Antibacterial and antifungal screening of natural products sourced from Australian fungi and characterisation of pestalactams D–F

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Abstract

Eighteen natural products sourced from Australian micro- or macro-fungi were screened for antibacterial and antifungal activity. This focused library was comprised of caprolactams, polyamines, quinones, and polyketides, with additional large-scale isolation studies undertaken in order to resupply previously identified compounds. Chemical investigations of the re-fermented culture from the endophytic fungus *Pestalotiopsis* sp. yielded three caprolactam analogues, pestalactams D–F, along with larger quantities of the known metabolite pestalactam A, which was methylated using diazomethane to yield 4-*O*-methylpestalactam A. The chemical structures of the previously undescribed fungal metabolites were determined by analysis of 1D/2D NMR and MS data. The structure of 4-*O*-methylpestalactam A was confirmed following single crystal X-ray diffraction analysis. The antibacterial and antifungal activity of all compounds was assessed, which identified three compounds, (1*S*,3*R*)-austrocortirubin, (1*S*,3*S*)-austrocortirubin and 1-deoxyaustrocortirubin with mild activity (100 μ M) against Gram-positive isolates and one compound, 2-hydroxy-6-methyl-8-methoxy-9-oxo-9*H*-xanthene-1-carboxylic acid, with activity against *Cryptococcus neoformans* and *Cryptococcus gattii* at 50 μ M.

Keywords

Pestalotiopsis; Amphisphaeriaceae; fungal natural products; pestalactams A–F; pestalactam; caprolactam; X-ray crystal structure; antibacterial; antifungal; MIC screening.

1. Introduction

Over the decades, fungi have been the sources of numerous bioactive and novel secondary metabolites (Turner, 1971; Turner and Aldridge, 1983) with much of this research focused on terrestrial macro- and micro-fungi. Fungi have also been a prominent source of therapeutic drugs (Wasser and Weis, 1999); in addition, many plant-derived drugs have subsequently been identified in fungi (Nicoletti et al., 2008) readily providing the opportunity to obtain pharmaceutical compounds through fermentation. It has only recently been discovered that endophytic fungi, that live asymptotically within plant tissues, can evolve to produce the same or similar bioactive compounds as their host plants (Zhao et al., 2011).

Research over the past two decades has revealed that fungi sourced from marine habitats as well as endophytic fungi derived from plants are proving to be a rich source of unique chemistry (Bugni and Ireland, 2004; Rateb and Ebel, 2011; Saleem et al., 2007; Schueffler and Anke, 2014; Tan and Zou, 2001). A recent analysis of bioactive natural products and drugs currently in the clinical pipeline suggest that 67-80% of therapeutically useful drugs have been derived from pre-existing drug-productive biota families (Zhu et al., 2011). As part of our continuing research on the bioactive chemistry of endophytic fungi from Australian plants (Davis, 2005; Davis et al., 2005a; Davis et al., 2010; Davis et al., 2008; Davis et al., 2005b; Healy et al., 2004) we recently reported the isolation and structure elucidation of three novel caprolactam natural products that we named pestalactams A–C (**4**, **6**, **7**) from *Pestalotiopsis* sp. (BRIP 39872) (Davis et al., 2010). Preliminary biological investigations of these secondary metabolites identified moderate cytotoxic and antimalarial activities (Davis et al., 2010). Although >55 caprolactam natural products have been reported in the literature (Dictionary of Natural Products, 2015) caprolactams with unsaturation between C-3/C-4 and C-6/C-7 are rare, and only seven metabolites are known to contain this functionality. These include pestalactams A–C (Davis et al., 2010), silvaticamide (Yamazaki et al., 1981), hygrocine B (Cai et al., 2005) and ceratamines A and B (Manzo et al., 2003).

Of the ~230 *Pestalotiopsis* species described to date only a fraction of these have been investigated for their chemistry or biological activity (Yang et al., 2012). The known chemistry spans a diverse variety of chemical classes including: terpenoids, coumarins, alkaloids, polyketides, quinones, phenolics and chromones (Yang et al., 2012). Of interest, in addition to the pestalactams, *Pestalotiopsis* spp. have afforded a variety of pharmacologically active natural products, such as the cytotoxic pestaloficiols I–L (Liu et al., 2009), pupukeanane derivatives (Liu et al., 2011) and (+)-torreyanic acid (Lee et al., 1996), along with the antifungal agents pestalachloride A (Li et al., 2008) and pestacin (Harper et al., 2003).

