# Discovery of antimicrobial compounds from *Lendenfeldia*, *Ircinia* and *Dysidea* sponges using bioassay guided fractionation of marine extracts

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*Supporting Information*

**ABSTRACT:** Multidrug resistant bacteria have emerged as a threat to public health all over the world. At the same time, the discovery of new bioactive small molecules with antimicrobial activity and suitable pharmacological properties has waned. Herein we report the screening of marine extracts to identify novel compounds with antimicrobial activity. Bioassay guided fractionation has enabled the discovery and identification of a family of simple amines with promising activity against methicillin resistant *Staphylococcus aureus* (MRSA). To confirm the natural product structures proposed, these compounds and analogues have been prepared synthetically. Several of the synthetic analogues showed promising bioactivity against the medically important pathogens MRSA (MICs to 12.5 µM), *Mycobacterium tuberculosis* (MICs to 0.02 µM), uropathogenic *Escherichia coli* (MIC 6.2 µM) and *Pseudomonas aeruginosa* (MIC 3.1 µM). Cross-referencing antimicrobial activity and toxicity show that these synthetic compounds display a favourable therapeutic index for their target pathogens.

## 1. INTRODUCTION

Marine ecosystems have long been a rich source of bioactive natural products, in the search for interesting molecules and novel therapeutic agents.[1-5] Many interesting and structurally diverse secondary metabolites have been isolated from marine sources and characterised over the last 70 years.[6-9] 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,[10] and sea squirt metabolite trabectedin in 2007 for treatment of soft-tissue sarcoma.[11] Interest in marine natural products has continued to grow since,[6, 8-9] spurred in part by the spread of antimicrobial resistant pathogens and the need for new drugs to combat them.[3]

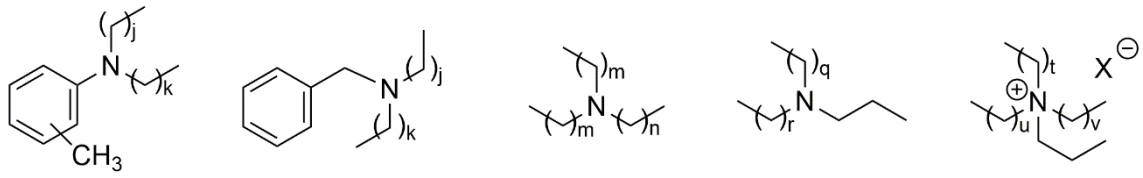
Human pathogens are associated with a variety of moderate to severe infections and the recent rise of multi-drug resistant pathogens makes treatment more difficult. The last two decades have seen the emergence of methicillin resistant *Staphylococcus aureus* (MRSA) strains resistant even to 'drugs of last resort' such as vancomycin,[12] and *Mycobacterium tuberculosis* resistant to all front-line drugs,[13-15] which highlights the urgent need to find new effective antibiotics. Natural products continue to offer a productive source of structural diversity and bioactivity, and are an important source for new drugs.[3-4, 6-7]

In the search for new antimicrobial agents, we screened a set of marine extracts [16] to determine activity against antibiotic resistant microorganisms using a high-throughput screening (HTS) assay. Fractionation and purification of active components by high-

79 performance liquid chromatography (HPLC) and structural elucidation using high
80 resolution and tandem mass spectrometry (MS) led us to a series of potential structures
81 for new, bioactive amine natural products (Figure 1).

82

83 To validate the proposed structures, and to explore the potential of this compound class
84 more broadly, analogues based on the general structures **1–5** were synthesised and
85 evaluated as antimicrobial agents against a panel of medicinally important
86 microorganisms: MRSA, *M. tuberculosis*, uropathogenic *Escherichia coli*, and
87 *Pseudomonas aeruginosa*.




88 **1** **2** **3** **4** **5**

89 **Figure 1.** Proposed structures of bioactive amine natural products identified as lead
90 compounds in this study; (j + k) = 14; m = 5, n = 9; (q + r) = 20; (t + u + v) = 19; X
91 = unidentified counterion.

92

## 2. RESULTS AND DISCUSSION

**2.1. Identification of Active Marine Extracts.** To identify marine samples with activity against MRSA, 1434 compounds from the AIMS Bioresources Library[16] (provided by the Queensland Compound Library,[17] now called Compounds Australia[18]) 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 (Figure S1, Supporting Information). 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 and Table S1). 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, S1, S2 and S3).

