Identification of Bioactive Compounds from Marine Natural Products and Exploration of Structure-Activity Relationships (SAR)

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Abstract: Marine natural products (MNPs) have been an important and rich source for antimicrobial drug discovery and an effective alternative to control drug resistant infections. Herein, we report 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: aniline 1, benzylyamine 2, tertiary amine 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 antibiotic activities of the compounds, and their isolation as natural products have not been reported before. Using tandem mass spectrometry (MS) analysis, potential structures of the bioactive fractions were assigned, leading to the hit validation of potential compounds through synthesis, and commercially available compounds. This method is a novel strategy to overcome insufficient quantities of pure material (NPs) for drug discovery and development which is a big challenge for pharmaceutical companies. The antibacterial screening of the marine extracts has shown several of the compounds exhibited potent in-vitro antibacterial activity, especially against methicillin-resistant Staphylococcus aureus (MRSA) with minimum inhibitory concentration (MIC) values between 15.6 to 62.5 microg mL⁻¹. Herein, we also report structure activity relationships of a diverse range of commercial structurally similar compounds. The structure-activity relationships (SAR) results demonstrate that modification of the amines through linear chain length, and inclusion of aromatic rings, modifies the observed antimicrobial activity. Several commercially available compounds, which are structurally related to the discovered molecules, showed broad-spectrum antimicrobial activity against different test pathogens with a MIC range of 50 to 0.01 µM. The results of cross-referencing antimicrobial activity and cytotoxicity establish that these compounds are promising potential molecules, with a favourable therapeutic index for antimicrobial drug development. Additionally, the SAR studies show that simplified analogues of the isolated compounds have increased bioactivity.

Keywords: marine natural products; tandem mass spectrometry (MS); structure—activity relationships (SAR); antimicrobial drug discovery; multidrug resistant bacteria; methicillin-resistant Staphylococcus aureus

1. Introduction

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 [1]. 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 [2], and Mycobacterium tuberculosis resistant to all first-line agents [3–5], 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 therefore hold the potential for the discovery of new and efficacious an-
timicrobial drugs [6–9]. Almost 70 percent of the Earth’s surface is covered by ocean, representing a huge reserve of natural biological and chemical diversity on our planet [10]. Marine ecosystems have long been a rich source of bioactive natural products in the search for interesting molecules and novel therapeutic agents [6,7,11–15]. Many interesting and structurally diverse secondary metabolites have been isolated from marine sources over the last 70 years [8,9,14,15]. In addition, the preclinical pharmacology of seventy-five compounds isolated from marine organisms have been reported to have biological activities [16]. Yet the first drugs from marine natural products were only approved in the early 2000s: the cone snail peptide ziconotide (ω-conotoxin MVIIA) in 2004 to alleviate chronic pain [17], and sea squirt metabolite trabectedin in 2007 for the treatment of soft-tissue sarcoma [18]. Marine natural products (MNPs) have displayed exceptional potency and potential as anticancer therapeutics [19]. The interest in MNPs has continued to grow [8,14,15], spurred in part by the spread of antimicrobial resistant pathogens and the need for new drugs to combat them [6].

The most prolific marine organisms are sponges [6], and the oldest metazoans on earth belong to the phylum Porifera [20]. The Demospongiae are the most abundant class of Porifera, representing 83% of described species [20,21], and has the largest number of bioactive compounds [19]. The genus Lendenfeldia is known as a source of sulfated sterols [22] and metabolites from the Lendenfeldia species have anti-HIV, anti-tumor [23], anti-inflammatory, antifouling [24] activities but they lack antimicrobial activity [22]. Secondary metabolites of the genus Ircinia and Dysidea are prime candidates for further study to unveil biological metabolites with antibacterial activity (Figure 1) [15,25–27].

![Figure 1. Ansalactams B–D displayed moderate antibacterial activity towards MRSA [15].](image-url)

In the search for new antimicrobial agents, we screened a set of marine extracts [28] to determine activity against antibiotic resistant microorganisms using a high-throughput screening (HTS) assay. The 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 (Figure 1 and Figures S20, S21 and S25).

Following on from these studies, we decided to screen commercially available compounds, with structural similarity to the amine scaffolds we identified, synthesised [29] and ascididemin-like compounds. Thus, we found these molecules to be an interesting potential structure for further development, focusing on increasing antimicrobial potency by investigating the structure—activity relationship (SAR). Based on these facts, in this work, small-molecule libraries of commercial analogues were studied to increase the structural variability and improving the pharmacological properties of the final drugs.