Replication of the chemical investigations of the static rice fermentation cultures of *Pestalotiopsis* sp. (BRIP 39872) yielded a further three previously undescribed caprolactam analogues pestalactams D–F (**1–3**), as well as the known fungal compounds pestalactam A (**4**) and tyrosol (**8**). The previously isolated pestalactams B and C were not identified in these repeat rice fermentations. Herein, we report the isolation and structure elucidation of pestalactams D–F (**1–3**) along with the antibacterial and antifungal evaluation of these compounds along with 15 other fungi-derived natural products, the majority of which have been isolated from Australian micro- or macro-fungal sources.

2. Results and discussion

The fungus *Pestalotiopsis* sp. (BRIP 39872) was re-fermented on static damp rice for 28 days before the culture was extracted with EtOAc. This crude extract was fractionated using semi-preparative C₁₈ HPLC (MeOH/H₂O/0.1% TFA) and all UV-active fractions from each purification step were analysed by LRESIMS and ¹H NMR spectroscopy. This afforded the new compounds pestalactams D–F (**1–3**), as well as the previously reported metabolites pestalactam A (**4**) and tyrosol (**8**) (Fig. 1).

Pestalactam D (**1**) was isolated as a white stable solid. The (–)-LRESIMS of **1** displayed two pseudo-molecular ions at m/z 228 and 230 in a relative intensity ratio of 3:1, indicating the molecule contained one chlorine atom. The ^1H and ^{13}C NMR data (**Table 1**) in combination with the (–)-HRESIMS data allowed a molecular formula of $\text{C}_{10}\text{H}_{12}\text{ClNO}_3$ to be assigned to **1**. The ^1H NMR spectrum displayed only six unique resonances, two of which (δ_{H} 11.40 and 11.07) were deemed to be exchangeable protons following addition of D_2O to a $\text{DMSO}-d_6$ solution of **1**. The remaining protons at δ_{H} 5.90, 2.27, 1.89 and 0.88 were attached to carbons resonating at δ_{H} 107.6, 45.4, 28.7 and 22.1, respectively, following HSQC data analysis. The ^{13}C NMR spectrum of **1** presented nine unique carbon signals and comparison of the ^1H and ^{13}C NMR data of **1** with those of pestalactam A (**4**) revealed a high degree of structural homology between the two metabolites. The only NMR spectroscopic differences noted were that the C-7 substituted side chain resonances for **4** [δ_{H} 1.16 (s, 6H), 2.27 (s, 2H), 4.98 (brs, 1H); δ_{C} 29.1 (2C), 48.3 (1C), 69.8 (1C)] had been replaced in **1** [δ_{H} 0.88 (s, 6H), 1.89 (m, 1H), 2.27 (d, 1H); δ_{C} 22.1 (2C), 28.7 (1C), 45.4 (1C)]. COSY data analysis of **1** readily established an isobutyl moiety that was subsequently linked to C-7 via HMBC and ROESY correlations (**Fig. 2**). Hence, 3-chloro-4-hydroxy-7-isobutyl-1*H*-azepine-2,5-dione or pestalactam D was assigned to structure **1**.

The minor metabolite, pestalactam E (**2**), was purified as a stable amorphous solid. Analysis of the LRESIMS data for **2** indicated that this compound was devoid of chlorine, and had a molecular weight that differed to **1** by 34 Da; these data suggested that the chlorine atom in **1** had been replaced with a hydrogen atom in **2**. This was further supported by the ^1H NMR data (**Table 1**) of **2** that contained one extra sp^2 proton (δ_{H} 6.12) compared with pestalactam D. (+)-HRESIMS and 2D NMR data analysis confirmed that **2** was the dechloro derivative of **1**, and was assigned as 4-hydroxy-7-isobutyl-1*H*-azepine-2,5-dione.