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

| Entry | AIMS Sample Code | QCL Sample Number | MIC ($\mu g\ mL^{-1}$) MRSA | Cytotoxicity (% cell survival) Hep G2 | A549 | HEK |
|---|---|---|---|---|---|---|
| **1** | 19033 | SN00733110 | 31.25 | 91 | 91 | 98 |
| **2** | 20608 | SN00760947 | 31.25 | 97 | 101 | 102 |
| **3** | 20608 | SN00760956 | 31.25 | 100 | 106 | 95 |
| **4** | 20608 | SN00760958 | 62.5 | 98 | 108 | 95 |
| **5** | 26051 | SN00731005 | 62.5 | 101 | 110 | 98 |
| **6** | 24307 | SN00730755 | 31.25 | 100 | 106 | 96 |

**2.2. 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 S4, Figures S2–S7), and tandem mass spectrometry (MS/MS) methods used to deduce structures (Table 2 and Supporting Information).[19-20] [21] Insufficient quantities were obtained for NMR analyses.

Active components were isolated and characterised for five of the six extracts shown in Table 1: aromatic amines **1**/**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); and aliphatic tertiary amine **4**/ quaternary amine salt **5** from *Ircinia gigantea* (AIMS Sample Code 26051, Table 1 entry 5). The active component of the other active Demospongiae extract (AIMS Sample Code 24307, Table 1 entry 6) could not be isolated.

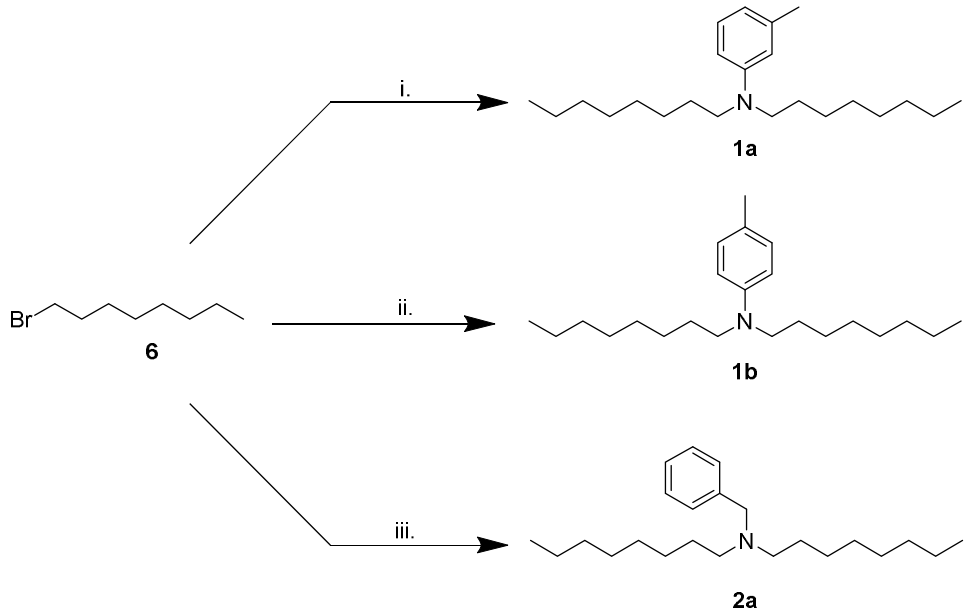
**Table 2. Key MS Data for Bioactive Samples, and Proposed Structures as Shown in Figure 1.**

| Sample | Molecular ion (m/z) | Molecular formula | Proposed structures | Spectra | MS/MS |
|---|---|---|---|---|---|
| 19033 | 326.37813 $[MH]^+$ | $[C_{22}H_{48}N]^+$ Calc. = 326.37802 ($\Delta$m = 0.11 ppm) RDBE = 0 | **3** | Figure S8 | Table S5 Figure S9 |
| 20608[†] | 332.33115 $[MH]^+$ | $[C_{23}H_{42}N]^+$ Calc = 332.33118 ($\Delta$m = 0.03 ppm) RDBE = 4[‡] | **1, 2** | Figure S10 | Table S6 Figure S11 |
| 26051 | 368.42508 $[MH]^+$ | $[C_{25}H_{54}N]^+$ Calc. = 368.42495 ($\Delta$m = 0.13 ppm) RDBE = 0 | **4,5** | Figure S12 | Table S7 Figure S13 |

[†] The same active species was observed for all three fractions SN00760947, SN00760956 and SN00760958.