2. Results and Discussion

2.1. Identification of Active Marine Extracts

To identify marine samples with activity against MRSA, 1434 compounds from the AIMS Biosources Library [28] (provided by the Queensland Compound Library, Brisbane, Australia [30], now called Compounds Australia [31]) 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. The 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 \( \mu \text{g mL}^{-1} \) (all Porifera samples), while another four samples returned MICs of 62.5 \( \mu \text{g mL}^{-1} \) (also all Porifera). Cytotoxicity screens against HepG2, HEK 293, A549 and THP-1 cell lines were performed with concentrations from 500 \( \mu \text{g mL}^{-1} \) to 7.8 \( \mu \text{g mL}^{-1} \) 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 (Table 1).

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     | 19,033           | SN00733110        | 31.25 ± 0.9 | 91 ± 1.2 | 91 ± 0.3 | 98 ± 1.6 |
| 2     | 20,608           | SN00760947        | 31.25 ± 1.3 | 97 ± 1.0 | 101 ± 2.9 | 102 ± 0.7 |
| 3     | 20,608           | SN00760956        | 31.25 ± 0.4 | 100 ± 4.4 | 106 ± 1.8 | 95 ± 1.0 |
| 4     | 20,608           | SN00760958        | 62.5 ± 2.2 | 98 ± 0.6 | 108 ± 14 | 95 ± 2.5 |
| 5     | 26,051           | SN00731005        | 62.5 ± 2.8 | 101 ± 1.0 | 110 ± 2.7 | 98 ± 2.0 |
| 6     | 24,307           | SN00730755        | 31.25 ± 1.0 | 100 ± 1.0 | 106 ± 1.0 | 96 ± 4.0 |
| 7     | 25,663           | SN00732222        | 15.6 ± 1.5 | 100 ± 1.6 | 89 ± 2.0 | 97 ± 0.0 |
| 8     | 26,104           | SN00734298        | 62.5 ± 1.0 | 98 ± 0.5 | 100 ± 1.1 | 98 ± 1.1 |
| 9     | 22,565           | SN00739718        | 31.25 ± 0.4 | 100 ± 0.0 | 99 ± 0.4 | 97 ± 1.0 |

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. The 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 and Figures S1–S10), NMR and tandem mass spectrometry (MS/MS) methods used to deduce potential structures for compounds (Table 2 and Supporting Information) [32–34]. Insufficient quantities were obtained for positive ion high resolution mass spectrometry (HRMS) analyses.
Table 2. Key high resolution mass spectrometry (HRMS) data for bioactive samples, and proposed structures as shown in the Supplementary Materials file 1.

| Sample | Molecular Ion (m/z) | Molecular Formula | Proposed Structures | Spectra | MS/MS | NMR Analyses |
|--------|---------------------|-------------------|---------------------|---------|-------|--------------|
| 19,033 † | 326.37813 [M + H]⁺ | [C_{22}H_{48}N]⁺ | Calc. = 326.37802 (Δm = 0.11 ppm) RDBE = 0 | 3 | Figure S1 | Table S2 Figure S11 - |
| 20,608 | 332.33115 [M + H]⁺ | [C_{23}H_{40}N]⁺ | Calc. = 332.33118 (Δm = 0.03 ppm) RDBE = 4 ‡ | 1,2 | Figure S3 | Table S3 Figure S13 - |
| 26,051 | 368.42508 [M + H]⁺ | [C_{25}H_{54}N]⁺ | Calc. = 368.42495 (Δm = 0.13 ppm) RDBE = 0 | 4,5 | Figure S5 | Table S4 Figure S15 - |
| 25,663 | 306.0635 [M+Na]⁺ | [C_{18}H_{6}ON_{3}]⁺ | Calc. = 306.0637 (Δm = 0.8 ppm) RDBE = 16 | 6 | Figure S7 | - Table S5 Figure S20 |
| 25,663 | 361.9925 [M + H]⁺ | [C_{18}H_{8}BrN_{3}O]⁺ | Calc. = 361.9923 (Δm = 0.5 ppm) RDBE = 16 | 7 | Figure S7 | - Table S6 Figure S21 |
| 26,104 | 467.117 [M + H]⁺ | [C_{30}H_{17}N_{2}O_{4}]⁺ | Calc. = 467.118 (Δm = 1.9 ppm) RDBE = 0 | 8 | Figure S9 | - Table S7 Figures S22–S25 |