Pestalactam F (**3**) was also obtained as a white amorphous solid. In a similar manner to **2**, LRESIMS data analysis showed that this molecule was also devoid of chlorine. The ^1H and ^{13}C NMR data (**Table 1**) in conjunction with the (+)-HRESIMS allowed a molecular formula of

C₁₁H₁₅NO₄ to be assigned to **3**. Comparison of the NMR data (**Table 1**) of **3** with pestalactam A (Davis et al., 2010) clearly identified that pestalactam F also contained a caprolactam ring system with a 2-hydroxy-2-methylpropyl side-chain attached at C-7. Additional NMR signals (δ_{H} 3.69 / δ_{C} 59.1) associated with a methoxy group were observed for **3**, and this was attached to C-3 of the caprolactam based on HMBC correlations from both δ_{H} 3.69 and 10.67, to δ_{H} 138.5 (C-3). Thus the chemical structure 4-hydroxy-7-(2-hydroxy-2-methylpropyl)-3-methoxy-1*H*-azepine-2,5-dione was assigned to pestalactam F.

With the larger quantities of pestalactam A (**4**, 19 mg) obtained from these studies we decided to generate a pestalactam analogue for inclusion in the biological evaluations of this project. Thus compound **4** was methylated using diazomethane in Et₂O and MeOH; followed by acidic work-up and semi-preparative C₈ HPLC (MeOH/H₂O/0.1% TFA) this afforded 4-*O*-methylpestalactam A (**5**, 3.2 mg, 21% yield) and unreacted pestalactam A (2.3 mg). The structure of **5** was established as 3-chloro-7-(2-hydroxy-2-methylpropyl)-4-methoxy-1*H*-azepine-2,5-dione by X-ray crystallography. An ORTEP-3 (Farrugia, 2012) representation of the molecule is shown in **Fig. 3**.

In the crystal lattice, the molecules form dimers disposed about a crystallographic inversion center through N-H...O and O-H...O hydrogen bonding between the amide group in the seven-membered ring and the hydroxyl group of the (CH₃)₂C(OH)CH₂- sidechain (**Fig. 4**).

Owing to the range of biological activities of *Pestalotiopsis*-derived metabolites we decided to add pestalactams A (**4**) and D (**1**), 4-*O*-methylpestalactam A (**5**), and tyrosol (**8**) to our developing fungi-based screening compound library, which consisted of the previously isolated and identified secondary metabolites pestalactams B (**6**) and C (**7**) (Davis et al., 2010), trichodermamide C (**9**) (Davis et al., 2008), 3-chloro-4-hydroxyphenylacetamide (**10**) (Davis et al., 2005b), 3-chloro-4-hydroxyphenylacetic acid (**11**) (Davis et al., 2005b), (-)-xylariamide A (**12**) (Davis, 2005), 2-hydroxy-6-methyl-8-methoxy-9-oxo-9*H*-xanthene-1-carboxylic acid (**13**) (Healy et al., 2004), 2-hydroxy-6-hydroxymethyl-8-methoxy-9-oxo-9*H*-xanthene-1-carboxylic acid (**14**) (Healy et al.,

2004), 2,8-dimethoxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ester (**15**) (Healy et al., 2004), pistillarin (**16**) (Capon et al., 2007; Choomuenwai et al., 2013; Holinsworth and Martin, 2009; Lee et al., 2011; Steglich et al., 1984), (1*S*,3*R*)-austrocortirubin (**17**) (Elsworth et al., 1999), (1*S*,3*S*)-austrocortirubin (**18**) (Gill et al., 1990; Gill and Steglich, 1987), 1-deoxyaustrocortirubin (**19**) (Gill et al., 1990; Gill and Steglich, 1987) and austrocortinin (**20**) (Archard et al., 1985). Compound **15** is a synthetic derivative of the natural product xanthone **13**, the synthesis of which has been previously reported (Healy et al., 2004).

This 20 membered library was screened for antibacterial activity against a panel of Gram-positive and Gram-negative bacterial isolates, and for antifungal activity against a panel of pathogenic yeasts (detailed in **Table S23**). The compound library was also back screened for cytotoxicity to further evaluate the potential of the antibacterial and antifungal active compounds. Most of the compounds were inactive against bacteria and fungi, but were non-toxic to the tested human cell lines, at the highest concentration tested of 100 μ M. However, four compounds showed mild antimicrobial potential (**Table 2**), one against fungi (**13** at 50 μ M) and three (**17–19** at 100 μ M) against Gram-positive bacteria. Interestingly there was no cross activity between the fungal active compound and the Gram-positive active compounds, which may suggest distinct modes of action of the compounds. Compound **13**, which shows therapeutic potential against *Cryptococcus neoformans* and *Cryptococcus gattii* was not toxic up to 50 μ M against HEK293 or HepG2 cell lines, but **17–19** that were active against Gram-positive strains, including clinical isolates, showed mild toxicity (**Table 2**).

