

The helicase-inhibitor (Compound 8) with biological activity, also detected from other natural sources such as specimens which isolated from marine organisms in Okinawa, Japan, Sorong and Indonesia, has been shown to play an essential role in inhibiting viral replication with IC$_{50}$ values of 3 $\mu$M 59. Halisulfate analogues with a range of different stereochemistries have been described before reported to have similar activity 59-61.

**CONCLUSIONS**

Commercial drugs vancomycin and rifampicin remain the main agents for the treatment of invasive MRSA and *M. tuberculosis* diseases, respectively. Yet the number of vancomycin-resistant *S. aureus* (VRSA) and rifampicin-resistant *M. tuberculosis* strains is on the rise. The emergence of antibiotic resistance highlights the need for novel, effective antibacterial agents which circumvent traditional resistance mechanisms.

Thus we assayed 1434 extracts from the AIMS Bioresources Library 28 against MRSA, finding five samples that have a promising combination of high antibacterial activity and low toxicity to mammalian cells: AIMS Sample Codes 20608, 26051, 25663, 26104 and 19033 (Table 1). Samples of three extracts (20608, 26051 and 19033) were subjected to HPLC purification and
bioassay guided fractionation, enabling bioactive components to be isolated in low yield (~1 mg). Then high-resolution MS and tandem MS analysis was used to decipher structures (Table 2, Figures 1). The proposed structures are all tertiary amines or quaternary amine salts: anilines 1 and benzylamines 2 (from *Lendenfeldia* sample number 20608), aliphatic amine 3 (from *Dysidea herbacea* sample number 19033), aliphatic tertiary amines 4 and quaternary amine salt 5 (from *Ircinia sp.* sample number 26051). The proposed structures from samples 25663 and 26104 are ascididemin 6 and 2-bromoascididemin 7 (from *Flavobranchia* sample number 25663), and halisulfate 8 (from *Ircinia* sample number 26104).

The compounds uncovered in this study add to the growing arsenal of antimicrobial agents from the sea,3–4 and offer interesting new avenues in the quest for new, effective agents to combat the growing scourge of multidrug resistant bacteria.

**EXPERIMENTAL SECTION**

**General.** Chemical reagents were purchased from BDH Chemicals and Sigma Aldrich (Castle Hill, Sydney, Australia) and used as supplied unless otherwise indicated.

**Natural Product Library.** Natural product extracts were provided by the Australian Institute of Marine Science (AIMS), Townsville, Queensland as part of the AIMS Bioresources Library,28 via the Queensland Compound Library,29 (now called Compounds Australia30). Crude extracts had been partially fractionated by AIMS/ QCL to generate a library of 1434 samples, supplied in DMSO (100%) solution and stored at -80 °C. Original concentrations as provided were 5 mg mL⁻¹. Stock solutions were made by diluting these samples by a factor of 1:10 in dH₂O and stored at -80 °C.

**Bacterial Inhibition Assays.** For screening of the AIMS library, each test sample (10 μL) was dispensed into a separate well of a 96 well microtiter plates (final sample concentration 0.5 mg
mL\(^{-1}\)) using sterile dH\(_2\)O. For determination of MIC, extracts (250 to 0.5 µg mL\(^{-1}\)) or synthesized compounds (100 to 0.0002 µM) were serially diluted in microtiter plates. Bacterial suspension (90 µL, OD600nm 0.001) was added to each well and plates were incubated at 37°C for either 18 hours (MRSA, P. aeruginosa PAO1, E. coli EC958) or 7 days for M. tuberculosis H37Rv as described previously.\(^{49-50}\) Resazurin (10 µL; 0.05% w/v) was added and plates were incubated for 3 h or 24 h (M. tuberculosis) at 37 °C. The inhibitory activity was calculated by visual determination of colour change within wells or detection of fluorescence at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany). Percentage survival was calculated in comparison to the average of untreated control wells after normalising for background readings.

