1 **Discovery of antimicrobial compounds from *Lendenfeldia*, *Ircinia* and** 2 ***Dysidea* sponges using bioassay guided fractionation of marine extracts**

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24 *Supporting Information*

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

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56 1. INTRODUCTION

57 Marine ecosystems have long been a rich source of bioactive natural products, in the
58 search for interesting molecules and novel therapeutic agents.¹⁻⁵ Many interesting and
59 structurally diverse secondary metabolites have been isolated from marine sources and
60 characterised over the last 70 years.⁶⁻⁹ Yet the first ‘drugs from the sea’ were only
61 approved in the early 2000s: the cone snail peptide ziconotide (ω -conotoxin MVIIA) in
62 2004 to alleviate chronic pain,¹⁰ and sea squirt metabolite trabectedin in 2007 for
63 treatment of soft-tissue sarcoma.¹¹ Interest in marine natural products has continued to
64 grow since,^{6, 8-9} spurred in part by the spread of antimicrobial resistant pathogens and the
65 need for new drugs to combat them.³

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

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76 In the search for new antimicrobial agents, we screened a set of marine extracts¹⁶ to
77 determine activity against antibiotic resistant microorganisms using a high-throughput
78 screening (HTS) assay. Fractionation and purification of active components by high-

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

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

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

93 **2. RESULTS AND DISCUSSION**

94 **2.1. Identification of Active Marine Extracts.** To identify marine samples with
 95 activity against MRSA, 1434 compounds from the AIMS Bioresources Library¹⁶
 96 (provided by the Queensland Compound Library,¹⁷ now called Compounds Australia¹⁸)
 97 were screened in a resazurin cell viability assay. Of the samples tested, 29 inhibited the
 98 growth of MRSA by greater than 50% compared to non-treated controls (Figure S1,
 99 Supporting Information). Minimum inhibitory concentrations (MICs) were determined
 100 for the 23 most promising samples, representing extracts and fractions from the phyla
 101 Porifera (90%), Echinodermata (5%) and Chordata (5% (Table 1 and Table S1). The
 102 five most active samples showed MICs at 31.3 $\mu\text{g mL}^{-1}$ (all Porifera samples), while
 103 another four samples returned MICs of 62.5 $\mu\text{g mL}^{-1}$ (also all Porifera).

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 105 Cytotoxicity screens against HepG2, HEK 293, A549 and THP-1 cell lines were
 106 performed to define the cytotoxicity profile of the most active samples. Pleasingly, all
 107 the samples most active against MRSA were also nontoxic to the cell lines tested
 108 (Tables 1, S1, S2 and S3).

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

Entry	AIMS Sample Code	QCL Sample Number	MIC ($\mu\text{g mL}^{-1}$)	Cytotoxicity (% cell survival)		
			MRSA	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

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124 **2.2. Isolation and Characterization of Bioactive Compounds.** Following the primary
125 screening of the AIMS library and selection of positive hits, HPLC was used to separate
126 and isolate active compounds, guided by bioassays against MRSA. Extracts were
127 fractionated by analytical HPLC (see Experimental section and Supporting Information
128 for further details), and fractions evaluated for bioactivity. Preparative scale HPLC was
129 carried out on each bulk sample to isolate the active component (Table S4, Figures S2–
130 S7), and tandem mass spectrometry (MS/MS) methods used to deduce structures (Table
131 2 and Supporting Information).^{19-20 21} Insufficient quantities were obtained for NMR
132 analyses.

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134 Active components were isolated and characterised for five of the six extracts shown in
135 Table 1: aromatic amines **1/2** from the *Lendenfeldia sp.* samples (AIMS Sample Code
136 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.

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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 (Δm = 0.11 ppm) RDBE = 0	3	Figure S8	Table S5 Figure S9
20608 [†]	332.33115 [MH] ⁺	[C ₂₃ H ₄₂ N] ⁺ Calc = 332.33118 (Δm = 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 (Δm = 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

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153 **2.3. Synthesis.** To validate the structures proposed for the natural products, and to
 154 explore the potential of these a compounds as bioactive agents, a series of tertiary amine
 155 derivatives of compounds **1–5** were synthesised from 1-bromooctane **6**, *m*-toluidine **7**,
 156 *p*-toluidine **8**, benzyl amine **9**, 1-iododecane **10**, *N,N*-dihexylamine **11** and *N,N,N*-
 157 trioctylamine **12** (Schemes 1 and 2). *o*-Toluidine is carcinogenic and therefore was not
 158 used in synthetic experiments.