† 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 eight of the nine extracts shown in Table 1: aniline 1 and benzylamine 2 from the Lendenfeldia sp. samples (AIMS Sample Code 20608, Table 1 entries 2–4, Figure 2); tertiary aliphatic amine 3 from the Dysidea herbacea extract (AIMS Sample Code 19033, Table 1 entry 1, Figure 2); aliphatic tertiary amine 4 and quaternary amine salt 5 from Ircinia gigantea (AIMS Sample Code 26051, Table 1 entry 5, Figure 2); ascididemin 6 and 2-bromoascididemin (2-Bromoleptoclinidinone) 7 from the Flavobranchia samples (AIMS Sample Code 25663, Table 1 entries 7, Figure 2 and Figures S26 and S27); and halisulfate 8 from Ircinia (AIMS Sample Code 26104, Table 1 entry 8, Figure 2 and Figures S26 and S27). The aniline/amines 1–5 have not previously been identified as natural products, nor have their antimicrobial activities been assessed.

Figure 2. Cont.
Compounds 6, 7 [35–37] and 8 [38–41] have previously been reported (Figure 2), and in the current study these structures were confirmed by comparison of data with HRMS, NMR and literature values. The antimycobacterial activity of compounds 6, 7 and 8 with wide range of MIC against different microorganisms have also been reported [42,43].

2.3. Structure—Activity Relationship

The SAR was investigated through commercially available compounds of three groups of molecules, namely the tertiary amines, benzyl substituted amines [29], and the ascididemin structural motifs. To investigate the number of carbons in the linear chains, commercially available amines compounds with structures similar to synthesised compounds 3 and 13 [29] (Figure 3), such as triethylamine, tributylamine and trihexylamine were screened; in addition, the primary and secondary amines hexylamine, dihexylamine and dioctylamine were screened. Biological activities of commercial compounds are based on the molecular weight of the proposed structures.
The results show a general trend of increased activity with increasing chain length of the tertiary amines (triethylamine, tributylamine, trihexylamine and trioctylamine), as seen in the bioactivity against MRSA, *E. coli* and *P. aeruginosa* (Table 3). In addition, the trend in the primary, secondary, and tertiary hexylamines, show increased activity with an increasing number of alkyl chains; hexylamine, showed activity against MRSA, *E. coli* and *P. aeruginosa* with MICs of 50 to 100 µM, which all improve to MICs of 3 to 6.5 µM for trihexylamine (Table 3). Interestingly, trioctylamine with eight carbon alkyl chains, showed strong activity against *M. tuberculosis* H37Rv (MIC of 0.02 µM). Furthermore, dicyclohexylamine has poorer bioactivity than dihexylamine, suggesting potential benefits of linear chains. This trend in structural modification did not improve activity against *M. tuberculosis* H37Rv or alter the toxicity to cell lines except HEK 293 (Table 3).

Further structures related to 1a, 1b and 2a [29] and the sequence of benzylamine, dibenzylamine, tribenzylamine, were investigated (Figure 4). Observations from this experiment correlate with the increased substitution leading to increased bioactivity against *P. aeruginosa*. Anti-*P. aeruginosa* activity was improved to MIC = 6.5 with tribenzylamine (Table 4).

![Figure 4. The commercially available amines with structures similar to 1a, 1b and 2a [29].](image-url)

The third group of compound structures we looked at for SAR analysis are related to the structural motifs of ascididemin (Figure 5), which is a pentacyclic aromatic alkaloid, bearing similarity to motifs of quinoline and phenanthroline. Therefore, isoquinoline, quinoline, 4,7-phenanthroline, 1,7-phenanthroline, 1,10-phenanthroline and bipyridine structures (Tables 4 and 5) were evaluated for bioactivity inspired by the ascididemin structure. The results show isoquinoline and quinoline displayed improved anti-microbial activity, especially against *M. tuberculosis* (Table 5). Furthermore, the fused tricyclic cores of 1,10-phenanthroline, when compared with the more flexible 2,2′-bipyridine, showed activity against *M. tuberculosis* with MIC = 0.1 but also displayed toxicity for 4 cell lines tested: HepG2, HEK 293 and THP-1. Interestingly, structures in which the two nitrogens were closer to each other have more activity against *M. tuberculosis* and *P. aeruginosa* while the opposite situation was seen with MRSA and *E. coli* activity; this can be observed by comparing the results of 1,10-phenanthroline with those observed for 4,7-phenanthroline. The toxicity assays determined that the A549 cell line is more resistant to 4,7-phenanthroline. Alongside the acceptable antimicrobial activity of 4,7-phenanthroline and 1,7-phenanthroline, also show limited toxicity against mammalian cells (Table 5). Meanwhile, 1,10-phenanthroline displayed variable toxicity and antimicrobial activity (Table 5).
Table 3. Screening of commercially available amines with structures similar to 3 and 13 (The biological activities are based on the molecular weight of the proposed structures).