3. Conclusion

In conclusion, this paper reports the isolation and structure elucidation of three previously undescribed caprolactam analogues, pestalactams D–F, from a *Pestalotiopsis* species along with the

synthesis and X-ray crystal structure of 4-*O*-methylpestalactam A (**5**). Pestalactams A–F, 4-*O*-methylpestalactam A, and 13 other natural products (or synthetic derivatives) from Australian micro- or macro-fungi were evaluated for antimicrobial activity. Compound **13** exhibited low toxicity and moderate antifungal activity against both *Cryptococcus* species tested.

4. Experimental

4.1 General

NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C chemical shifts were referenced to the solvent peak for DMSO- d_6 at δ_{H} 2.49 and δ_{C} 39.5. LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Daltonics Apex III 4.7e fourier-transform mass spectrometer. Melting points were determined using a Gallenkamp digital melting point apparatus and were uncorrected. IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer and a Jasco V650 UV spectrophotometer, respectively. Optical rotations were recorded on a Jasco P-1020 polarimeter. A Waters 600 pump equipped with a Waters 966 PDA detector and a Waters 717 Plus Autosampler connected to a Gilson FC204 fraction collector were used for semi-preparative HPLC separations. Alltech C₁₈ bonded silica, 35-75 μm , 150 Å and Alltech stainless steel guard cartridges (10 $\times$ 30 mm) were used for pre-adsorption work. A Phenomenex Luna C₁₈ 5 μm 100 Å (21.2 $\times$ 250 mm) column and a Hypersil C₈ BDS 5 μm 143 Å column (10 mm $\times$ 250 mm) were used for semi-preparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade, and the H₂O was Millipore Milli-Q PF filtered. All fungal culture media was purchased from Difco. All synthetic reagents and solvents were obtained from Sigma–Aldrich and used without further purification.

4.2 Collection and identification of fungus

Pestalotiopsis sp. was isolated from a surface sterilised piece of stem (~ 2 mm × 10 mm) from the plant *Melaleuca quinquenervia* collected from Toohey Forest, Queensland, Australia during February of 2003. Sterilisation of the stem fragment was performed by sequential immersion in 95% EtOH (1 min), 2.1% NaOCl (5 min) and 95% EtOH (1 min). The sterilised stem was immediately transferred to a potato dextrose agar (PDA) plate and incubated at 25 °C for 3 days. Developing mycelial colonies were subcultured to fresh PDA plates. Colonies of *Pestalotiopsis* sp. were persistently isolated and one of these cultures was retained (BRIP 39872) for further study. Members of *Pestalotiopsis* are easily recognised by their distinctive conidial morphology, although the determination and delimitation of species in this genus is particularly difficult (Jeewon et al., 2003). The identification of *Pestalotiopsis* sp. was based on the presence of acervular conidiomata that produced 4-septate conidia, 22–30 × 6–8 µm, with second, third and fourth cells pale to mid-brown; basal and apical cells hyaline, appendages on the apical cells mostly 2, unbranched, up to 20 µm long; basal appendage, when present, single, up to 10 µm long. A voucher specimen (BRIP39872) and living cultures were deposited at the Department of Employment, Economic Development and Innovation (DEEDI), 41 Boggo Rd, Dutton Park, QLD 4102, Australia.

4.3 Extraction and isolation

The fungal isolate (BRIP 39872) was initially grown on potato dextrose agar, until a prominent growth of mycelia was achieved. The mycelia covered agar was then cut into ~1 cm³ portions, and then transferred to six conical flasks (500 mL) each containing sterilised damp white rice (50 g rice plus 100 mL H₂O). The fermentation was allowed to proceed under static conditions at 25 °C for 28 days after which time the rice and mycelia were extracted with EtOAc (3 × 150