[‡] RDBE = ring or double bond equivalents

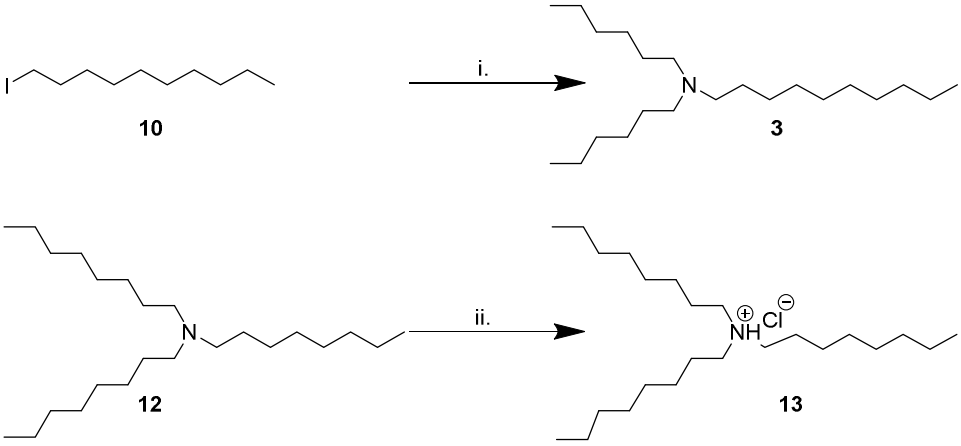
**2.3. Synthesis.** To validate the structures proposed for the natural products, and to explore the potential of these a compounds as bioactive agents, a series of tertiary amine derivatives of compounds **1**–**5** were synthesised from 1-bromooctane **6**, *m*-toluidine **7**, *p*-toluidine **8**, benzyl amine **9**, 1-iododecane **10**, *N*,*N*-dihexylamine **11** and *N*,*N*,*N*-trioctylamine **12** (Schemes 1 and 2). *o*-Toluidine is carcinogenic and therefore was not used in synthetic experiments.




**Scheme 1. Synthesis of Amines Related to Natural Products 1 and 2.** i. *m*-toluidine **7**, $K_2CO_3$, KI, MeCN, 60 °C, overnight, 8 %; ii. *p*-toluidine **8**, $K_2CO_3$, KI, MeCN, 60 °C, overnight, 6 %; iii. benzylamine **9**, $K_2CO_3$, MeCN, 82 °C, overnight, 19 %.

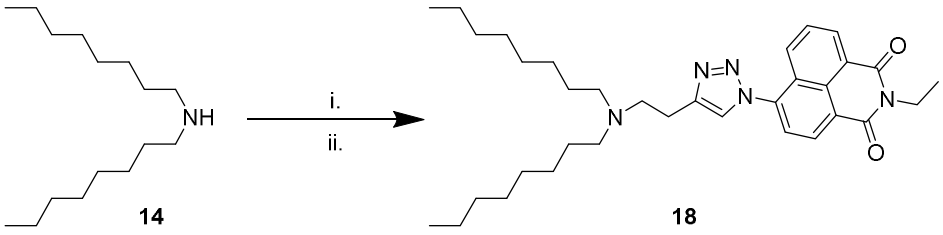
Compounds **1a**, **1b** and **2a** were prepared using 1-bromooctane **6** to alkylate *m*-toluidine **7**, *p*-toluidine **8**, benzyl amine **9** respectively (Scheme 1), giving three compounds based on the active component of AIMS sample 20608. Compound **3**, the active component of AIMS sample 19033, was prepared by reacting 1-iododecane **10** with *N*,*N*-dihexylamine **11** (Scheme 2), while *N*,*N*,*N*-trioctylamine hydrochloride **13** was

prepared from the free amine **12** as a simple and readily accessible analogue of the natural product structures **4** and **5** that had been isolated from AIMS sample 26051.




**Scheme 2. Synthesis of Amines Related to Natural Products 3–5**. i. *N*,*N*-dihexylamine **11**, $K_2CO_3$, MeCN, 82 °C, overnight, 21 %; ii. HCl in 1,4-dioxane, 5 min, 99%.

Finally, to broaden the scope of this work, we sought to combine the tertiary amine structures elucidated in this study with a triazolyl naphthalimide pendant, recently shown to be an important component of a new class of anti-tubercular agents.[23-24] Thus *N*,*N*-dioctylamine **14** was alkylated with 4-bromo-1-butyne **15**, and the resulting alkyne product **16** 'clicked'[25] with 6-azido-2-ethyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione **17**[26] to afford the naphthalimide derivative **18** (Scheme 3).




**Scheme 3. Synthesis of Naphthalimide Derivative 18.** i. 4-bromo-1-butyne **15**, $K_2CO_3$, KI, MeCN, 60 °C, overnight, 2 %; ii. 6-azido-2-ethyl-1*H*-

186 benzo[*de*]isoquinoline-1,3(2*H*)-dione **17**, $CuSO_4$, sodium ascorbate, $^{t}$BuOH/ $H_2O$,
187 60 °C, overnight, 1 %.

188 While the yields of many synthetic steps were low, sufficient quantities of material were
189 nonetheless isolated to enable characterisation, and biological evaluation, so the
190 synthetic reactions were not further optimised.