| Name and CAS no. | Structures | MRSA | E. coli | P. aeruginosa | M. tuberculosis | HepG2 | HEK 293 | A549 | THP-1 |
|-----------------|------------|------|---------|---------------|----------------|-------|---------|------|-------|
| Hexylamine 111-26-2 | | >100 | >100 | 50 | >100 | >100 | >100 | >100 | >100 |
| Dihexylamine 143-16-8 | | 12.5 | 12.5 | 6.2 | >100 | >100 | >100 | >100 | >100 |
| Dioctylamine 1120-48-5 | | 6.2 | 6.2 | 3 | >100 | >100 | >100 | >100 | >100 |
| Triethylamine 121-44-8 | | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| Tributylamine 102-82-9 | | >100 | >100 | 50 | >100 | >100 | >100 | >100 | >100 |
| Trihexylamine 102-86-3 | | 3 | 3 | 6.5 | >100 | >100 | 50 | >100 | >100 |
| Trioctylamine 1116-76-3 | | 12.5 | 25 | 25 | 0.02 | >100 | 6 | 50 | 3.1 |
Table 4. Screening of commercially available amines with structures related to 1a, 1b and 2a.

| Name and CAS no.       | Structures | MRSA | E. coli | P. aeruginosa | M. tuberculosis | HepG2 | HEK 293 | A549 | THP-1 |
|------------------------|------------|------|---------|---------------|----------------|-------|---------|------|-------|
| Benzylamine 100-46-9   | ![Benzylamine](image) | >100 | >100    | >100          | >100           | >100  | >100    | >100 | >100  |
| Dicyclohexylamine 101-83-7 | ![Dicyclohexylamine](image) | >100 | >100    | 12.5          | >100           | >100  | >100    | >100 | >100  |
| Dibenzylamine 103-49-1 | ![Dibenzylamine](image) | >100 | >100    | 12.5          | >100           | >100  | >100    | >100 | >100  |
| Tribenzylamine 620-40-6| ![Tribenzylamine](image) | >100 | >100    | 6.5           | >100           | >100  | >100    | >100 | >100  |
| 2,2'-Bipyridine 366-18-7 | ![2,2'-Bipyridine](image) | >100 | >100    | >100          | 0.1            | 3     | 3       | >100 | 50    |
Figure 5. The commercially available compounds with structures inspired by ascididemin.

To further study the phenanthroline compounds (Figure 6) we evaluated 4,7-dimethyl-1,10-phenanthroline, neocuproine hemihydrate, 5,6-dimethyl-1,10-phenanthroline, 3,4,7,8-tetramethyl-1,10-phenanthroline, 1,10-phenanthroline-5,6-dione, 5-nitro-1,10-phenanthroline, bathophenanthroline, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, and bathocuproine disulfonic acid for bioactivity (Table 6). Modifying the three aromatic rings further, by adding methyl groups as in 4,7-dimethyl-1,10-phenanthroline, neocuproine hemihydrate and 5,6-dimethyl-1,10-phenanthroline (Figure 7) improved the anti-bacterial activity of the compounds but also increased their toxicity effect on all cell lines tested (Table 6). Maintaining one of the methyl groups in the rings as in 5,6-dimethyl-1,10-phenanthroline reduced the activity against *P. aeruginosa* (Table 6).

Figure 6. The compounds with structures inspired by ascididemin which include three aromatic rings.

Figure 7. The compounds with structures inspired by ascididemin which include three aromatic rings plus further modifications.
Table 5. Screening of commercially available compounds with structures inspired by ascididemin.

| Name and CAS no.         | Structures | MRSA | E. coli | P. aeruginosa | M. tuberculosis | HepG2 | HEK 293 | A549 | THP-1 |
|--------------------------|------------|------|---------|---------------|----------------|-------|---------|------|-------|
| Isoquinoline 119-65-3    | ![Isoquinoline](image) | >100 | >100    | >100          | 0.1            | 3     | >100    | 50   | 3     |
| Quinoline 91-22-5        | ![Quinoline](image) | >100 | >100    | >100          | 0.1            | 3     | 50      | 50   | 3     |
| 4,7-phenanthroline 230-07-9 | ![4,7-phenanthroline](image) | 1.5  | 1.5     | 2.5           | 2.5            | >100  | >100    | >100 | >100  |
| 1,7-phenanthroline 230-46-6 | ![1,7-phenanthroline](image) | 25   | 25      | 2.5           | >100           | >100  | >100    | >100 | >100  |
| 1,10-phenanthroline 66-71-7 | ![1,10-phenanthroline](image) | >100 | 50      | 0.3           | 0.1            | 0.1   | 50      | 3    | 0.1   |
Table 6. Screening of compounds with structures inspired by ascididemin which include three aromatic rings.

