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Natural Product Research
Formerly Natural Product Letters

ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: Unang Supratman, Takuma Suzuki, Tomoki Nakamura, Yasuhiro Yokoyama, Desi Harneti, Rani Maharani, Supriatno Salam, Fajar Fauzi Abdullah, Takuya Koseki & Yoshihito Shiono (2019): New metabolites produced by endophyte *Clonostachys rosea* B5−2, Natural Product Research, DOI: 10.1080/14786419.2019.1656629

To link to this article: https://doi.org/10.1080/14786419.2019.1656629

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2019.1656629

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New metabolites produced by endophyte *Clonostachys rosea* B5 – 2

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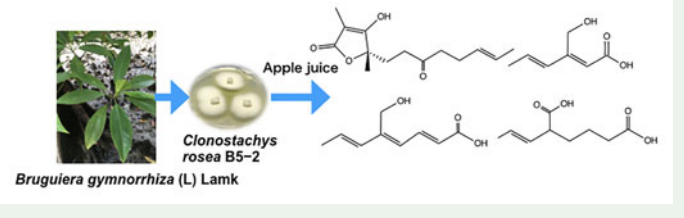
ABSTRACT

The endophytic fungus, *Clonostachys rosea* B5-2 was isolated from mangrove plants and subjected to the one strain many compounds (OSMAC) methodology. By this approach, it was found that modification of the culture media enhanced the production of secondary metabolites by *C. rosea* B5-2. The apple juice supplemented solid rice media led to significant changes in the secondary metabolism of the fungus, *C. rosea* B5-2, and induced the production of four new compounds, (-)-dihydrovertinolide (**2**), and clonostach acids A (**3**), B (**4**), and C (**5**) together with the known compound, (-)-vertinolide (**1**). The new compound (-)-dihydrovertinolide (**2**) exhibited phytotoxicity against lettuce seedlings at a concentration of 50 mg L^{-1}.



ARTICLE HISTORY
Received 1 July 2019
Accepted 6 August 2019

KEYWORDS
Endophyte; Clonostachys rosea; OSMAC; (-)-Vertinolide; phytotoxicity

1. Introduction

Endophytes are a potential abundant source of bioactive natural products with interesting biological properties (Gouda et al. 2016). Indeed, a large number of biologically active molecules have been discovered from endophytes in the last two decades. However, it has been reported that most of the biosynthetic genes from various endophytes are silent under standard cultivation conditions. Thus, the biosynthetic potential of the endophytes is not being effectively exploited and there exists a vast

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Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2019.1656629.

potential to produce far more secondary metabolites (Brakhage 2013, Hautbergue et al. 2018). In order to gain access to these untapped biosynthetic gene clusters, the biosynthetic pathways must be induced. Previous studies utilized an approach referred to as OSMAC, which is based on the activation of cryptic biosynthesis genes by altering various culture parameters such as media composition, temperature, and pH (Bode et al. 2002, Li et al. 2019). Up to now, this strategy was applied to maximize the generation of new bioactive secondary metabolites from a single strain. This study was initiated in order to explore secondary metabolite production by endophytes when cultured under different media compositions. In our previous studies with an endophyte, *Cosmospora vilior* isolated from a mangrove plant, we found that addition of NaCl to the culture medium containing unpolished rice led to the synthesis of new biologically active compounds, which were not detected when the strain was cultured under the same conditions without additional NaCl (Shiono et al. 2016).

In an ongoing search for bioactive compounds from natural sources, we isolated the endophyte *C. rosea* B5-2. *C. rosea* B5-2 was subjected to the OSMAC approach, utilizing rice solid media supplemented with apple juice, a soy source, or a metal solution (Figure S1). *C. rosea* B5-2 cultures, grown under conditions of apple juice supplementation to the rice media were analysed for secondary metabolite production. Under these conditions, four new compounds (**2–5**) were found together with the known compound (-)-vertinolide (**1**) (Figure 1). This study also provides the fermentation, isolation, and structural elucidation of compounds **2–5**.

2. Results and discussion

The *C. rosea* B5-2 strain was cultured on unpolished rice with or without the following media: an apple juice, a soy source, or a metal solution. The HPLC results illustrated that an increased number of peaks was found in cultures containing apple juice medium in comparison to other media types (Figure S1). Moreover, the MeOH extract of cultures grown with apple juice medium showed several compounds that were not observed in cultures grown with other media. In order to isolate these compounds, *C. rosea* B5-2 grown on solid rice medium with apple juice was subjected to scale-up fermentation. The resulting crude MeOH extract was concentrated and then chromatographed over different stationary phases (silica gel and ODS), which afforded compounds **1–5**. Based on both NMR and HRESITOFMS data as well as comparison to previously reported data, the known compound **1** was identified as (-)-vertinolide (Trifonov et al. 1982).

