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# Waspergillamide A, a Nitro *depsi*-Tetrapeptide Diketopiperazine from an Australian Mud Dauber Wasp-Associated *Aspergillus* sp. (CMB-W031)

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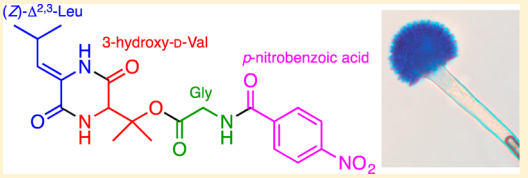
Supporting Information

**ABSTRACT:** Chemical profiling of extracts from a mud dauber wasp-associated fungus, *Aspergillus* sp. (CMB-W031), revealed a remarkably diverse array of secondary metabolites, with many biosynthetic gene clusters being transcriptionally responsive to specific culture conditions. Chemical fractionation of a jasmine rice cultivation yielded many known fungal metabolites, including the highly cytotoxic (−)-stephacidin B and an unprecedented nonribosomal peptide synthase derived nitro *depsi*-tetrapeptide diketopiperazine, waspergillamide A (**1**). All structures were assigned by detailed spectroscopic analysis and, where appropriate, chemical degradation and Marfey's analysis.




Phylogenetic analysis of a fungus, *Aspergillus* sp. (CMB-W031), isolated from a mud dauber wasp (order Hymenoptera, genus *Sceliphron*) collected from an urban environment in Brisbane, Australia, revealed it to be an undocumented strain most closely related to *Aspergillus westerdijkiae* NRRL 3174 (Figure S2). To explore the secondary metabolite capabilities of CMB-W031, we subjected it to analytical-scale (1.5 mL) cultivations under 38 different cultivation conditions, ranging across multiple media, shaken and static broth, as well as solid phase. Chemical profiling (HPLC-DAD-ESIMS) of the resulting solvent extracts revealed a remarkably diverse array of secondary metabolites. For example, jasmine rice, Czapek agar, and YES broth cultivations revealed complex secondary metabolite profiles with minimal overlap. Not surprisingly, biological profiles were also highly varied, with a jasmine rice grain cultivation exhibiting particularly potent cytotoxicity against three human carcinoma cell lines. Encouraged by these observations, we elected to investigate jasmine rice grain cultivations of CMB-W031.

Solvent extraction and partitioning followed by reversed-phase fractionation of a scaled-up jasmine rice cultivation of CMB-W031 yielded the known fungal metabolites penicillic acid,[1] dehydropenicillic acid,[2] (−)-stephacidin B,[3] (−)-versicolamide B,[4,5] (+)-preussin,[6,7] cycloanthranylproline,[8] circumdatin E,[8] and circumdatin G[9] together with an unprecedented nitro *depsi*-tetrapeptide diketopiperazine, waspergillamide A (**1**). Structures were assigned to all known metabolites by detailed spectroscopic analysis and, where appropriate, chemical degradation, Marfey's analysis, and literature comparisons (Supporting Information). The known fungal metabolite (−)-stephacidin B was deemed responsible for the observed cytotoxicity of the jasmine rice grain extract against human colon (SW620, $IC_{50}$ 3.2 $\mu$M), lung (NCI-H460, $IC_{50}$ 15 $\mu$M), and cervical (KB-3-1, $IC_{50}$ 6.4 $\mu$M) carcinoma cells. In addition to the known fungal metabolites, CMB-W031 yielded an unprecedented diketopiperazine, the structure elucidation of which is presented below.

HRESI(+)MS measurements on **1** revealed a sodiated molecular ion ($[M + Na]^+$) attributed to the molecular formula $C_{20}H_{24}N_4O_7$ (Δppm +1.4). Analysis of the 1D NMR (MeOH-$d_4$) data (Table 1) confirmed the presence of a trisubstituted olefin, four amide/ester carbonyls, and a *p*-disubstituted benzene. A distinctive UV−vis spectrum ($\lambda_{max}$ 275 nm, log $\varepsilon$ 3.73) and diagnostic NMR data supported assignment of $\Delta^{2,3}$-Leu, 3-OH-Val, Gly, and *p*-nitrobenzene residues. These observations account for all but one double-bond equivalent. Key 2D NMR (MeOH-$d_4$) HMBC and COSY correlations permitted assembly of the planar structure for **1** as indicated (Figure 1). Consideration of the predicted lowest energy Newman projection about C-3‴ to C-4‴ in **1** is consistent with a *trans*-antiparallel-like orientation for H-3‴ and H-4‴, as evidenced by $J_{3‴,4‴}$ (10.4 Hz) (Figure S15). Similarly, a 2D NMR (DMSO-$d_6$) ROESY correlation between H-4‴ and 1‴NH supported a *Z* $\Delta^{2‴,3‴}$ configuration (Figure 1). To support the proposed structure assignment, a sample of **1** (50 $\mu$g) was hydrolyzed in 6 M HCl at 100 °C overnight to deliver a hydrolysate, from which HPLC-DAD-ESIMS analysis detected the predicted ester hydrolysis products *cyclo*-((*Z*)-$\Delta^{2,3}$-Leu-3-OH-valine) and glycinyl-*p*-nitrobenzamide, with the