| Name and CAS no.                  | Structures | MRSA | E. coli | P. aeruginosa | TB. H37Rv | HepG2 | HEK 293 | A549 | THP-1 |
|-----------------------------------|------------|------|---------|---------------|-----------|-------|---------|------|-------|
| 4,7-Dimethyl-1,10-Phenanthroline 3248-05-3 | ![Structure](image1) | 0.3  | 0.3     | 0.1           | 0.1       | 0.1   | 0.1     | 0.1  | 0.1   |
| Neocuproine hemihydrate 34302-69-7 | ![Structure](image2) | 0.09 | 0.3     | 0.04          | 0.1       | 0.1   | 0.1     | 6.2  | 12    |
| 5,6-Dimethyl-1,10-phenanthroline 3002-81-1 | ![Structure](image3) | 0.3  | 0.78    | >100          | 0.1       | 0.1   | 0.1     | 0.1  | 0.1   |
| 3,4,7,8-Tetramethyl-1,10-phenanthroline 1660-93-1 | ![Structure](image4) | 0.3  | 0.3     | 0.3           | 0.1       | 0.1   | 0.1     | 0.1  | 0.1   |
| 1,10-Phenanthroline-5,6-dione 27318-90-7 | ![Structure](image5) | 0.04 | 0.04    | 0.04          | 0.1       | 0.1   | 0.1     | 0.1  | 0.1   |
Increasing the number of methyl substituents to four (3,4,7,8-tetramethyl-1,10-phenanthroline) or incorporating carbonyl groups (1,10-phenanthroline-5,6-dione) or a nitro group (5-nitro-1,10-phenanthroline) instead of the methyl groups enhanced the activity of the compounds (Tables 6 and 7). A different behaviour was only observed against *P. aeruginosa* in the presence of the nitro group. The inclusion of two additional aromatic rings as pendants on the phenanthroline core (bathophenanthroline) showed impressive antibacterial activity with MIC = 0.09 µM for MRSA, MIC = 0.019 µM for *E. coli*, MIC = 0.03 µM for *P. aeruginosa* and MIC = 0.01 µM for *M. tuberculosis*. Furthermore, cross-referencing the biological activity and toxicity of these molecules demonstrated they are more potent against the microorganisms than against mammalian cells. In addition, adding two methyl groups or two sulfonate groups, as in 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline and bathocuproine disulfonic acid, respectively, resulted in the reduced activity of molecules against bacteria, but also a reduction in toxicity against the A549 and THP-1 cells. However, including the sulfonate groups improved the activity against the *P. aeruginosa* with MIC = 0.19 µM (Table 7).

A summary of all activities of the commercially available amines that were screened is presented in Table 8. The potential antimicrobial activity of commercial materials which are structurally related to ascididemin (Tables 6 and 7) was identified by bioactivity screening. 1,10-phenanthroline-5,6-dione at MIC 0.04 µM was found to be a potent inhibitor of MRSA, while bathophenanthroline at MIC 0.019 µM, MIC 0.03 µM and MIC 0.01 µM showed strong activity against *E. coli*, *P. aeruginosa* and *M. tuberculosis*, respectively. These results reinforce the relationship between structures and anti-microbial activity for the synthesized compounds. For example, in the third group of compounds tested (Tables 6 and 7) bioactivity was optimal for 1,10-phenanthroline-5,6-dione containing carbonyl groups, and biological activity depends on structural properties such as the length of the aliphatic linker, and the side chain of these compounds. The results of these experiments further suggest that hydrophobic compounds, such as dioctylamine (Table 3) and bathophenanthroline (Table 7), have a more pronounced effect on the inhibition of bacterial growth, as has been reported previously [41–47].

