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SHORT COMMUNICATION

Oryzanigral: a new polyketide from an endophytic fungus *Nigrospora oryzae* isolated from *Coccinia grandis*

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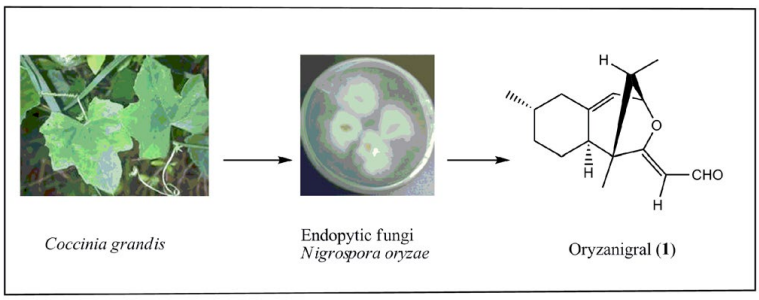
ABSTRACT

A new heptaketide named oryzanigral (**1**) was isolated from the fermentation extract of the endophytic fungus *Nigrospora oryzae* isolated from the leaves of *Coccinia grandis*, along with five known compounds, (*R*)-mellein, (*R*)-*O*-methylmellein, (3*R*,4*R*)-4-hydroxymellein, (3*R*,4*S*)-4-hydroxymellein and abscisic acid. The structure of oryzanigral was elucidated by spectroscopic analyses including 2D-NMR. A plausible biosynthetic pathway involving Diels-Alder reaction was proposed for compound **1** and related polyketides previously reported. In addition, structure revision of the double bond geometry for coicenal A was described.



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Oryzanigral; *Nigrospora oryzae*; *Coccinia grandis*; endophytic fungus; heptaketide; coicenal A

1. Introduction

Endophytic fungi are playing a vital role in the production of secondary metabolites. Considering the importance of endophytic fungi from medicinal plants, this study focused on the isolation of an endophyte fungi from the leaves of *C. grandis* (Cucurbitaceae family) and secondary metabolites produced by the endophyte. *C.*

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grandis is a well-known herb for diabetic treatments. Various parts of the plant have been shown to have medicinal values such as analgesic, antipyretic, anti-inflammatory, antimicrobial, antiulcer, antidiabetic, antioxidant, hypoglycemic, hepatoprotective, antimalarial, antidyslipidemic, anticancer, antitussive and mutagenic (Pekamwar et al. 2013). We have previously reported several bioactive compounds produced by endophytes isolated from Sri Lankan plants (Bandara et al. 2015; Piyasena et al. 2015; Siriwardane et al. 2015; Thanabalasingam et al. 2015; Qader et al. 2016, 2017, 2018; Munasinghe et al. 2017, 2021; Padmathilake et al. 2017; Kehelpannala et al. 2018; Rathnayake et al. 2018, 2019; Sritharan et al. 2019; Dissanayake et al. 2020). In this paper we report on the isolation and structure elucidation of a new heptaketide named oryzanigral (**1**) obtained from fermentation extract of the endophytic fungus *Nigrospora oryzae* isolated from the leaves of *Coccinia grandis*, along with five known compounds, (*R*)-mellein, (*R*)-*O*-methylmellein, (3*R*,4*R*)-4-hydroxymellein, (3*R*,4*S*)-4-hydroxymellein and abscisic acid (Figure 1).

2. Results and discussion

Compound **1** was isolated from cultured *N. oryzae* along with five known compounds, (*R*)-mellein (**2**), (*R*)-*O*-methylmellein (**3**), (3*R*,4*R*)-4-hydroxymellein (**4**), (3*R*,4*S*)-4-hydroxymellein (**5**) and abscisic acid (**6**) (Table S1, Supplementary material). Compound **1** was obtained as a colorless oil. The molecular formula of **1** was established to be $C_{16}H_{22}O_2$ (6 degrees of unsaturation) on the basis of positive HR-FAB-MS at *m/z* 247.1681 $[M+H]^+$ (calcd for $C_{16}H_{23}O_2^+$, 247.1698). The presence of a conjugated carbonyl chromophore was suggested by the absorption at 1656 cm^{-1} in the IR spectrum and the absorption at 273 nm in the UV spectrum. The ^1H NMR spectrum (Figure S1, Supplementary material) showed two doublet methyls at δ_H 0.92 and 0.98, one singlet methyl at δ_H 1.15, two olefinic protons at δ_H 5.83 (brd, $J = 5.8$ Hz) and 5.11 (d,