191 **2.4. Structural Comparison of Synthetic Compounds to Natural Products.**
192 Synthetic compounds were investigated using mass spectrometry (MS/MS and accurate
193 mass) and analysis of biological activity. Comparing the major ions in the mass spectra
194 of synthetic **1a** and **1b** (Table S8), **2a** (Table S9), **3** (Table S10) and **13** (Figure S11)
195 with the natural products shows good correlation (Tables S6, S5 and S7, and Figures S9,
196 S11 and S13 respectively). Some minor differences are apparent, which most likely arise
197 due to differences in the amounts of material analysed (which are significantly greater
198 for the synthesized products), and differences in the instrumentation used.
199

**2.5. Antibacterial Activity and Toxicity of Synthetic Compounds.** Synthetic compounds were assessed for bioactivity against MRSA, *P. aeruginosa*, uropathogenic *E. coli* and *M. tuberculosis*. Interestingly, all synthetic derivatives compounds showed similar MICs against MRSA (the organism against which the original natural product screening assays had been conducted), typically around 12.5 μM. The simple amine salt **13** proved the most effective of the synthetic compounds against *M. tuberculosis* with an MIC of 0.02 μM, and showed moderate inhibitory activity against the other bacteria. Compound **3** displayed broad activity, with low MICs against *P. aeruginosa* (MIC 3.1 μM), E. coli (6.2 μM) and *M. tuberculosis* (3.1 μM). The naphthalimide derivative **18** displayed good selective activity against *E. coli* (MIC 1.5 μM).

**Table 3. Anti-Bacterial Activity of Synthetic Compounds Against MRSA, *P. Aeruginosa, E. Coli* and *M. Tuberculosis*.**

| Synthetic compound | MIC (μM)[†‡] | | | |
|---|---|---|---|---|
| | MRSA | *M. tuberculosis* | *E. coli* | *P. aeruginosa* |
| **1a** | 12.5 | 1.5 | 12.5 | 6.2 |
| **1b** | 100 | 25 | 100 | 100 |
| **2a** | 12.5 | 1.5 | 50 | 50 |
| **3** | 12.5 | 3.1 | 6.2 | 3.1 |
| **13** | 12.5 | 0.02 | 25 | 25 |
| **18** | 12.5 | 12.5 | 1.5 | 12.5 |

[†] Samples were diluted from 100 μM to 0.0002 μM and incubated with bacteria (starting concentration $OD_{600}$=0.001) under optimum assay conditions as described in the Experimental section.

[‡] Values represents averages from three independent repeats.

The potential toxicity of the synthetic compounds was also evaluated, against A549, THP-1, HepG2 and HEK 293 and cell lines (Table 3). None of the synthetic compounds showed significant toxicity against HepG2 or A549 cells. Synthetic compounds **1a**, **2a**, **3**, **13** and **18** all showed some toxicity against THP1 and/or HEK 293 cells, with Minimum Toxicity Concentrations (MTC) as low as 3.1 μM. Compound **1b** showed low toxicity against all four of these cell lines, but also low activity (Table 4). Compound **1a** showed only mild effects on all cell lines tested (MTC 50–100 μM), while also displayed a broad antibacterial profile, suggesting this compound may be a candidate for further investigation.

**Table 4. Toxicity of Synthetic Compounds to Cell Lines.**

| Synthetic samples | Minimum Toxicity Concentration of Drug (MTC μM)[†‡§] | | | |
|---|---|---|---|---|
| | A549 | THP1 | HepG2 | HEK 293 |
| **1a** | > 100 | 50 | > 100 | 50 |
| **1b** | > 100 | >100 | > 100 | > 100 |
| **2a** | > 100 | 6.5 | > 100 | 50 |
| **3** | >100 | 50 | > 100 | 25 |
| **13** | 50 | 3.1 | > 100 | 6.5 |
| **18** | > 100 | > 100 | > 100 | 3.1 |

[†] Cells seeded at a concentration of $2 \times 10^5$ cells/ well; samples diluted 100 μM to 0.0002 μM.
[‡] Cells incubated with sample under humidified incubation of 37 °C with 5 % $CO_2$.
[§] Values represents averages of three independent repeats.

Comparing data from the antimicrobial activity and cytotoxicity assays shows that the active concentration ranges for these synthetic compounds against bacteria are substantially lower than active concentration ranges against the mammalian cells tested,

239 particularly HepG2 and A549 cells. THP1 cells and the primary cells appeared more
240 sensitive to these compounds. Cross-referencing the biological activity and toxicity data
241 for these compounds suggest that they have some potential for further development.

242 Compounds **1a**, **13** and **18**, which showed the most promising activity against *M.*
243 *tuberculosis*, were assessed against single and multi-drug resistant *M. tuberculosis*
244 strains (Table 5). Compound **13** exhibited strong inhibitory activity against all resistant
245 strains with MICs as low as 0.24 µm; Compound **1a** showed moderate and variable
246 activity against all assay strains (MIC 6.7–20.0 µm) and **18** showed similar activity
247 against all resistant strains with an MIC of 2.2 µm.