Furthermore, the side chain length significantly affects the hydrophobicity of these compounds, and subsequently the logP values, which are important molecular descriptors in determining biological activity. The presence of aromatic rings with short pendant groups and nitrogen-containing rings in 2,2'-bipyridine significantly reduced the activity of the molecules against the Gram-positive and Gram-negative pathogens tested, however the compounds were active against mycobacteria (Table 4). This suggests that the mechanism of action may be based on targeting complex cell wall structures, such as those present in mycobacteria. Many factors influence the activity of a molecule, including side chain length, symmetry, length of the alkyl chain, the presence of charged pyridinium moiety, the presence and position of the carboxylate in the structure, the position of the methyl group, the distance between the two pyridinium moieties and the chain linker between the two pyridinium rings. Thus, the mechanism of action of these molecules may be based on the space between the rings and distance between charged moieties, which possibly manifests through interaction with membrane sites. These results are in agreement with previous reports [48,49]; however, to clarify such hypotheses and determine important factors in bioactivity further pharmacological and biophysical studies are necessary to determine the mechanisms of action.
| Name and CAS no.                                      | Structures | MRSA  | E. coli | P. aeruginosa | TB. H37Rv | HepG2 | HEK 293 | A549 | THP-1 |
|-------------------------------------------------------|------------|-------|---------|---------------|------------|-------|---------|------|-------|
| 5-Nitro-1,10-phenanthroline 4199-88-6                 | ![Structure](image1.png) | 0.09  | 0.78    | >100          | 0.1        | 0.1   | 0.1     | 12   | 0.1   |
| Bathophenanthroline 1662-01-7                         | ![Structure](image2.png) | 0.09  | 0.019   | 0.03          | 0.01       | 1.5   | 25      | 3    | 13    |
| 2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline 4733-39-5 | ![Structure](image3.png) | 25    | 0.78    | 50            | 50         | 0.1   | 0.1     | >100 | >100  |
| Bathocuproinedisulfonic acid sodium salt 1257642-74-2 | ![Structure](image4.png) | >100  | >100    | 0.19          | 1.2        | 50    | 0.1     | >100 | >100  |
Table 8. Summary of anti-bacterial activity and toxicity of compounds inspired by the natural products characterised in this project.

| Sample Name, structure and CAS no | Minimum Inhibitory/Toxicity Concentration of Drug (µM) |
|----------------------------------|-------------------------------------------------------|
|                                  | MRSA  | *E. coli*  | *P. aeruginosa* | *M. tuberculosis H37Rv* | HepG2 | HEK 293 | A549 | THP-1 |
| Hexylamine                        |       |            |                |                        |       |         |      |       |
| ![Hexylamine Structure](image)    | >100  | >100       | 50             | >100                    | >100  | >100     | >100 | >100  |
| Dihexylamine                      |       |            |                |                        |       |         |      |       |
| ![Dihexylamine Structure](image)  | 12.5  | 12.5       | 6.2            | >100                    | >100  | 100      | >100 | >100  |
| Dioctylamine                      |       |            |                |                        |       |         |      |       |
| ![Dioctylamine Structure](image)  | 6.2   | 6.2        | 3              | >100                    | >100  | >100     | >100 | >100  |
| Triethylamine                     |       |            |                |                        |       |         |      |       |
| ![Triethylamine Structure](image) | >100  | >100       | >100           | >100                    | >100  | >100     | >100 | >100  |
| Tributylamine                     |       |            |                |                        |       |         |      |       |
| ![Tributylamine Structure](image) | 3     | 3          | 6.5            | >100                    | >100  | 50       | 100  | >100  |
| Trihexylamine                     |       |            |                |                        |       |         |      |       |
| ![Trihexylamine Structure](image) |       |            |                |                        |       |         |      |       |
| Triocylamine                      |       |            |                |                        |       |         |      |       |
| ![Triocylamine Structure](image)  |       |            |                |                        |       |         |      |       |
| Sample Name, structure and CAS no | Minimum Inhibitory/Toxicity Concentration of Drug (µM) |
|------------------------------------|-----------------------------------------------------|
|                                   | MRSA       | E. coli | P. aeruginosa | M. tuberculosis H37Rv | HepG2 | HEK 293 | A549 | THP-1 |
| Benzylamine 100-46-9              | >100       | >100    | >100          | >100               | >100  | >100    | >100 | >100 |
| Dicyclohexylamine 101-83-7        | >100       | >100    | 12.5          | >100               | >100  | >100    | >100 | >100 |
| Dibenzylamine 103-49-1            | >100       | >100    | 12.5          | >100               | >100  | >100    | >100 | >100 |
| Tribenzylamine 620-40-6           | >100       | >100    | 6.5           | >100               | >100  | >100    | >100 | >100 |
| 2,2′-Bipyridyl 366-18-7           | >100       | >100    | >100          | 0.1                | 3     | 3       | >100 | 50   |
Table 8. Cont.