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Table 1. NMR Spectroscopic Data (600 MHz, MeOH-$d_4$) for Waspergillamide A (1)

| position | $\delta_C$ | $\delta_H$, mult ($J$ in Hz) | COSY | HMBC | $\delta_H$, mult ($J$ in Hz) DMSO-$d_6$ | ROESY DMSO-$d_6$ |
|---|---|---|---|---|---|---|
| 1 | 168.9 | | | | | |
| 2 | 151.7 | | | | | |
| 3, 7 | 130.3 | 8.07, d (9.0) | 4/6 | 1, 2, 3, 7 | 8.04, d (9.0) | |
| 4, 6 | 125.0 | 8.33, d (9.0) | 3/7 | 2, 4, 5, 6 | 8.30, d (9.0) | |
| 5 | 140.8 | | | | | |
| 1′ | 170.0 | | | | | |
| 2′ | 43.6 | 4.02, s | | 1 | 3.85, d (3.6) | |
| 1″ | 165.6 | | | | | |
| 2″ | 63.7 | 4.34, s | | 1″, 3″, 6″ | 4.07, d (3.5) | |
| 3″ | 86.4 | | | | | |
| 4″ | 23.8 | 1.63, s | | 2″, 3″, 5″ | 1.46, s | |
| 5″ | 23.0 | 1.54, s | | 2″, 3″, 4″ | 1.41, s | |
| 1‴ | 163.9 | | | | | |
| 2‴ | 126.9 | | | | | |
| 3‴ | 128.2 | 5.85, d (10.4) | 4‴ | | 5.59, d (10.4) | |
| 4‴ | 26.4 | 2.75, m | 6‴, 5‴, 3‴ | 5‴, 1‴ | 2.80, m | 2‴-NH |
| 5‴ | 22.7 | 1.07, d (6.6) | 4‴ | 2‴ | 0.92 (6.5) | |
| 6‴ | 22.9 | 1.05, d (6.6) | 4‴ | 4‴ | 0.89 (6.5) | |
| 2‴-NH | | | | | 10.2 | 4‴ |

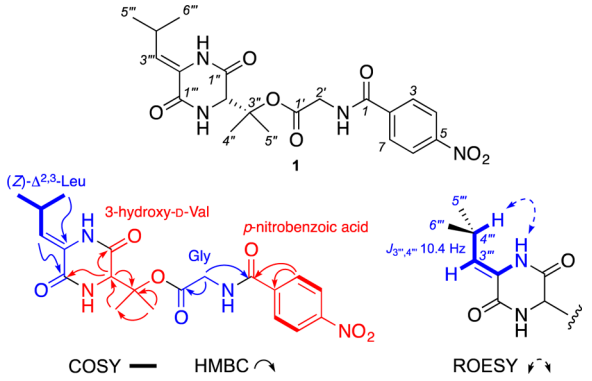


**Figure 1.** Structure and diagnostic NMR (MeOH-$d_4$) COSY and HMBC and (DMSO-$d_6$) ROESY correlations for **1**.

latter confirmed by coelution with a synthetic authentic standard (Figure S3). Of note, long-term (>6 month) storage of **1** in DMSO at −30 °C resulted in a comparable level of hydrolysis.