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160 **Scheme 1. Synthesis of Amines Related to Natural Products 1 and 2.** i. *m*-
 161 toluidine **7**, K₂CO₃, KI, MeCN, 60 °C, overnight, 8 %; ii. *p*-toluidine **8**, K₂CO₃, KI,
 162 MeCN, 60 °C, overnight, 6 %; iii. benzylamine **9**, K₂CO₃, MeCN, 82 °C, overnight,
 163 19 %.

164 Compounds **1a**, **1b** and **2a** were prepared using 1-bromooctane **6** to alkylate *m*-toluidine
 165 **7**, *p*-toluidine **8**, benzyl amine **9** respectively (Scheme 1), giving three compounds
 166 based on the active component of AIMS sample 20608. Compound **3**, the active
 167 component of AIMS sample 19033, was prepared by reacting 1-iododecane **10** with
 168 *N,N*-dihexylamine **11** (Scheme 2), while *N,N,N*-trioctylamine hydrochloride **13** was

169 prepared from the free amine **12** as a simple and readily accessible analogue of the
170 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**, K₂CO₃, MeCN, 82 °C, overnight, 21 %; ii. HCl in 1,4-dioxane, 5 min, 99%.

175 Finally, to broaden the scope of this work, we sought to combine the tertiary amine
176 structures elucidated in this study with a triazolyl naphthalimide pendant, recently
177 shown to be an important component of a new class of anti-tubercular agents.²³⁻²⁴ Thus
178 *N,N*-dioctylamine **14** was alkylated with 4-bromo-1-butyne **15**, and the resulting alkyne
179 product **16** ‘clicked’²⁵ with 6-azido-2-ethyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione
180 **17**²⁶ to afford the naphthalimide derivative **18** (Scheme 3).

Scheme 3. Synthesis of Naphthalimide Derivative 18. i. 4-bromo-1-butyne **15**, K₂CO₃, KI, MeCN, 60 °C, overnight, 2 %; ii. 6-azido-2-ethyl-1*H*-

186 benzo[*de*]isoquinoline-1,3(2*H*)-dione **17**, CuSO₄, sodium ascorbate, ^tBuOH/ H₂O,
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.

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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	<i>M. tuberculosis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
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 OD₆₀₀=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.

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Table 4. Toxicity of Synthetic Compounds to Cell Lines.

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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

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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.**

<i>M. tb</i> 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;

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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.*

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

259

260 Thus we assayed 1434 extracts from the AIMS Bioresources Library¹⁶ against MRSA,
261 finding three samples that have a promising combination of high antibacterial activity
262 and low toxicity to mammalian cells: AIMS Sample Codes 20608, 26051 and 19033
263 (Table 1). Samples of these extracts were subjected to HPLC purification and bioassay
264 guided fractionation, enabling bioactive components to be isolated in low yield (<1 mg).
265 Then high resolution MS and tandem MS analysis was used to decipher structures
266 (Table 2, Figure 1). The proposed structures are all tertiary amines or quaternary amine
267 salts: aromatic amines **1/2** (from *Lendenfeldia* sample number 20608), aliphatic amine **3**
268 (from *Dysidea herbacea* sample number 19033), the aliphatic tertiary amine **4** and
269 quaternary amine salt **5** (from *Ircinia sp.* sample number 26051).

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

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

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

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284 **4. EXPERIMENTAL METHODS**

285 **4.1. General.** Chemical reagents were purchased from BDH Chemicals and Sigma
286 Aldrich (Castle Hill, Sydney, Australia) and used as supplied unless otherwise indicated.