**Evaluating Toxicity of AIMS Extract Library.** Human alveolar epithelial cells (A549),\(^{62}\) Madin-Darby canine kidney epithelial cells (MDCK),\(^{63}\) human leukaemia cells (THP-1),\(^{64}\) human hepatocellular carcinoma cells (Hep-G2),\(^{65}\) and human embryonic kidney cells 293 (HEK293)\(^{66}\) were grown and differentiated in complete RPMI (Roswell Park Memorial Institute Medium) and DMEM (Dulbecco's Modified Eagle's medium) tissue culture media (RPMI\(_c\) and DMEM\(_c\)). To determine toxicity of the AIMS extract library, \(2 \times 10^5\) of each cell type were added to a 96-well plate and left for 48 h at 37 °C to adhere. Extract samples at a final concentration of 0.5 mg mL\(^{-1}\) were added to the wells, then incubated for 7 days in a humidified 5% CO\(_2\) incubator at 37 °C. Then resazurin (10 µL of 0.05% w/v) was added and after 4 h, fluorescence measured as described previously. Cell viability was calculated as percentage fluorescence relative to untreated cells.

**Purification of Natural Products from Extracts and Structure Elucidation**
**High Performance Liquid Chromatography (HPLC) Purification.** Samples were separated using analytical (Waters 2695 Alliance pump with Waters 2996 PDA, Sunfire reversed-phase column, and WFIll fraction collector) and preparative (Waters 600 HPLC pump, Phenomenex reversed-phase column, Waters 2487 UV detector and WFIll fraction collector) HPLC systems with UV detectors at 254 and 280 nm, employing a gradient of solvents A (dH2O) and B (acetonitrile) with trifluoroacetic acid (0.1%). Extract mixtures were kept at 4 °C until injection, then extract sample (100 μL) was injected onto an analytical Waters X-bridge C18 100 Å (4.6 × 250 mm, 5 μm) reversed-phase column on the same analytical HPLC system described above. The mobile phase was obtained using binary gradients of solvents A and B at a flow rate of 1 ml min⁻¹ at 30 °C over 80 min. Fractions, separated every 60 s, were collected. Purified fractions were flash-frozen in liquid nitrogen then freeze-dried overnight. The resulting fractionated extracts were re-suspended in DMSO and antibacterial activity versus MRSA was determined as described above.

Fractions identified as active against MRSA were further purified on the preparative HPLC unit described above, using a Phenomenex C18 100 Å (250 × 21.2 mm, 10 μm) reversed-phase column with UV detection at 254 and 280 nm, 7 mL min⁻¹ flow rate with water/acetonitrile gradient containing 0.1% trifluoroacetic acid.

The gradient for AIMS extracts 19033, 20608, 26104, 25641, and 26051 was 0% B initially, increased to 40% B over 20 min, then to 100% over 40 min, held at 100% for 10 min. For AIMS extracts 25663 gradient was 0% B initially, increased to 40% B over 60 min, then to 100% over 30 min, held at 100% for 10 min. Compounds thus purified were evaluated for biological activity and analysed by MS to determine potential structures for the bioactive components.
Identification and Structure Elucidation

Identification and elucidation of purified compounds from sample numbers 19033, 20608 and 26051. Purified compounds were identified and characterised using MS and NMR. High resolution ESI mass spectra (HRMS) were recorded on a Bruker Apex Qe 7T Fourier Transform ion cyclotron resonance mass spectrometer with an Apollo II ESI MTP ion source with samples (in CH₃CN:H₂O 1:1) infused using a Cole Palmer syringe pump at 180 µL h⁻¹. Where required, low resolution ESI tandem MS was performed on a Bruker amaZon SL ion trap via syringe infusion or by injection into a constant flow stream with a rheodyne valve and an Alltech HPLC pump (mobile phase methanol, flow rate 0.3 mL min⁻¹) connected to an Apollo II ESI MTP ion source in positive ion mode. Tandem mass spectra of the [M+H]⁺ parent ion were obtained manually up to MS⁵ (depending on sensitivity). Spectra were acquired in positive ion mode using a 1–4 Da isolation window, with the excitation amplitude manually optimized for each spectrum to have the selected mass at ~10% of the height of the largest fragment. Data analysis was performed for both high resolution MS and low resolution tandem MS data using Bruker Data Analysis 4.0 with smart formula assuming C, H, N, O, Na (0-1), mass error <2 ppm, C:H ratio 3 maximum, even electron (or both for tandem MS data). The results of high resolution MS data analysis were further refined manually by comparing isotopic fine structures of simulations where possible (resolving power > 200,000) to further eliminate potential formulae within the 2ppm mass error window (particularly ¹⁵N, ¹⁸O, ²H, ¹³C and ¹³C₂ isotopes and confirm no ³⁴S presence).