mL). The organic extracts were all combined and the solvent removed in vacuo to yield a dark brown gum (5.1 g). This crude extract was divided into 17 equal portions (~ 300 mg $\times$ 17), and each portion pre-adsorbed to C₁₈-bonded silica (~ 1 g), packed into a guard cartridge then attached to a semi-preparative C₁₈ Luna HPLC column. The HPLC solvent separation conditions consisted of isocratic conditions of 10% MeOH (0.1% TFA) / 90% H₂O (0.1% TFA) for the first min, followed by a linear gradient to 25% MeOH (0.1% TFA) / 75% H₂O (0.1% TFA) over 10 min, then another linear gradient to 75% MeOH (0.1% TFA) / 25% H₂O (0.1% TFA) over 100 min, then another linear gradient to MeOH (0.1% TFA) over 15 min; isocratic conditions of MeOH (0.1% TFA) were held for 15 min, all at a flow rate of 9 mL/min. One hundred and forty fractions (140 $\times$ 1 min) were collected for each run. From the 17 HPLC separations, similar fractions were combined then analysed by (+)-LRESIMS and ¹H NMR spectroscopy. Fractions 26–27 afforded tyrosol (**8**, 27.2 mg), fraction 28 afforded pestalactam F (**3**, 7.4 mg), fraction 38 yielded pestalactam A (**4**, 24.8 mg), fraction 56 afforded pestalactam E (**2**, 0.5 mg) and fraction 69 contained pestalactam D (**1**, 15.0 mg).

4.3.1 Identification of known compounds

Compounds **4** and **8** were identified as the previously reported natural products, pestalactam A (**4**) (Davis et al., 2010) and tyrosol (**8**) following NMR and MS data analysis and comparison with literature values.

4.3.2 Pestalactam D

White amorphous solid; UV λ_{max} (MeOH) (log ϵ) 268 (3.77), 323 (3.15), 368 (2.85) nm; IR ν_{max} (KBr) 3237 (br), 1665, 1582, 1549, 1530, 1493, 1462, 1411, 1333, 1248, 1168 cm⁻¹; ¹H (600 MHz) and ¹³C (150 MHz) NMR, see Table 1 & Table S25; (+)-LRESIMS m/z (rel. int.) 230 (100%) [³⁵Cl: M+H]⁺, 232 (33%) [³⁷Cl: M+H]⁺; (–)-LRESIMS m/z (rel. int.) 228 (100%) [³⁵Cl: M–H][–], 230 (33%) [³⁷Cl: M–H][–]; (+)-HRESIMS m/z 230.0517 (C₁₀H₁₃³⁵ClNO₃ [M+H]⁺ requires 230.0584).

4.3.3 Pestalactam E

White amorphous solid; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 1 & Table S26; (+)-LRESIMS m/z (rel. int.) 196 (100%) $[\text{M}+\text{H}]^+$; (+)-HRESIMS m/z 218.0739 ($\text{C}_{10}\text{H}_{13}\text{NO}_3\text{Na}$ $[\text{M}+\text{Na}]^+$ requires 218.0788).

4.3.4 Pestalactam F

White amorphous solid; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 1 & Table S27; (–)-LRESIMS m/z (rel. int.) 240 (100%) $[\text{M}-\text{H}]^-$; (+)-LRESIMS m/z (rel. int.) 242 (100%) $[\text{M}+\text{H}]^+$, 264 $[\text{M}+\text{Na}]^+$; (+)-HRESIMS m/z 264.0845 ($\text{C}_{11}\text{H}_{15}\text{NO}_5\text{Na}$ $[\text{M}+\text{Na}]^+$ requires 264.0842).

4.4 Methylation of pestalactam A

Pestalactam A (**4**, 14.0 mg, 0.058 mmol) was dissolved in dry MeOH (1.5 mL) and Et_2O (1.5 mL) then treated with excess $\text{CH}_2\text{N}_2\text{-Et}_2\text{O}$ at 0 °C for 30 min. The reaction was allowed to warm to rt overnight then the solvent were evaporated and the residue was purified using semi-preparative C_8 Hypersil HPLC and linear gradient from H_2O (0.1% TFA) to MeOH (0.1% TFA) in 20 min at a flowrate of 4 mL/min. Unreacted pestalactam A (**4**, 2.3 mg) eluted between 13.0 to 13.2 min while 4-*O*-methylpestalactam A (**5**, 3.2 mg, 21% yield) eluted at 14.1–14.3 min. Compound **5** formed crystalline material during the slow evaporation of a MeOH solution at 4 °C.