248

249 **Table 5. Activity of synthetic compounds against drug resistant *M. tuberculosis***
250 **strains.**

| ***M. tb* strain**[†] | **Minimum inhibitory concentration (MIC) Drug (µM)** | | |
|---|---|---|---|
| | **1a** | **13** | **18** |
| sensitive | 20 | 0.74 | 2.2 |
| Inh | 20 | 0.74 | 6.7 |
| Rif, Inh | 20 | 0.74 | 6.7 |
| Rif, Inh, Eth | 20 | 0.74 | 6.7 |

251 [†] Rif = resistant to rifampicin; Inh = resistant to isoniazid; Eth = resistant to ethambutol;

252

253 **3. CONCLUSIONS**

254 Commercial drugs vancomycin and rifampin remain the reference standard for the
255 treatment of invasive MRSA and *M. tuberculosis* infections respectively. Yet the
256 number of vancomycin-resistant *S. aureus* (VRSA) and rifampicin-resistant *M.*

*tuberculosis* strains is on the rise. The emergence of antibiotic resistance brings a need for novel, effective antibacterial agents that are resistant to antimicrobial resistance.

Thus we assayed 1434 extracts from the AIMS Bioresources Library[16] against MRSA, finding three samples that have a promising combination of high antibacterial activity and low toxicity to mammalian cells: AIMS Sample Codes 20608, 26051 and 19033 (Table 1). Samples of these extracts 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, Figure 1). The proposed structures are all tertiary amines or quaternary amine salts: aromatic amines **1**/**2** (from *Lendenfeldia* sample number 20608), aliphatic amine **3** (from *Dysidea herbacea* sample number 19033), the aliphatic tertiary amine **4** and quaternary amine salt **5** (from *Ircinia sp.* sample number 26051).

Synthetic compounds based on the natural product structures **1**–**5** was prepared to validate and expand these findings. Synthetic compounds **1a**, **1b**, **2a**, **3** and **13** showed tandem MS fragmentation patterns consistent with the natural product samples, thus supporting the structures proposed for those samples, and promising bioactivity/ toxicity profiles. Naphthalimide derivative **18** was prepared as a hybrid of the amines uncovered in this study, and naphthalimide-amine derivatives we have previously reported as potent anti-mycobacterial agents.[23-24]

The compounds uncovered in this study add to the growing arsenal of antimicrobial agents from the sea,[2-3] and offer interesting new avenues for further investigation in the

quest for new, effective agents to combat the growing scourge of multidrug resistant bacteria.

## 4. EXPERIMENTAL METHODS

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

**4.2. 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,[16] via the Queensland Compound Library,[17] (now called Compounds Australia[18]). 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 $dH_2O$ and stored at -80 °C.

**4.3. Screening the AIMS Extract Library Against MRSA.** 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 $dH_2O$. Bacterial suspension (90 µL, $OD_{600nm}$ 0.001) was added to each well and plates were incubated at 37 $^{o}$C for 18 hours. To determine MIC of samples, crude extracts were added to wells in sequential 2-fold dilutions and incubated with diluted bacteria as described previously.[23-24] Resazurin (10 µL; 0.05% w/v) was added and plates were incubated for 3 h at 37 °C. The bioactivity of extracts 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).

**4.4. Evaluating Toxicity of AIMS Extract Library.** Human alveolar epithelial cells (A549),[27] Madin-Darby canine kidney epithelial cells (MDCK),[28] human leukaemia cells (THP-1),[29] human hepatocellular carcinoma cells (Hep-G2),[30] and human embryonic kidney cells 293 (HEK293)[31] were grown and differentiated in complete RPMI (Roswell Park Memorial Institute Medium) and DMEM (Dulbecco's Modified Eagle's medium) tissue culture media (RPMIc and DMEMc). 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.

### 4.4. Purification of Natural Products from Extracts and Structure Elucidation

**4.4.1. High Performance Liquid Chromatography (HPLC) Purification.** Samples were separated using analytical (Waters 2695 Alliance with Waters 2996 PDA, Sunfire reversed-phase column, and WFIII fraction collector) and preparative (Waters 600 HPLC pump, Phenomenex reversed-phase column, Waters 2487 UV detector and WFIII fraction collector) HPLC systems with UV detectors at 254 and 280 nm, employing a gradient of solvents A ($dH_2O$) and B (acetonitrile) with trifluoroacetic acid (0.01%). Extract mixtures were kept at 4 °C until injection, then extract sample (100 µL) was injected onto an analytical Waters X-bridge C18 100 A (4.6 × 250 mm, 5 µm) reversed-phase column on the same analytical HPLC system described above. The mobile phase was obtained using 100% acetonitrile and 0% water at a flow rate of 1 ml $min^{-1}$ at 30 ºC 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 C18 100 A (250 × 21.2 mm, 10 µm) reversed-phase column (Phenomenex) 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, and 26051 was 0% B initially, increased to 40% B over 20 min, then to 100% at 60 min, held at 100% for 10 min, and finally a

linear decrease to 0% B over 5 min and held at 0% B for 10 min prior to the next run. Compounds thus purified were evaluated for biological activity and analysed by MS to determine potential structures for the bioactive components.