To assign an absolute configuration to **1**, we took inspiration from a prior study of the cytotoxic marine sponge peptides yaku'amides A and B.[10] In assigning absolute configurations to 3-OH-Val residues, Ueoka et al. derivatized an authentic synthetic standard of 3-OH-L-Val with both enantiomers of Marfey's reagent, *Nα*-(2,4-dinitro-5-fluorophenyl)-D-alaninamide (D-FDAA) and *Nα*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA), and confirmed that under $C_{18}$ HPLC conditions 3-OH-L-Val-L-FDAA (chemically equivalent to 3-OH-D-Val-D-FDAA) eluted prior to 3-OH-L-Val-D-FDAA (chemically equivalent to 3-OH-D-Val-L-FDAA). In our hands, an acid hydrolysate of **1** (100 μg) was partitioned into two aliquots, each derivatized with either L-FDAA or D-FDAA. As the resulting 3-OH-Val-D-FDAA eluted prior to 3-OH-Val-L-FDAA (16.8 min versus 25.1 min), this confirmed that **1** incorporated a 3-OH-D-Val residue (Figure S4).

Despite a distinctive UV−vis spectrum, it is instructive to observe that **1** was not detected in analytical HPLC-DAD traces of jasmine rice grain cultivations of CMB-W031. Indeed, as **1** was only detected during fractionation of the scaled-up cultivation, this raised the prospect that other cultivation conditions may be capable of producing **1** and/or related metabolites. To test this hypothesis, we prepared serial dilutions of a pure sample of **1** and subjected these to HPLC-ESIMS-SIE (i.e., single-ion extraction) analysis of $m/z$ 433 ($[M + H]^+$) to construct a quantitative calibration curve over the range 1−30 μg (Figure S8). We then analyzed an array of different liquid (shaken and static) and solid cultivation media and conditions. Under the majority of cultivation conditions (Figure S9) **1** was either not detected (<1 μg/mL), or detected at very low levels (1−10 μg/mL). By contrast we observed a significant, 10−100-fold enhancement in jasmine rice and red rice grain cultivations (90−100 μg/mL) (Figure S9). In an effort to improve on these yields we turned our attention to the biosynthesis of **1**.

Waspergillamide A (**1**) is particularly unusual in that it incorporates an aromatic nitro moiety. Biosynthetic options for introducing such moieties into secondary metabolites are generally limited to either (a) nitric oxide synthase (NOS)-mediated production of nitric oxide (NO), leading to aromatic nitration, or (b) oxidation of aromatic amines.[11−13] To establish whether the biosynthesis of **1** was NO dependent, we added the NOS inhibitor aminoguanidine (AG) to CMB-W031 cultivations and also examined CMB-W031 cultivations under fluorescent microscopy in the presence of an NO-selective fluorescent dye.[14] These studies provided conclusive evidence that NO was not implicated in the biosynthesis of **1**. Turning to the alternate biosynthetic hypothesis, chemical profiling of CMB-W031 extracts successfully detected low levels of the aromatic amine and putative biosynthetic *p*-aminobenzoic acid (PABA). Significantly, addition of the known PABA biosynthesis inhibitors L-Trp and L-Phe[15] to CMB-W031 cultivations resulted in a significant decrease in the production of **1** (Figures S10 and S11), implicating PABA in the biosynthesis of **1**.

On the basis of the above we propose that the biosynthesis of **1** proceeds by a nonribosomal peptide synthase (NRPS) employing PABA as the starter unit. In this hypothesis PABA is sequentially conjugated with Gly, L-Val, and L-Leu residues, which are further elaborated by epimerization (L-Val to D-Val)


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and oxidation (PABA to *p*-nitrobenzoic acid (PNBA); L-Val to 3-OH-L-Val; L-Leu to $\Delta^{2,3}$-Leu). The biosynthesis of **1** is concluded by 2,5-diketopiperazine cyclization–mediated termination (Figure 2). A failure to detect PNBA in CMB-W031

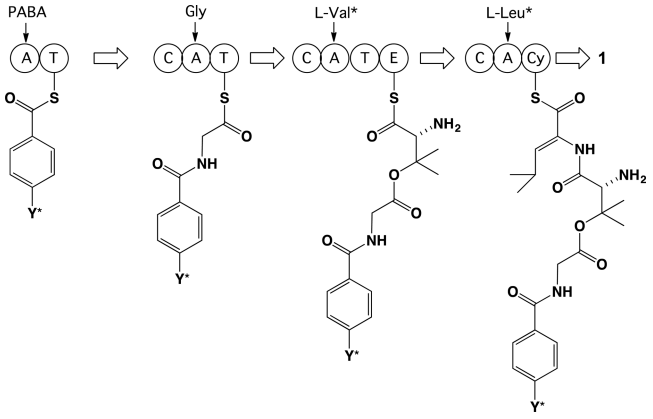


**Figure 2.** Schematic of a plausible NRPS for **1**. Domains: A = adenylation, T = thiolation, C = condensation, E = epimerization, and Cy = cyclization (termination). *Oxidases that sequentially and/or simultaneously transform Y = $NH_2$ to Y = $NO_2$, L-Val to 3-OH-L-Val, and L-Leu to $\Delta^{2,3}$-L-Leu.