4.4.1 4-*O*-Methylpestalactam A

Stable red needles (MeOH); mp >200 °C; UV λ_{max} (MeOH) (log ϵ) 250 (4.12), 324 (3.49), 341 sh (3.34) nm; IR ν_{max} (KBr) 1703, 1668, 1564, 1456, 1386, 1233, 1130, 1063 cm^{-1} ; ^1H NMR ($\text{DMSO-}d_6$, 600 MHz): δ_{H} 10.83 (1H, brs, 1-NH), 5.81 (1H, s, H-6), 5.00 (1H, brs, 9-OH), 3.87 (3H, s, 4-OMe), 2.49 (2H, s, H-8), 1.16 (6H, s, H-10, H-11); ^{13}C NMR ($\text{DMSO-}d_6$, 125 MHz): δ_{C}

177.1 (C-5), 161.1 (C-4), 159.9 (C-2), 146.5 (C-7), 128.0 (C-3), 111.7 (C-6), 69.8 (C-9), 60.0 (4-OMe), 47.8 (C-8), 29.1 (2C, C-10, C-11); (–)-LRESIMS m/z (rel. int.) 258 (100%) [^{35}Cl : M–H] $^-$, 260 (33%) [^{37}Cl : M–H] $^-$; (+)-LRESIMS m/z (rel. int.) 260 (100%) [^{35}Cl : M+H] $^+$, 262 (33%) [^{37}Cl : M+H] $^+$; (+)-HRESIMS m/z 260.0685 ($\text{C}_{11}\text{H}_{15}^{35}\text{ClNO}_4$ [M+H] $^+$ requires 260.0684).

4.5 X-ray crystallography studies on 4-O-methylpestalactam A

X-ray diffraction data for 4-O-methylpestalactam A (**5**) were measured at 295(2) K using a Rigaku AFC7R four circle diffractometer (ω -2 θ scan mode, graphite monochromated Mo- $\text{K}\alpha$ radiation $\lambda = 0.71069$ Å). The structure was solved by the direct methods and refined by full matrix least squares refinement on F^2 using the WinGX software package (Farrugia, 2012) incorporating SHELXL-2013. Anisotropic thermal parameters were refined for non-hydrogen atoms; (x , y , z , U_{iso}) $_{\text{H}}$ on C and O atoms were included and constrained at estimated values. The amine proton was refined freely with isotropic thermal parameters. Conventional residuals at convergence are quoted; statistical weights were employed. ORTEP-3 (Farrugia, 2012) and PLATON (Spek, 2003) software was utilised to prepare material for publication. Full .cif deposition resides with the Cambridge Crystallographic Data Centre (CCDC No. 1416198). Copies can be obtained free of charge on application at the following address: <http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi>.

4.5.1 Crystal data for 4-O-methylpestalactam A

$\text{C}_{11}\text{H}_{14}\text{ClNO}_4$. $M = 259.68$, triclinic, space group $P-1$, $a = 5.358(3)$, $b = 11.117(4)$, $c = 11.152(3)$ Å, $\alpha = 105.26(2)$, $\beta = 102.96(3)$, $\gamma = 101.80(3)^\circ$, $U = 599.5(5)$ Å 3 , $Z = 2$, $D_c = 1.44$ g cm $^{-3}$, $\mu = 0.32$ mm $^{-1}$, Crystal size: $0.40 \times 0.20 \times 0.15$ mm. $T_{\text{min/max}} = 0.88, 0.95$. 2478 reflections collected, 2106 unique ($R_{\text{int}} = 0.018$), $R = 0.044$ [1621 reflections with $I > 2\sigma(I)$], $wRF^2 = 0.129$ (all data).