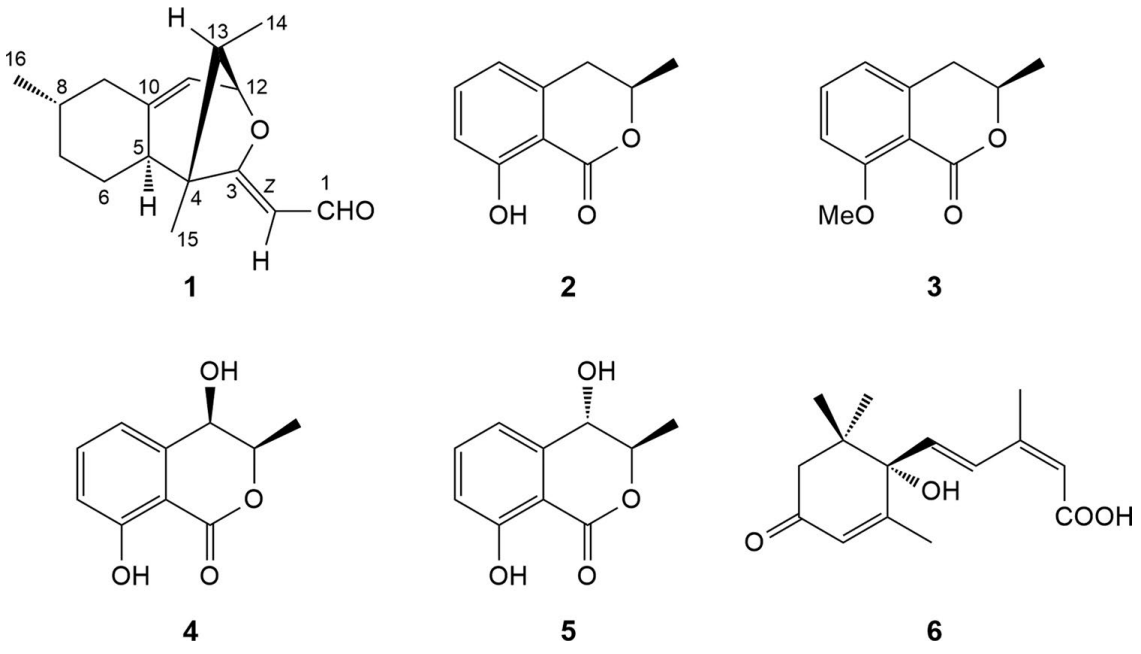

Figure 1. Structures of compounds **1**–**6**.

$J = 8.5$ Hz), and an aldehydic proton at δ_H 9.92 (d, $J = 8.5$ Hz). The ^{13}C, DEPT and HSQC spectra (Figure S2, S3 and S5, Supplementary material) showed 16 signals, including an aldehydic carbonyl at δ_C 190.1, two olefinic methines at δ_C 101.2 and 120.6, two quaternary olefinic carbons at δ_C 185.5 and 146.4 and one oxymethine at δ_C 82.8. Consideration into the chemical shift (δ_C 185.5) for one of the olefinic carbons and the presence of a conjugated aldehyde suggests that it could be assigned to an oxygen-substituted quaternary olefinic carbon β to the aldehydic carbonyl since the proton of the carbon (δ_C 101.2) α to the aldehyde showed spin-spin coupled to aldehydic proton with $J = 5.8$ Hz. The other two olefinic carbons could be assigned to tri-substituted olefin. The rest of carbon signals were observed in an aliphatic region: three methyls, three methylenes, three methines and one quaternary carbon. These analyses of the NMR data (two double bonds and one aldehydic carbonyl) indicated that there are three ring units in the molecule. The planer structure of **1** was established based on the HMBC correlations shown in Figure S6, Supplementary material. The C-5–C-6–C-7 linkage was deduced from spin systems of H-5–H_2-6–H_2-7, which was evidenced by the H-H COSY spectrum (Figure S4, Supplementary material). Compound **1** possesses a unique 6-oxabicyclo[3,2,1]oct-3-ene scaffold and the presence of three ring system and β-oxygenated conjugated aldehyde was confirmed. The cyclohexane fused 6-oxabicyclo[3,2,1]oct-3-ene nuclei can be seen in the structure of compound **1**.