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288 **4.2. Natural Product Library.** Natural product extracts were provided by the
289 Australian Institute of Marine Science (AIMS), Townsville, Queensland as part of the
290 AIMS Bioresources Library,¹⁶ via the Queensland Compound Library,¹⁷ (now called
291 Compounds Australia¹⁸). Crude extracts had been partially fractionated by AIMS/ QCL
292 to generate a library of 1434 samples, supplied in DMSO (100%) solution and stored at -
293 80 °C. Original concentrations as provided were 5 mg mL⁻¹. Stock solutions were made
294 by diluting these samples by a factor of 1:10 in dH₂O and stored at -80 °C.

295

296 **4.3. Screening the AIMS Extract Library Against MRSA.** Each test sample (10 μL)
 297 was dispensed into a separate well of a 96 well microtiter plates (final sample
 298 concentration 0.5 mg mL^{-1}) using sterile dH_2O . Bacterial suspension ($90 \text{ }\mu\text{L}$, $\text{OD}_{600\text{nm}}$
 299 0.001) was added to each well and plates were incubated at 37°C for 18 hours. To
 300 determine MIC of samples, crude extracts were added to wells in sequential 2-fold
 301 dilutions and incubated with diluted bacteria as described previously.²³⁻²⁴ Resazurin (10
 302 μL ; 0.05% w/v) was added and plates were incubated for 3 h at 37°C . The bioactivity of
 303 extracts was calculated by visual determination of colour change within wells or
 304 detection of fluorescence at 590 nm using a FLUOstar Omega microplate reader (BMG
 305 Labtech, Germany).

306
 307 **4.4. Evaluating Toxicity of AIMS Extract Library.** Human alveolar epithelial cells
 308 (A549),²⁷ Madin-Darby canine kidney epithelial cells (MDCK),²⁸ human leukaemia
 309 cells (THP-1),²⁹ human hepatocellular carcinoma cells (Hep-G2),³⁰ and human
 310 embryonic kidney cells 293 (HEK293)³¹ were grown and differentiated in complete
 311 RPMI (Roswell Park Memorial Institute Medium) and DMEM (Dulbecco's Modified
 312 Eagle's medium) tissue culture media (RPMIc and DMEMc). To determine toxicity of
 313 the AIMS extract library, 2×10^5 of each cell type were added to a 96-well plate and left
 314 for 48 h at 37°C to adhere. Extract samples at a final concentration of 0.5 mg mL^{-1} were
 315 added to the wells, then incubated for 7 days in a humidified $5\% \text{ CO}_2$ incubator at 37°C .
 316 Then resazurin ($10 \text{ }\mu\text{L}$ of 0.05% w/v) was added and after 4 h, fluorescence
 317 measured as described previously. Cell viability was calculated as percentage
 318 fluorescence relative to untreated cells.

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320 **4.4. Purification of Natural Products from Extracts and Structure Elucidation**

321 **4.4.1. High Performance Liquid Chromatography (HPLC) Purification.** Samples

322 were separated using analytical (Waters 2695 Alliance with Waters 2996 PDA, Sunfire
323 reversed-phase column, and WFIII fraction collector) and preparative (Waters 600
324 HPLC pump, Phenomenex reversed-phase column, Waters 2487 UV detector and WFIII
325 fraction collector) HPLC systems with UV detectors at 254 and 280 nm, employing a
326 gradient of solvents A (dH₂O) and B (acetonitrile) with trifluoroacetic acid (0.01%).
327 Extract mixtures were kept at 4 °C until injection, then extract sample (100 µL) was
328 injected onto an analytical Waters X-bridge C18 100 A (4.6 × 250 mm, 5 µm) reversed-
329 phase column on the same analytical HPLC system described above. The mobile phase
330 was obtained using 100% acetonitrile and 0% water at a flow rate of 1 ml min⁻¹ at 30 °C
331 over 80 min. Fractions, separated every 60 s, were collected. Purified fractions were
332 flash-frozen in liquid nitrogen then freeze-dried overnight. The resulting fractionated
333 extracts were re-suspended in DMSO and antibacterial activity versus MRSA was
334 determined as described above.