**4.4.2. Identification and Structure Elucidation.**

Purified compounds were identified and characterised using MS. 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 $CH_3CN$:$H_2O$ 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 $MS^5$ (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 DataAnalysis 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 $^{15}N$, $^{18}O$, $^{2}H$, $^{13}C$ and $^{13}C_2$ isotopes and confirm no $^{34}S$ presence).

366

367 **4.5. Synthesis**

368 **4.5.1. *N,N*-Dioctyl-3-methylaniline 1a.** To a solution of 1-bromooctane 6 (6.3 mL, 36.6
369 mmol) in acetonitrile (50 mL) was added potassium carbonate (25.3 g, 183 mmol), KI
370 (6.08 g, 36.6 mmol) and m-toluidine 7 (1.96 mL, 18.3mmol) stirred at 60 °C overnight.
371 The suspension was filtered and washed with acetonitrile (3 × 50 mL), then
372 concentrated by rotary evaporation to yield the crude product (1.50 g). Purification by
373 automated column chromatography (100 g cartridge, 100% petroleum benzine over 12
374 CV) then by preparative TLC (10% ethyl acetate:petroleum benzine) afforded the pure
375 compound 1a as a yellow oil (0.46 g, 8 %). $^{1}$H NMR (500 MHz , $CDCl_3$): δ 7.13 – 7.07
376 (m, 1 H), 6.50 – 6.44 (m, 3 H), 3.28 – 3.22 (m, 4 H), 2.32 (s, 3 H), 1.64 – 1.53 (m, 4 H),
377 1.38 – 1.24 (m, 20 H), 0.94 – 0.87 (m, 6 H); $^{13}$C NMR (125 MHz , $CDCl_3$): δ 148.3,
378 138.7, 129.0, 116.0, 112.4, 109.0, 51.0, 31.8, 29.5, 29.3, 27.3, 27.2, 22.7, 22.0, 14.1;
379 LRMS (ESI+): m/z 332.33 $[M+H]^+$, 100%; HRMS (ESI): m/z calculated for $[C_{23}H_{42}N]^+$,
380 $[M + H]^+$ 332.3317, found 332.33054.

381

382 **4.5.2. *N,N*-Dioctyl-4-methylaniline 1b.** To a solution of 1-bromooctane 6 (6.3 mL, 36.6
383 mmol) in acetonitrile (50 mL) was added potassium carbonate (25.3 g, 183 mmol), KI
384 (6.08 g, 36.6 mmol) and p-toluidine 8 (1.96g, 18.3 mmol) stirred at 60 °C overnight.
385 The suspension was filtered then concentrated by rotary evaporation to yield the crude
386 product (1.65 g). Purification by automated column chromatography (100 g cartridge,
387 100% petroleum benzine over 4 CV, ramping to 100% ethyl acetate over 4 CV) gave the
388 product 1b as a yellow oil (0.39 g, 6 %). $^{1}$H NMR (500 MHz, $CDCl_3$): δ 7.02 (d, J = 8.2