extracts supports the hypothesis that biosynthesis of **1** is initiated by PABA and not preformed PNBA. Disappointingly, supplementing CMB-W031 cultivations with PABA did not increase the yield of **1**, nor was the biosynthetic pathway influenced by the addition of *p*-nitro, *o*-nitro, *m*-nitro, or *o,p*-dinitro benzoic acids.

Waspergillamide A (**1**) is the first example of a natural diketopiperazine incorporating either a 3-OH-Val or a *p*-nitrobenzamide residue and, to the best of our knowledge, the first example of a natural *depsi*-tetrapeptide diketopiperazine. Importantly, natural nitro-substituted diketopiperazines are rare, with a noteworthy example being the phytotoxic thaxtomins produced by *Streptomyces* strains responsible for scab disease in potatoes.[16,17] By contrast, waspergillamide A (**1**) was inactive in growth inhibition assays against a panel of Gram-negative and Gram-positive bacteria and human colon (SW620), lung (NCI-H460), and cervical (KB-3-1) carcinoma cells.

## EXPERIMENTAL SECTION

**General Experimental Details.** Specific rotations ($[\alpha]_D$) were measured on a JASCO P-1010 polarimeter in a 100 × 2 mm cell at room temperature. UV–vis spectra were obtained on a Varian Cary 50 UV–vis spectrophotometer with 1 cm quartz cells. NMR spectra were acquired on a Bruker Avance 600 MHz spectrometer with either a 5 mm PASEL 1H/D-13C Z-gradient probe or 5 mm CPTCI 1H/19F-13C/15N/DZ-gradient cryoprobe, controlled by TopSpin 2.1 software. In all cases spectra were acquired at 25 °C (unless otherwise specified) in solvents as specified in the text, with referencing to residual $^1$H or $^{13}$C signals in the deuterated solvents. Electrospray ionization mass spectrometry (ESIMS) experiments were carried out on an Agilent 1100 series LC/MSD (quadrupole) instrument in both positive and negative modes. High-resolution ESIMS spectra were obtained on a Bruker micrOTOF mass spectrometer by direct injection in MeCN at 3 μL/min using sodium formate clusters as an internal calibrant. Liquid chromatography-diode array-mass spectrometry (HPLC-DAD-ESIMS) data were acquired on an Agilent 1100 series separation module equipped with an Agilent 1100 series HPLC/MSD mass detector and diode array multiple-wavelength detector. Semipreparative and preparative HPLCs were performed using Agilent 1100 series HPLC instruments with corresponding detectors, fraction collectors, and software inclusively.

**Fungal Strain Collection.** The fungus CMB-W031 was isolated in 2011 from internal (homogenized) tissues of a mud dauber wasp (order Hymenoptera, genus *Sceliphron*) collected in Brisbane, Queensland. The fresh wasp was transported to the laboratory, where it was rinsed in sterile water for 1 min and subjected to surface sterilization in 70% ethanol (v/v) for 30 s, after which it was washed with sterile water to remove traces of EtOH. Subsequently, the sample was homogenized in a mortar and pestle under aseptic conditions and placed on ISP-2 agar plates (comprising 0.4% glucose, 0.4% yeast extract, 1% malt extract, and 2% agar in distilled water). The plates were sealed with parafilm and incubated at 26.5 °C for 3–4 weeks. A pure culture of fungus CMB-W031 was obtained by single-colony serial transfer on agar plates and then cryopreserved at −80 °C in 15% aqueous glycerol. The fungus initially formed a white colony, with yellow spores after 10 d of incubation at 26.5 °C. Taxonomic analysis identified CMB-W031 as *Aspergillus* sp. (see Supporting Information).