335

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

340

341 The gradient for AIMS extracts 19033, 20608, and 26051 was 0% B initially, increased
342 to 40% B over 20 min, then to 100% at 60 min, held at 100% for 10 min, and finally a

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

346 **4.4.2. Identification and Structure Elucidation.**

347 Purified compounds were identified and characterised using MS. High resolution ESI
348 mass spectra (HRMS) were recorded on a Bruker Apex Qe 7T Fourier Transform ion
349 cyclotron resonance mass spectrometer with an Apollo II ESI MTP ion source with
350 samples (in CH₃CN:H₂O 1:1) infused using a Cole Palmer syringe pump at 180 µL h⁻¹.
351 Where required, low resolution ESI tandem MS was performed on a Bruker amaZon SL
352 ion trap via syringe infusion or by injection into a constant flow stream with a rheodyne
353 valve and an Alltech HPLC pump (mobile phase methanol, flow rate 0.3 mL min⁻¹)
354 connected to an Apollo II ESI MTP ion source in positive ion mode. Tandem mass
355 spectra of the [M+H]⁺ parent ion were obtained manually up to MS⁵ (depending on
356 sensitivity). Spectra were acquired in positive ion mode using a 1–4 Da isolation
357 window, with the excitation amplitude manually optimized for each spectrum to have
358 the selected mass at ~10% of the height of the largest fragment. Data analysis was
359 performed for both high resolution MS and low resolution tandem MS data using
360 Bruker DataAnalysis 4.0 with smart formula assuming C, H, N, O, Na (0-1), mass error
361 <2 ppm, C:H ratio 3 maximum, even electron (or both for tandem MS data). The results
362 of high resolution MS data analysis were further refined manually by comparing
363 isotopic fine structures of simulations where possible (resolving power > 200,000) to
364 further eliminate potential formulae within the 2ppm mass error window (particularly
365 ¹⁵N, ¹⁸O, ²H, ¹³C and ¹³C₂ isotopes and confirm no ³⁴S presence).

366

367 4.5. Synthesis

368 **4.5.1. *N,N*-Diocetyl-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 %). ¹H NMR (500 MHz, CDCl₃): 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); ¹³C NMR (125 MHz, CDCl₃): 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 [C₂₃H₄₂N]⁺,
380 [M + H]⁺ 332.3317, found 332.33054.

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382 **4.5.2. *N,N*-Diocetyl-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 %). ¹H NMR (500 MHz, CDCl₃): 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 $[\text{M}+\text{H}]^+$, 100%; HRMS (ESI): m/z calculated for $[\text{C}_{23}\text{H}_{42}\text{N}]^+ [\text{M}+\text{H}]^+$
 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 °C 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 benzene over 10 CV) yielded N-benzyl-N-octyloctan-1-amine 2a as a
 403 colourless oil (1.15 g, 19 %). ^1H 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 $[\text{M}+\text{H}]^+$, 100%; HRMS (ESI): m/z calculated for $[\text{C}_{23}\text{H}_{42}\text{N}]^+ [\text{M} + \text{H}]^+$ 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 %). 1H 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). 1H 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%). ¹H NMR (500 MHz, CDCl₃):
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); ¹³C
440 NMR (125 MHz, CDCl₃): 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 C₂₀H₄₀N⁺ [MH]⁺ 294.3161, found 294.3157.

443

444 **4.5.7. 6-(4-(2-(Diocetyl-amino) ethyl)-1H-1,2,3-triazol-1-yl)-2-ethyl-1H-**
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-1H-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 60°C 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 chromatography (100g cartridge, 0%–20% methanol in
452 dichloromethane over 8 CV). Purified fractions were re-purified by automated reversed
453 phase chromatography (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 ¹H NMR (500 MHz, CDCl₃): 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); ¹³C NMR (126 MHz, CDCl₃): 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 C₃₄H₅₀N₅O₂⁺ [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 dH₂O. 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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485

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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

515 spectrometry; MS/MS, tandem mass spectrometry; MTC, minimum toxic concentration;
 516 NMR, nuclear magnetic resonance; RPMI, Roswell Park Memorial Institute Medium;
 517 THP-1, human leukaemia cells; Hep-G2, human hepatocellular carcinoma cells; VRSA,
 518 vancomycin-resistant *Staphylococcus aureus*.

519

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