The relative stereochemistry at C-5, C-8 and C-13 stereogenic centers as well as the geometry of C-2–C-3 double bond were elucidated as follows. The (*Z*)-configuration for the C-2–C-3 double bond was determined on the basis of an intense NOE correlation from H_3-15 to H-2 (Figure S7, Supplementary material). If the double bond has (*E*)-configuration, the special distance between H_3-15 and H-2 will be much larger than that of the (*Z*)-isomer. Coicenal A is a known structurally similar compound and (*E*)-geometry was assigned for the double bond in contrast to that of compound **1** (Wang et al. 2013). However, compound **1** and coicenal A were expected to have the same double bond geometry since the ^1H and ^{13}C NMR data around the double bond of the two compounds are in good agreement from each other. Indeed, the NOESY spectrum of coicenal A indicated a clear NOESY cross peak corresponding to that from H-2 to H_3-15 of compound **1** (personal communication from Prof. Hongwei Liu). Therefore, the double bond geometry of coicenal A (also coicenals B-D) must be corrected from (*E*) to (*Z*). The stereochemistry at C-8 of **1** was determined based on the finding that H-8 (δ_H 1.38 (m)) collapsed by decoupling H_3-16 to a triplet of triplets ($J = 11.5$, 3.7 Hz) typical of an axial proton surrounded by two adjacent methylene groups in the chair form of cyclohexane rings. Thus, H_3-16 was located in the equatorial position as shown in Figure S8. The stereochemistry at C-13 was determined to be as shown in Figure S8 based on the $J_{H\text{-}12,H\text{-}13}$ value (nearly 0 Hz), which implied that the dihedral angle, H-12–C-12–C-13–H-13, is close to 90 degree. The C-13 stereochemistry was supported by no NOE correlation was detected between H_3-14 to any of the cyclohexane ring protons. Finally, the stereochemistry at C-5 was assigned as shown in Figure S8 on the basis of an NOE correlation from H-2 (δ_H 5.11 (d, $J = 8.5$ Hz)) to H-5 (δ_H 1.98 (brd, $J = 12.3$ Hz)). In addition, a weak NOE correlation was observed from H_3-15 to H-6 (δ 1.92 (brd, $J = 12.4$ Hz, equatorial proton)). Other NOE correlations observed are shown in Figure S8, which agreed with the structure of **1**.

The absolute configuration of **1** was not investigated in the present study, but can be assumed as 4*R*,5*S*,8*S*,12*S*,13*R* as shown in Figure 1, since compound **1** ($[\alpha]_D$ +314 (c, 0.17, MeOH)) showed a large positive specific optical rotation, which was similar to that of coicenal A ($[\alpha]_{D+}$ 318.2 (c 0.10, MeOH) with known absolute configuration (Wang et al. 2013).

Oryzanigral (**1**) is a unique tricyclic heptaketide with a 6-oxabicyclo[3,2,1]oct-3-ene scaffold. Fungal metabolites, coicenals A-C, erroneously reported as diterpenoids, have the same scaffold (Wang et al. 2013). Aspermytin A (Tsukamoto et al. 2004) and viridicatumones A-C (Wang et al. 2019) are heptaketides with a bicyclic structure but with the same carbon skeleton as oryzanigral. It should be noted that the stereochemistries at the stereogenic centers of oryzanigral are consistent with those reported for coicenal A, aspermytin A and viridicatumone A. Consideration of the structures of these secondary metabolites allowed us to propose a common biosynthetic pathway involving an intramolecular Diels-Alder reaction (Figure 2). The linear heptaketide assembled by PKS is likely to undergo an enzymatic Diels-Alder reaction to form a decalin intermediate **A**. The intermediate **A** undergoes hydroxylation with concomitant double bond migration. Intramolecular nucleophilic attack on the C-3 carbonyl of the hydroxyl group followed by dehydration could afford the 6-oxabicyclo[3,2,1]oct-3ene scaffold of **1**. Intermediate **A** can be regarded as a common key precursor for aspermytin A and viridicatumone A. Oryzanigral (**1**) showed moderate toxicity in brine shrimp lethality assay with the LC_{50} value of 9.7 µM (Meyer et al. 1982). (*R*)-mellein (**2**) and (*R*)-*O*-methylmellein (**3**) inhibited 90% and 99% of α-glucosidase activities at a concentration of 100 µg/mL, showing IC_{50} values of 22.4 µM and 3.6 µM, respectively (Sathya et al. 2020).