| Sample Name, structure and CAS no | Minimum Inhibitory/Toxicity Concentration of Drug (µM) |
|---------------------------------|------------------------------------------------------|
|                                 | MRSA |  E. coli |  P. aeruginosa |  M. tuberculosis H37Rv |  HepG2 |  HEK 293 |  A549 |  THP-1 |
| Isoquinoline 119-65-3            | >100 | >100 | >100 | 0.1 | 3 | 3 | >100 | 50 |
| Quinoline 91-22-5                | >100 | >100 | >100 | 0.1 | 3 | 3 | 50 | 50 |
| 4,7-phenanthroline 230-07-9      | 1.5 | 1.5 | 2.5 | 2.5 | >100 | >100 | >100 | >100 |
| 1,7-phenanthroline 230-46-6      | 25 | 25 | 2.5 | >100 | >100 | >100 | >100 | >100 |
| 1,10 phenanthroline 66-71-7      | >100 | 50 | 0.3 | 0.1 | 0.1 | 0.1 | 50 | 3 |
Table 8. Cont.

| Sample Name, structure and CAS no | Minimum Inhibitory/Toxicity Concentration of Drug (µM) |
|----------------------------------|--------------------------------------------------------|
|                                  | MRSA | E. coli | P. aeruginosa | M. tuberculosis H37Rv | HepG2 | HEK 293 | A549 | THP-1 |
| 4,7-Dimethyl-1,10-Phenanthroline | 0.3  | 0.3     | 0.1           | 0.1                    | 0.1   | 0.1     | 0.1  | 0.1   |
| Neocuproine hemihydrate         | 0.09 | 0.3     | 0.04          | 0.1                    | 0.1   | 0.1     | 6.2  | 12    |
| 5,6-Dimethyl-1,10-Phenanthroline| 0.3  | 0.78    | >100          | 0.1                    | 0.1   | 0.1     | 0.1  | 0.1   |
| 3,4,7,8-Tetramethyl-1,10-Phenanthroline| 0.04 | 0.04   | 0.04         | 0.1                    | 0.1   | 0.1     | 0.1  | 0.1   |
| 5-Nitro-1,10-Phenanthroline     | 0.09 | 0.78    | >100          | 0.1                    | 0.1   | 0.1     | 12   | 0.1   |
| Sample Name, structure and CAS no                             | Minimum Inhibitory/Toxicity Concentration of Drug (µM) |
|---------------------------------------------------------------|------------------------------------------------------|
|                                                              | MRSA  | *E. coli* | *P. aeruginosa* | *M. tuberculosis H37Rv* | HepG2 | HEK 293 | A549 | THP-1 |
| Bathophenanthroline                                          | 0.09  | 0.019     | 0.03           | 0.01                  | 1.5   | 25      | 3    | 13    |
| 1662-01-7                                                    |       |           |                |                       |       |         |      |       |
| 2,9-Dimethyl-1,7-diphenyl-1,10-phenantroline 4733-39-5      | 25    | 0.78      | 50             | 50                    | 0.1   | 0.1     | >100 | >100  |
| Bathocuproinedisulfonic acid sodium salt 1257642-74-2       | >100  | >100      | 0.19           | 1.2                   | 50    | 0.1     | >100 | >100  |
3. Materials and Methods

3.1. General

Chemical reagents were purchased from BDH Chemicals and Sigma Aldrich (Castle
Hill, Sydney, Australia) and used as supplied unless otherwise indicated. Commercial
amines of >97% purity were purchased from Sigma Aldrich and were used in this study
(CAS numbers are added in the SI table).

3.2. Natural Product Library

Natural product extracts were provided by the Australian Institute of Marine Sci-
ence (AIMS), Townsville, Australia as part of the AIMS Bioresources Library [28], via
the Queensland Compound Library, Brisbane, Australia [29], (now called Compounds
Australia [30]). 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.

3.3. 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 dH2O. For determination of MIC, extracts (250 to 0.5 µg mL−1) or synthesised com-
pounds (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 h (MRSA, P. aeruginosa PAO1, E. coli EC958) or 7 days for M. tuberculosis H37Rv
as described previously [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, Ortenberg, Germany).
Percentage survival was calculated in comparison to the average of untreated control wells
after normalising for background readings.

3.4. Evaluating Toxicity of AIMS Extract Library

Toxicity of AIMS Extract Library. Human alveolar epithelial cells (A549) [51], Madin-
Darby canine kidney epithelial cells (MDCK) [52], human leukaemia cells (THP-1) [53],
human hepatocellular carcinoma cells (Hep-G2) [54] and human embryonic kidney cells 293
(HEK293) [55] 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, from the Thermo Fisher Scientific, Waltham, MA, USA) by adding
10% FBS, 200 µM L-glutamine and 1 mM HEPES buffer solution. To determine toxicity
of the AIMS extract library, 2 × 105 of each cell type were added to a 96-well plate and
left for 48 h in a humidified 5% CO2 incubator at 37 °C to adhere. Extract samples at a
final concentration of 0.5 mg mL−1 were added to the wells, then the plates incubated for
4 more days in a humidified 5% CO2 incubator at 37 °C. Then, resazurin (10 µL of 0.05% w/v)
was added and after 4 h, fluorescence measured as described previously. The cell
viability was calculated as percentage fluorescence relative to untreated cells.