4.6 Antibacterial assays

The tested bacteria strains (detailed in **Table S23**) were cultured in Muller Hinton broth (MHB) (Bacto laboratories, Cat. no. 211443) at 37 °C overnight with shaking (200 RPM). A sample of each culture was then diluted 40-fold in fresh MHB broth and incubated with shaking (200 RPM) at 37 °C for 2-3 h. Compounds were first tested for their potential against a panel of four Gram-negative strains and MRSA. For those active at the single concentration of 100 µM (n=2) a dose response MIC (Minimum Inhibitory Concentration) assay was performed to further elucidate their activity. In this instance, bacteria was cultured in the same manner and the compounds were serially diluted two-fold across the wells of 96-well plates (Corning; Cat. No 3370, polystyrene plates) with concentrations ranging from 0.1–200 µM, and plated in duplicate (n=2). The resultant mid-log phase cultures were diluted to a concentration of 5×10^5 CFU/mL, then 50 µL was added to each well of the compound-containing plates, giving a final compound concentration range of 0.05–100 µM. Plates were covered and incubated at 37 °C for 18 h. MICs were determined visually, being defined as the lowest concentration showing no visible growth. Vancomycin (Sigma 861987), daptomycin (Molekula 64342447) and colistin sulfate (Sigma C4661) were used as positive inhibitor controls.

4.7 Antifungal assays

Broth microdilution assays were performed in accordance with the CLSI M27-A2 guidelines (Clinical and Laboratory Standards Institute 2002) modified for *Cryptococcus neoformans*. The yeast strains tested (detailed in **Table S23**) were cultured in Yeast Nitrogen Base medium (Becton Dickinson; 233520) supplemented with ammonium sulfate and 2% glucose, final inoculum concentration of $(1.5 \pm 1.0) \times 10^3$ cells/ml and incubated at 35 °C for 72 h (Ghannoum et

al. 1992). A 96-well plate format (Thermo Scientific; 167008) was employed for initial single compound concentrations (100 or 50 μ M) of the 20 compounds to identify those which exhibited antifungal activity. Compounds that inhibited growth of one or more species were investigated in greater detail via MIC assay, with concentrations ranging from 0.05–50 μ M; the MIC was defined as the concentration that prevented any discernible growth. Fluconazole (Sigma, F8929) was used as a positive control.

4.8 Cytotoxicity assay

HEK293 and HepG2 cells were seeded at 3000 and 5000 cells per well respectively, in a 384-well plate to a final volume of 20 μ L in DMEM medium (GIBCO-Invitrogen #11995-073), in which 2% of FBS was added. Cells were incubated for 24 h at 37 °C, 5% CO₂ to allow cells to attach to the plates. Test compounds were prepared as a stock solution at 10 mM in 100% DMSO. The highest possible concentration to test the compounds was 50 μ M, in order to keep the DMSO end-concentration at 0.5%. A 1:3 fold dilution series in serum free medium was created, and 20 μ L of each dilution was added to the cell culture wells, resulting in 1% FBS as the final concentration. The cells were incubated with the test compounds for 24 h at 37 °C, 5% CO₂. After the incubation, 10 μ M resazurin solution was added to each well and incubated for 2 h at 37 °C, 5% CO₂. Experiments were repeated at least three times. The fluorescence intensity (FI) was read using a Polarstar Omega fluorimeter with excitation/emission 560/590 nm. The data was analysed by GraphPad Prism 6 software. Results are presented as the average percentage of control $\pm$ SD for each set of duplicate wells using the following equation: Percentage Viability = $(FI_{\text{TEST}} - FI_{\text{Negative}}/FI_{\text{UNTREATED}} - FI_{\text{Negative}})*100$.

Acknowledgments

The authors acknowledge the National Health and Medical Research Council (NHMRC) for financial support (Grant APP1024314 to R.A.D, APP1049716 to J.A.F. and AF511105 to M.A.C.) and thank the Australian Research Council (ARC) for support towards NMR and MS equipment (Grant LE0668477 and LE0237908) and financial support (Grant LP120200339 to R.A.D.). We thank the Central Analytical Research Facility, Queensland University of Technology for access to single crystal X-ray diffraction facilities. We acknowledge G. MacFarlane (University of Queensland) for acquiring the HRESIMS measurements. All strains and cell lines were sourced from either ATCC (<http://www.atcc.org/>) or NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*) via BEI Resources (www.beiresources.org).

Appendix A. Supplementary data

Supplementary data [1D and 2D NMR spectra for pestalactams D–F (**1–3**) and 4-*O*-methylpestalactam A (**5**), a table containing the detailed information of bacteria strains and cell lines assayed against, a table containing single point antibacterial and antifungal screening (μM), and cell line cytotoxicity (μM), NMR data tables for pestalactams D–F (**1–3**)] associated with this article can be found in the online version at doi:

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