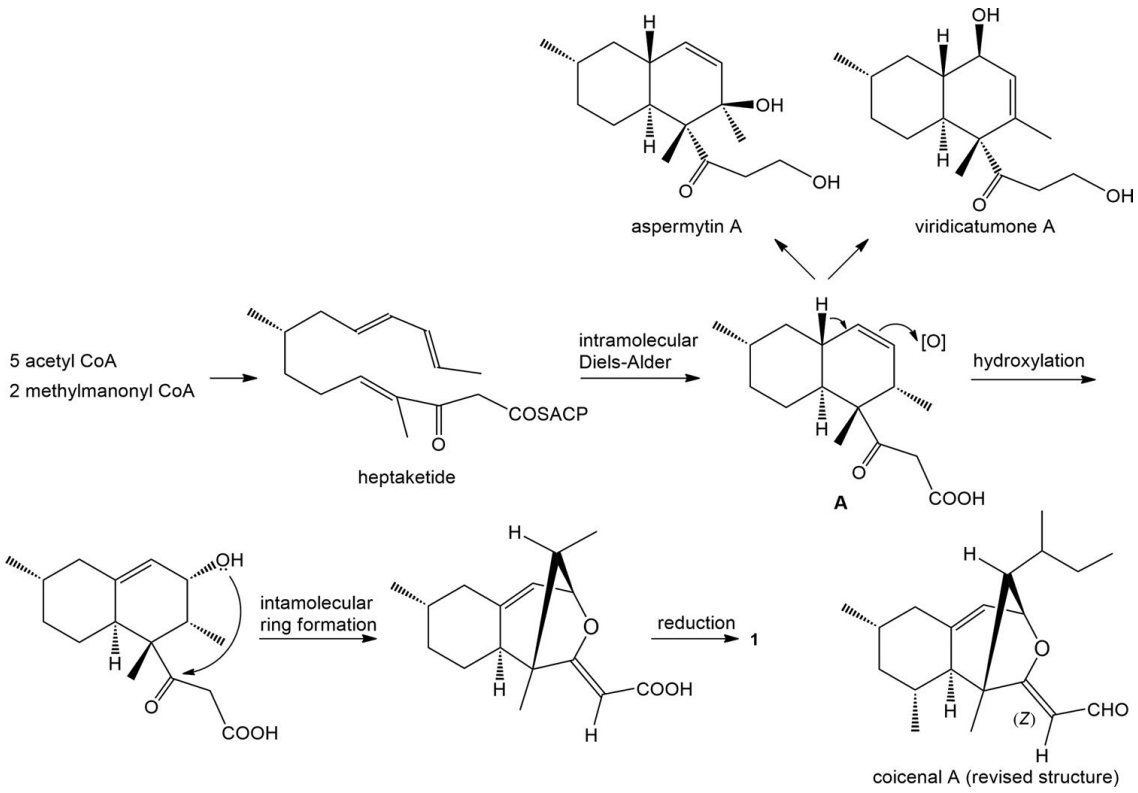

Figure 2. Plausible biosynthesis of oryzanigral (**1**) and the structures of related compounds.

3. Experimental

3.1. General

Extractions were performed using a sonicator (VWR Ultrasound cleaner, model-USC 1700D). Analytical TLC was carried out with silica gel 60 F_{254} precoated aluminum sheets (Merck Art. 1.05554). Compounds on TLC were located using a UV lamp and by heating after spraying with acidic anisaldehyde. Silica gel (Fluka 60741, Merck Art. 7734 and 9385) and Sephadex LH-20 were used for column chromatography. Preparative thin layer chromatography (PTLC) was carried out using silica gel 60 F_{254} precoated glass plates (Merck Art 1.05715). HPLC was performed Hitachi L-6000 pump and Hitachi L-4200 UV–vis detector (215 nm) using an ODS column (Inertsil ODS-3, 25 cm × 4.6 mm i.d., GL Sciences), ^{1}H NMR were recorded on a JEOL JNM-ECP500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) or JEOL JMN-AL300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer in $CDCl_3$ or CD_3OD solution at 20 °C. Optical rotations were measured on a JASCO P-2200 polarimeter. IR spectra were measured on a JASCO FT/IR-460 spectrometer. UV spectra were recorded on a JASCO MD-4017 photodiode array detector. HR-FAB-MS spectra were obtained with a JEOL JMS-700 spectrometer.

3.2. Isolation and identification of the endophytic fungus

Healthy leaves of *C. grandis* were collected from a home garden in Kandy, Central Province of Sri Lanka. Leaves were washed with running water and triple sterilized with ethanol, 5% Nicoll and distilled water. Few segments (ca. 10 mm × 5 mm) of the sterilized leaves were placed on PDA media in Petri dishes and incubated in the dark at room temperature. After 5 days, emerging fungi were serially transferred to PDA media on Petri dishes to obtain pure culture of endophytic fungus. The fungus was identified as *N. oryzae* by sequence analysis of the ITS region of the rDNA gene. DNA was extracted using Promega, Wizard Genomic DNA purification Kit (A1120) and amplification of the ITS region was carried out using the universal eukaryotic primers of ITS1 and ITS4. These experiments were performed by the GeneTech Institute, Sri Lanka. BLAST search indicated that the sequence of the ITS region had 100% similarity to that of *N. oryzae* F12-F (GenBank Accession No. KF516962.1). Pure culture of the fungal strain (NIFS-DEF2) and the photographic evidence of the leaves of the *C. grandis* and the fungal strain are deposited at the National Institute of Fundamental Studies.