3.5. Purification of Natural Products from Extracts and Structure Elucidation

3.5.1. High Performance Liquid Chromatography (HPLC) Purification

The samples were separated using analytical (Waters 2695 Alliance pump 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, employ-
ning 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. The 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.

3.5.2. 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 CH\(_3\)CN:H\(_2\)O 1:1) infused using a Cole Palmer syringe pump at 180 µL h\(^{-1}\). Where required, low resolution ESI tandem MS data were obtained 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 \(^{15}\)N, \(^{18}\)O, \(^{2}\)H, \(^{13}\)C and \(^{13}\)C\(_2\) isotopes and confirm no \(^{34}\)S presence).

Identification and Elucidation of Fractions 44 from Sample Number 25663 (Compound 6)

\(^{1}\)H NMR (500 MHz, DMSO-\(d_6\)): \(\delta\) 9.20 (d, \(J = 5.6\), 1H, H-21), 9.10 (dd, \(J = 4.5, 1.7, 1H, H-17\)), 9.00 (dd, \(J = 8.0, 1.1, 1H, H-6\)), 8.92 (d, \(J = 5.6, 1H, H-19\)), 8.63 (dd, \(J = 7.9, 1.7, 1H, H-15\)), 8.44 (d, \(J = 8.0, 1H, H-3\)), 8.09 (ddd, \(J = 8.0, 7.2, 1.1, 1H, H-2\)), 8.02 (ddd, \(J = 8.0, 7.2, 1.1, 1H, H-1\)), 7.80 (dd, \(J = 7.9, 4.5, 1H, H-16\)). HRMS (ESI): \(m/z\) 306.0635; the molecular formula C\(_{18}\)H\(_9\)ON\(_3\) gives an expected [M + Na]\(^{+}\) ion at 306.0637 (err 0.8 ppm). This molecular formula gives a rdbe 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 ascididemin \([36,37,55]\).

Identification and Elucidation of Fractions 58 from Sample Number 25663 (Compound 7)

\(^{1}\)H NMR (500 MHz, DMSO-\(d_6\)): \(\delta\) 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 rdbe 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 ascididemin \([36,37,55]\).
bonds. The NMR and mass data agree with those in the literature for the known compound 2-bromoascididemin [36,37].

Identification and Elucidation of Purified Compounds from Sample Number 26104 (Compound 8)

\(^{1}H\) NMR (500 MHz, DMSO-\(d_6\)): \(\delta\) 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 agree with those in the literature for the known compound halisulfate [55–58].

4. Conclusions

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, Brisbane, Australia [28] against MRSA, finding five samples that have a promising combination of high antibacterial activity and low toxicity to mammalian cells. The NMR, high-resolution MS and tandem MS analysis was used to decipher structures. The proposed structures form samples 20608, 26051 and 19033 are all tertiary amines or quaternary amine salts: aniline 1 and benzylamine 2 (from \(Lendenfeldia\) sample number 20608), aliphatic amine 3 (from \(Dysidea herbacea\) sample number 19033), aliphatic tertiary amine 4 and quaternary amine salt 5 (from \(Ircinia\) sp. sample number 26051). The proposed structures from samples 25663 and 26104 are acididemin 6 and 2-bromoascididemin 7 (from \(Flavobranchia\) sample number 25663), and halisulfate 8 (from \(Ircinia\) sample number 26104). The compounds discovered in this study add to the growing arsenal of antimicrobial agents from the sea [6,12], and offer interesting new avenues in the quest for new, effective agents to combat the growing scourge of multidrug resistant bacteria.

The structure activity relationship (SAR), close analogues studies have demonstrated active scaffolds of isolated natural products and synthetic derivatives compounds. These natural product analogues may be optimized further, inspiring further studies in the search for new lead compounds to fight against microbial pathogens. They will be helpful in broadening the understanding of the biological effects of the synthetic analogues of ascididemin and therefore may aid the development of novel anticancer and antimicrobial agents. Often the natural properties of natural compounds, such as molecular weight, a large number of chiral centres, and/or complex 3D structures can hamper the drug development process and make them unsuitable for synthesis or non-drug-like [59,60]. Interestingly, the simple molecules which have been found in this study may be suitable as potential drug leads.

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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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