Identification and elucidation of fractions 44 from sample number 25663 (Compound 6). ¹H NMR (500 MHz, DMSO-d₆): δ 9.20 (d, J=5.6, 1H, H-21), 9.10 (dd, J=4.5, 1.7, 1H, H-17), 9.00
Identification and elucidation of fractions 58 from sample number 25663 (Compound 7). $^1$H NMR (500 MHz, DMSO-d$_6$): δ 9.25 (d, J=5.3, 1H, H-21), 9.13 (d, J=4.3, 1H, H-17), 8.98 (d, J=8.8, 1H, H-6), 8.93 (m, 1H, H-19), 8.68 (s, 1H, H-15), 8.64 (d, J=7.6, 1H, H-3), 8.19 (d, J=8.8 1H, H-1), 7.81 (dd, J=4.3, 7.6, 1H, H-16). HRMS (ESI): $m/z$ 361.9925; the molecular formula C$_{18}$H$_8$BrN$_3$O would give an expected [M+H]$^+$ ion at 361.9923 (err 0.5 ppm). This molecular formula gives a rde of 16, requiring many rings or double bonds. The NMR and mass data are in agreement with those in the literature for the known compound 2-bromoascididemin $^{68}$. 

Identification and elucidation of purified compounds from sample number 26104 (Compound 8). $^1$H NMR (500 MHz, DMSO-d$_6$): δ 8.47 (br s, 1H, H-8), 8.46 (br s, 1H, H-7), 6.54 (d, J=8.4, 1H, H-6), 6.44 (d, J=3.2, 1H, H-3), 6.35 (dd, J=3.2, 8.4, 1H, H-5), 5.29 (m, 1H, H-25), 5.22 (t, J=7.3, 1H, H-10), 4.19 (d, J=11.4, 1H, H-18), 3.12 (d, J=7.3, 1H, H-9), 2.19 (m, 1H, H-21), 2.13 (m, 1H, H-16), 1.95 (t, J=7.4, 1H, H-13), 1.91 (m, 1H, H-24), 1.8 (m, 1H, H-24'), 1.66 (d, J=13.1, 1H, H-27), 1.62 (s, 3H, H-12), 1.6 (s, 3H, H-31), 1.42 (m, 1H, H-14), 1.41 (m, 1H, H-28), 1.37 (m, 1H, H-29), 1.34 (m, 1H, H-20), 1.21 (m, 1H, H-15), 1.13 (m, 1H, H-23), 1.12 (m, 1H, H-29'), 1.06 (m, 1H, H-20'), 1.03 (m, 1H, H-27'), 1.03 (m, 1H, H-15'), 0.84 (s, 3H,
H-33), 0.83 (s, 3H, H-34), 0.78 (d, J= 6.9, 1H, H-26), 0.66 (s, 3H, H-32). LRMS (APCI): m/z 467.3, [M+H]^+; HRMS (APCI): m/z 467.1, [M+H]^+; the molecular of formula C_{30}H_{17}N_{2}O_{4} calculated at 467.118, err 1.9 ppm. This results in rdbe calculation of 24 indicating a high level of unsaturation and rings. The NMR and mass data are in agreement with those in the literature for the known compound halisulfate 69-72.

**ASSOCIATED CONTENT**

Supporting Information

Bioactivity and toxicity screening data, HPLC fractionation and purification protocols, plus mass spectrometry data (HRMS spectra, tables of daughter ions, and proposed fragmentation pathways) for natural products and synthetic compounds.

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**Author Contribution Statement**

MD conceived and designed the experiments; performed the experiments; analyzed the data; MD wrote the paper.

**Notes**

The authors declare no competing financial interest

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ABBREVIATIONS
A549, human alveolar epithelial cells; AIMS, Australian Institute for Marine Science; DMEM, Dulbecco's Modified Eagle's medium; HEK293, human embryonic kidney cells 293; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; MDCK, Madin-Darby canine kidney epithelial cells; MIC, minimum inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus*; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MTC, minimum toxic concentration; NMR, nuclear magnetic resonance; RPMI, Roswell Park Memorial Institute Medium; THP-1, human leukaemia cells; Hep-G2, human hepatocellular carcinoma cells; VRSA, vancomycin-resistant *Staphylococcus aureus*.

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