3.3. Fermentation of fungus, extraction, bioassays and isolation of compounds

The fungus *N. oryzae* was cultured by inoculating pure culture grown on PDA, in eighty 1L-conical flasks each containing 400 mL of PDB medium. The flasks were allowed to stand at room temperature for initial 10 days, and shaken every other day on a laboratory shaker (100 rpm). The fermentation medium was filtered after one month and the filtrate was extracted with EtOAc. The residual mycelium was extracted with EtOAc using sonicator. The two EtOAc extracts were combined (11.5 g) since they showed almost identical TLC pattern. Chromatographic separation of the combined

EtOAc extract was carried out over silica gel (n-hexane-EtOAc-MeOH/n-hexane -CH_2Cl_2-MeOH) and Sephadex LH-20 (MeOH), and by PTLC to furnish six compounds, oryzanigral (**1**) (2.5 mg), (*R*)-mellein (**2**) (15 mg) (Kuramochi et al. 2010), (*R*)-*O*-methylmellein (**3**) (10 mg) (Glauser et al. 2009), (3*R*,4*R*)-4-hydroxymellein (**4**) (6 mg) (Asha et al. 2004; Takesue et al. 2014), (3 *R*,4*S*)-4-hydroxymellein (**5**) (2 mg) (Asha et al. 2004) and abscisic acid (**6**) (3 mg) (Smith et al. 2006).

3.4. Oryzanigral (1)

Colorless oil; Rf 0.47 (hexane-EtOAc 4:1); $[\alpha]_D^{25}$ +314 (c, 0.17, MeOH); UV λ_{max} (MeOH): 273 nm; IR ν_{max} (KBr): 2922, 2852, 1661, 1630, 1461, 1396, 1156, 1037, and 947 cm^{-1}; HR-FAB-MS *m/z* 247.1681 $[M+H]^+$ (calcd for $C_{16}H_{23}O_2^+$, 247.1698); ^1H NMR (500 MHz, $CDCl_3$) δ 9.92 (1H, d, *J* = 8.5 Hz, H-1), 5.11 (1H, d, *J* = 8.5 Hz, H-2), 1.98 (1H, brd, *J* = 12.3 Hz, H-5), 1.22 (1H, m, H-6a), 1.92 (1H, brd, *J* = 12.4 Hz, H-6b), 1.82 (1H, brd, *J* = 13.3 Hz, H-7a), 1.08 (1H, m, H-7b), 1.39 (1H, m, H-8), 1.60 (1H, brt, *J* = 12.9 Hz, H-9a), 2.17 (1H, brd, *J* = 12.9 Hz, H-9b), 5.83 (1H, brd, *J* = 5.8 Hz, H-11), 4.36 (1H, d, *J* = 5.8 Hz, H-12), 2.23 (1H, q, *J* = 6.9 Hz, H-13), 0.98 (3H, d, *J* = 6.9 Hz, H_3-14), 1.15 (3H, s, H_3-15), 0.92 (3H, d, *J* = 6.4 Hz, H_3-16); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.1 (C-1), 101.2 (C-2), 185.5 (C-3), 50.8 (C-4), 50.3 (C-5), 28.6 (C-6), 34.6 (C-7), 34.9 (C-8), 43.3 (C-9), 146.6 (C-10), 120.6 (C-11), 82.8 (C-12), 40.7 (C-13), 13.5 (C-14), 17.1 (C-15), 22.2 (C-16).

4. Conclusion

In this study we isolated and identified the endophytic fungus *N. oryzae* from the medicinal plant *Coccinia grandis*. Chromatographic separation of the secondary metabolites produced by the fungus led to the isolation of a new polyketide named oryzanigral together with five known compounds. The structure of oryzanigral was determined to be a unique tricyclic heptaketide with a 6-oxabicyclo[3,2,1]oct-3-ene scaffold. A plausible biosynthetic pathway of oryzanigral and related compounds, including intramolecular Diels-Alder reaction of the linear heptaketide, was proposed. Furthermore, the double bond geometry of coicenals A-D was corrected from (*E*) to (*Z*).

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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