**Scaled-up Fermentation and Isolation of Waspergillamide A (1).** A spore suspension of *Aspergillus* sp. (CMB-W031) was prepared by diluting a loop of spores into a flask (250 mL) containing sterile water (50 mL) and homogenized by shaking at 200 rpm at 26.5 °C for 10 min. Forty flasks (250 mL) containing sterile jasmine rice (20 g) inoculated with aliquots of homogenized spore suspension (5 mL) were incubated at 26.5 °C for 15 d, after which they were extracted with acetone (40 × 85 mL). The combined acetone extract was concentrated *in vacuo* at 40 °C to give an aqueous suspension, which was partitioned against EtOAc (2 × 1200 mL), which after drying *in vacuo* yielded EtOAc (22.5 g) and aqueous (20.9 g) partitions. The EtOAc partition was subsequently dissolved in 90% MeOH/$H_2O$ (300 mL) and partitioned against hexane (2 × 300 mL), to yield hexane (2.7 g) and MeOH/$H_2O$ (19.8 g) partitions.

An aliquot of the MeOH/$H_2O$ partition (1.0 g) was subjected to solid-phase extraction (SPE) (GracePure $C_{18}$Max, 10% stepwise gradient elution from 10% MeCN/$H_2O$ to 100% MeCN, with constant 0.01% TFA) to yield 17 fractions. Further chromatographic resolution of selected fractions yielded the known fungal metabolites penicillic acid,[1] dehydropenicillic acid,[2] (−)-stephacidin B,[3] (−)-versicolamide B,[4,5] (+)-preussin,[6,7] cycloanthranylproline,[8] circumdatin E,[8] and circumdatin G[9] (see Supporting Information). The SPE fraction eluting at 30% $H_2O$/MeCN (82.5 mg) was subsequently subjected to HPLC fractionation (Phenomenex Luna $C_8$ column, 250 × 21.2 mm, 10 μm, 20 mL/min, with isocratic elution at 25% MeCN/$H_2O$ (0.01% TFA) for 5 min followed by gradient elution from 25% to 50% MeCN/$H_2O$ in 45 min with constant 0.01% TFA) to yield waspergillamide A ($t_R$ = 24.6 min, 3.2 mg, 0.32%) (% yield was determined on a mass-to-mass basis against the weight of the MeOH/$H_2O$ partition).

*Waspergillamide A (1):* white solid $[\alpha]^{21}_D$ +13.7 (*c* 0.21, MeOH); UV–vis (MeOH) $\lambda_{max}$ (log $\varepsilon$) 275 (3.73) nm; NMR (600 MHz, MeOH-$d_4$) see Table S1 and Figures S13 and S14; ESI(+)MS *m/z* 433 $[M + H]^+$; ESI(−)MS *m/z* 431 $[M - H]^-$; HRESI(+)MS *m/z* 455.1523 $[M + Na]^+$ (calcd for $C_{20}H_{24}N_4O_7Na^+$, 455.1537).

**Acid-Mediated Hydrolysis of Waspergillamide A (1).** An aliquot of **1** (50 μg) in 6 M HCl was heated at 100 °C for 16 h, after which the hydrolysate was dried under $N_2$ at 40 °C and redissolved in MeOH (50 μL) prior to HPLC-DAD-ESI MS analysis (Zorbax SB-$C_8$ column, 150 × 4.6 mm column, 5 μm, 1 mL/min gradient elution from 90% $H_2O$/MeCN to 100% MeCN over 15 min, with constant 0.05% $HCO_2H$/MeCN modifier).

**$C_3$ Marfey's Analysis of Waspergillamide A (1).** An aliquot of **1** (100 μg) in 6 M HCl (100 μL) was heated at 110 °C for 24 h, after which the hydrolysate was dried under $N_2$ at 40 °C. The resulting hyrolysate was divided into two samples (2 × 50 μg), which were treated with either 1 M $NaHCO_3$ (20 μL) and L-FDAA (1% solution in acetone, 50 μL) or with 1 M $NaHCO_3$ (20 μL) and D-FDAA (1% solution in acetone, 50 μL) at 40 °C for 1 h. Samples were neutralized with 1 M HCl (20 μL), diluted with MeCN (810 μL), and analyzed by HPLC-ESIMS (Agilent Zorbax SB-$C_{18}$ column, 5 μm, 150 × 4.6 mm,


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50 °C, 1 mL/min, 55 min linear gradient elution from 15% MeOH/$H_2O$ to 60% MeOH/$H_2O$ with a 5% isocratic 0.05% $HCO_2H$/MeCN modifier).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b01062.

General experimental details, microbial taxonomy, tabulated NMR data and spectra, $C_3$ Marfey's analysis, and bioassay results (PDF)

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### Notes

The authors declare no competing financial interest.

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