389 Hz, 2 H), 6.68 – 6.47 (m, 2 H), 3.33 – 3.10 (m, 4 H), 2.25 (s, 3 H), 1.58 - 1.52 (m, 4 H),
390 1.40 – 1.20 (m, 20 H), 0.95 – 0.82 (m, 6 H); $^{13}$C NMR (125 MHz , $CDCl_3$): δ 146.2,
391 129.7, 124.3, 112.2, 51.3, 31.9, 29.5, 29.4, 27.3, 27.2, 22.7, 20.1, 14.1; LRMS (ESI+):
392 m/z 332.33 $[M+H]^+$, 100%; HRMS (ESI): m/z calculated for $[C_{23}H_{42}N]^+$ $[M+H]^+$
393 332.3317, found 332.33095.
394
395 **4.5.3. *N*-Benzyl-*N*-octyloctan-1-amine 2a.** To a solution of 1-bromooctane 6 (6.3 mL,
396 36.6 mmol) in acetonitrile (50 mL) were added potassium carbonate (25.3 g, 183 mmol)
397 and benzylamine 9 (2 mL, 18 mmol) then stirred at 60 ºC overnight. The mixture, a clear
398 colourless solution, was concentrated using the rotary evaporator, to give a white solid.
399 The solid was triturated with DCM (1 × 50 mL, 1 × 25 mL) and the DCM solution
400 concentrated on the rotary evaporator to yield yellow oil (5.1 g). Purification by
401 automated column chromatography (100 g cartridge, 0–40% ethyl acetate (EtOAc) in
402 petroleum benzine over 10 CV) yielded N-benzyl-N-octyloctan-1-amine 2a as a
403 colourless oil (1.15 g, 19 %). $^1$H NMR (500 MHz, $CDCl_3$): δ 7.36 – 7.29 (m, 4H), 7.26 –
404 7.21 (m, 1H), 3.56 (s, 2H), 2.47 – 2.35 (m, 4H), 1.54 – 1.41 (m, 4H), 1.36 – 1.21 (m,
405 20H), 0.90 (t, J = 7.0 Hz, 6H); $^{13}$C NMR (125 MHz, $CDCl_3$): δ 140.3, 128.8, 128.0,
406 126.6, 58.6, 53.8, 31.9, 29.6, 29.3, 27.5, 27.0, 22.7, 14.1; LRMS (ESI+): m/z 332.33
407 $[M+H]^+$, 100%; HRMS (ESI): m/z calculated for $[C_{23}H_{42}N]^+$ $[M + H]^+$ 332.3317, found
408 332.3306.
409
410 **4.5.4. *N,N*-Dihexyldecan-1-amine 3.** To a solution of 1-iododecane 10 (2.75 mL, 12.9
411 mmol) in acetonitrile (60 mL) was added potassium carbonate (16.5 g, 129 mmol) and

412 dihexylamine 11 (3 mL, 12.9 mmol), then the mixture was stirred at reflux overnight.
413 The suspension was filtered to remove $K_2CO_3$ and washed with acetonitrile (3 × 50 mL),
414 then concentrated by rotary evaporation to yield the crude product (7.8 g). The crude
415 product was purified by automated column chromatography (100 g cartridge, 100%
416 petroleum benzene 2CV, then 0–60% ethyl acetate in petroleum benzene over 10 CV) to
417 yield the product as an oil (0.89 g, 21 %). $^1$H NMR (500 MHz , $CDCl_3$): δ 2.41 – 2.37
418 (m, 6 H), 1.38– 1.46 (m, 6 H), 1.35 – 1.21 (m, 28 H), 0.91 – 0.86 (m, 9 H); $^{13}$C NMR
419 (125 MHz , $CDCl_3$): δ 54.2,54.2, 31.9, 31.9, 29.7, 29.6, 29.6, 29.3, 27.7, 27.4, 26.9,
420 22.7, 14.1, 14.1; LRMS (ESI+): m/z 326.41 $[M+H]^+$, 100%; HRMS (ESI): m/z
421 calculated for $[C_{22}H_{48}N]^+$ $[M + H]^+$ 326.3787, found 326.3776.
422 **4.5.5. *N,N,N*-Trioctylammonium chloride 13.** To a solution of *N,N,N*-trioctylamine 12
423 (1.0 g, 2.80 mmol) in 1,4-dioxane (1.0 mL) was added 4M HCl in dioxane (2.80 mL,
424 11.2 mmol) in an ice bath. Instantaneously a precipitate formed and after 5 min this was
425 collected by vacuum filtration to yield a white solid (1.08 g, 99 % yield). $^1$H NMR (500
426 MHz, $CDCl_3$) δ 11.40 (br s, 1 H), 2.90 (td, J = 4.7, 12.5 Hz, 6 H), 1.74 – 1.66 (m, 6 H),
427 1.29 – 1.13 (m, 30 H), 0.79 (t, J = 7.0 Hz, 9 H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 52.2,
428 31.4, 28.8, 28.7, 26.6, 23.0, 22.3, 13.8.
429
430 **4.5.6. *N*-(But-3-yn-1-yl)-*N*-octyloctan-1-amine 16.** To a flask charged with potassium
431 carbonate (4.4 g, 31.8 mmol) and potassium iodide (880 mg, 5.30 mmol) was added
432 acetonitrile (72 mL) followed by dioctylamine 14 (8 mL, 26.5 mmol) and 4-bromo-1-
433 butyne 15 (2.74 mL, 29.2 mmol). The suspension was stirred at 60°C for 18 h,
434 then filtered and washed with acetonitrile (3 × 50 mL), and concentrated by rotary

435 evaporation to yield the crude product. This was purified by automated column
436 chromatography (100 g cartridge, 0%–15% ethyl acetate in petroleum benzine over 10
437 CV) to yield the product as a yellow oil (0.15 g, 2%). $^{1}$H NMR (500 MHz, $CDCl_3$):
438 δ 2.64 - 2.57 (m, 2 H), 2.39 - 2.31 (m, 4 H), 2.23 (dt, J = 2.7, 7.6 Hz, 2 H), 1.88 (t, J =
439 2.6 Hz, 1 H), 1.41 - 1.31 (m, 4 H), 1.27 - 1.14 (m, 20 H), 0.81 (t, J = 7.0 Hz, 6 H); $^{13}$C
440 NMR (125 MHz , $CDCl_3$): δ 83.3, 68.7, 54.0, 52.7, 31.8, 29.6, 29.3, 27.6, 27.2, 22.6,
441 16.7, 14.1; LRMS (ESI+): m/z 294.29 $[M+H]^+$, 100%; HRMS (ESI): m/z calculated for
442 $C_{20}H_{40}N^+$ $[MH]^+$ 294.3161, found 294.3157.
443
444 **4.5.7. 6-(4-(2-(Dioctylamino) ethyl)-1*H*-1,2,3-triazol-1-yl)-2-ethyl-1*H*-**
445 **benzo[de]isoquinoline-1,3(*2H*)-dione 18.** To a solution of 16 (400 mg, 1.24 mmol) and
446 6-azido-2-ethyl-1*H*-benzo[de]isoquinoline-1,3(*2H*)-dione 17 (365 mg, 1.61 mmol) in
447 tert-butanol: water (12.4 mL) were added copper sulphate hexahydrate (31.0 mg, 0.12
448 mmol) and ascorbic acid sodium salt (73.8 mg, 0.37 mmol) then the solution was stirred
449 at 60ºC overnight. Precipitants were removed by filtration and washed with acetonitrile
450 (3 x 50 mL), then the filtrate was concentrated by rotary evaporation and purified by
451 automated column chromotography (100g cartridge, 0%–20% methanol in
452 dichloromethane over 8 CV). Purified fractions were re-purified by automated reversed
453 phase chromotography (30g cartridge C18 silica (Biotage), 0%–90% acetonitrile in
454 water over 17 CV) and concentrated to yield the product as a yellow solid (10 mg, 1%).
455 $^{1}$H NMR (500 MHz, $CDCl_3$): δ 8.73 (d, J = 7.6 Hz, 2 H), 8.21 (dd, J = 0.9, 8.5 Hz, 1 H),
456 8.09 (s, 1 H), 7.89 - 7.81 (m, 2 H), 4.29 (q, J = 7.1 Hz, 2 H), 3.67 - 3.56 (m, 2 H), 3.49 -
457 3.37 (m, 2 H), 3.25 - 3.06 (m, 4 H), 1.87 - 1.70 (m, 4 H), 1.38 (t, J = 7.0 Hz, 3 H), 1.34 -

458 1.23 (m, 10 H), 0.89 (t, J = 7.0 Hz, 6 H); $^{13}$C NMR (126 MHz, $CDCl_3$): δ 162.4, 161.9,
459 142.1, 136.8, 131.2, 129.6, 128.1 (128.09), 128.1 (128.08), 127.7, 125.4, 124.1, 123.2,
460 122.6, 122.1, 51.6, 51.3, 34.8, 30.6, 28.7, 28.0, 25.7, 22.0, 21.5, 20.1, 13.0, 12.3; LRMS
461 (ESI+): m/z 560.38 $[M+H]^+$, 100%; HRMS (ESI): m/z calculated for
462 $C_{34}H_{50}N_5O_2^+$ $[MH]^+$ 560.3965, found 560.3950.
463
464 **4.6. Screening of Synthetic Compounds.** MRSA (provided by Dr John Merlino at
465 Concord Hospital, Sydney), *P. aeruginosa* PAO1 (provided by Dr Jim Manos,
466 University of Sydney) and *E. coli* EC958 (provided by Professor Mark Schembri,
467 University of Queensland) were grown in LB media. *M. tuberculosis* H37Rv was grown
468 in Middlebrook 7H9 media (Bacto, Australia) containing albumin, dextrose, and
469 catalase (ADC), 20% Tween 80, and 50% glycerol (Sigma-Aldrich, Australia). The
470 synthetic samples were suspended in DMSO and diluted in series to final concentrations
471 of 100 µM – 0.001µM, using sterile $dH_2O$. Antibacterial activities and toxicity were
472 determined for each compound via broth dilution resazurin assay as described above.
473
474 ■ **ASSOCIATED CONTENT**
475 **Supporting Information**
476
477 Bioactivity and toxicity screening data, HPLC fractionation and purification protocols,
478 plus mass spectrometry data (HRMS spectra, tables of daughter ions, and proposed
479 fragmentation pathways) for natural products and synthetic compounds.
480
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## ■ Author Contribution Statement

PJR, JAT, MD, NP (mass spectrometry) and MPS (synthesis) conceived and designed the experiments; MD, NP (mass spectrometry), MPS (synthesis) and GT (bioassays with virulent *M. tuberculosis*) performed the experiments; MD, NP, MPS, GT, JAT and PJR analyzed the data; MD, PJR, MPS and JAT 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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