(-)-Dihydrovertinolide (**2**) was isolated as a white amorphous powder. It was assigned the molecular formula $C_{14}H_{20}O_4$, as deduced from the HRESITOFMS data. The UV spectrum showed absorption maximums at 231 and 260 nm, indicating that a conjugated double bond was likely present in compound **2**. The IR spectrum displayed absorption bands diagnostic of a hydroxyl at 3300 cm^{-1} and a C = O stretching at 1666 cm^{-1}, which is within the expected range for an α,β-unsaturated ester. The α,β-unsaturated γ-lactone linkage predicted by the molecular formula was showed to be between C-1 and C-4 based on the similar ^{1}H and ^{13}C NMR chemical shift values of compound **2** compared to those of compound **1.** The ^{1}H NMR and COSY spectral

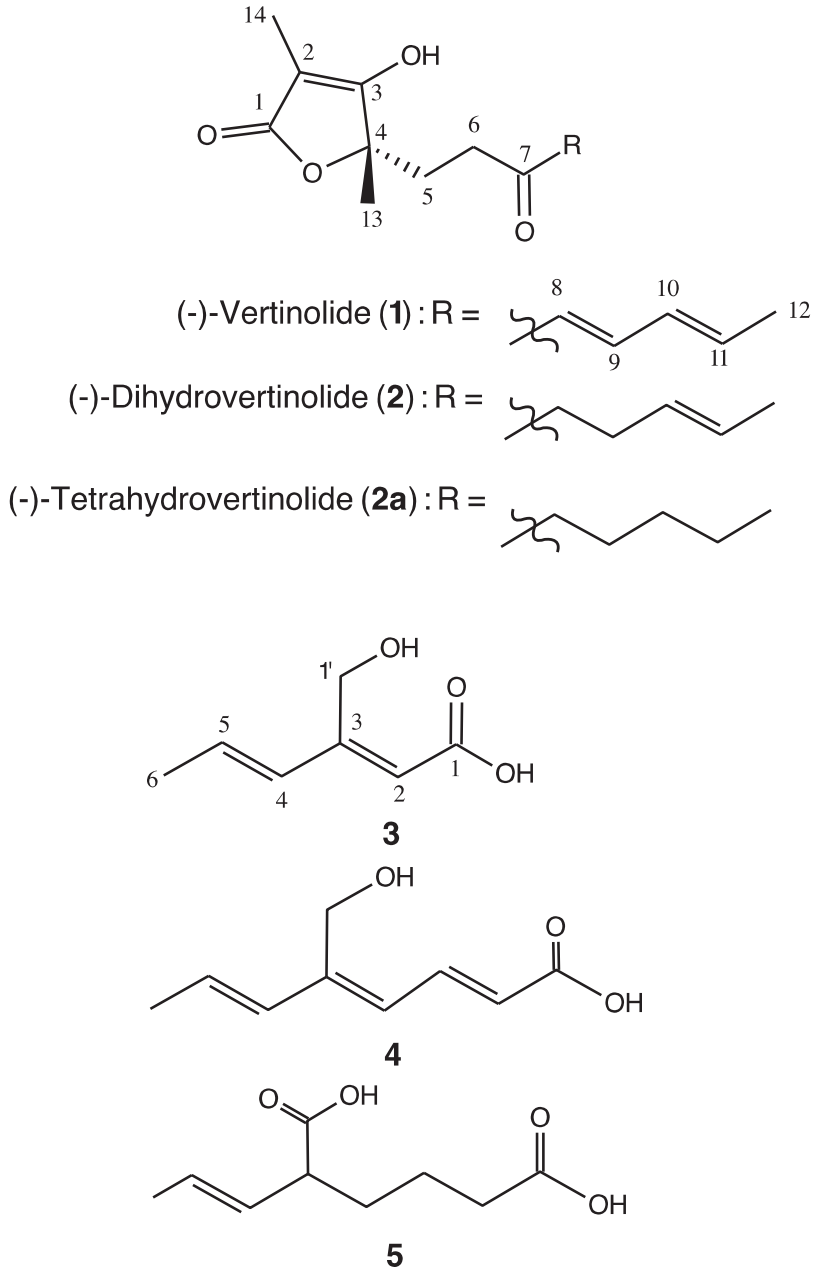

Figure 1. Structures of Compounds **1** -**5**.

data indicated that compound **2** had two additional methylene groups [δ_H 2.44 (2 H, t, $J = 7.8$ Hz, H-8) and 2.15 (2 H, m, H-9)], which suggested that the C8 = C9 double bond was saturated. The carbon skeleton was deduced from the results of an HMBC experiment (Figure S2). The HMBC correlations clearly connected spin systems A and B, which comprised a 6-octen-3-one moiety. Furthermore, the octane moiety was attached to C-4 based on HMBC correlations from Me-13 to C-4 and C-5. The NOE

(nuclear Overhauser effect) correlations between H-10 and Me-12 indicated the *trans* geometry of the double bond (Figure S2). Furthermore, the ^{1}H and ^{13}C NMR spectra as well as the positive optical rotation of the tetrahydrovertinolide that was obtained by hydrogenolysis of compound **2** with Pd/C corresponded to those of compound **2a** reported previously (Takaiwa and Yamashita 1983). Based upon the above data, the elucidated structure revealed that it is a new dihydro derivative.

Clonostach acid A (**3**) was isolated as a colourless oil. Its molecular formula was determined as $C_7H_{10}O_3$ on the basis of the HRESITOFMS analysis, which indicated that compound **3** has three degrees of unsaturation. The IR absorption bands at 3282 and 1700 cm^{-1} indicated the presence of OH and C=O groups, respectively. The presence of a conjugated dienone unit was evident from the UV spectrum (λ_{max} 260 nm). Carbon resonances observed at δ_C 168.9 (C-1), along with those at δ_C 18.1 (C-6), 126.3 (C-5), 131.7 (C-4), 153.3 (C-3), and 112.7 (C-2) in the ^{13}C NMR and DEPT spectra, confirmed the presence of the expected $\alpha,\beta,\gamma,\delta$-unsaturated carboxylic acid. The HMBC correlations from H-1′ to C-2, C-3, and C-4 indicated that the oxygenated methylene group was attached at C-4 (Figure S2). The $J_{4,5}$ ($J = 16.2$ Hz) value indicated the *trans* geometry of the double bond at C-4 and C-5. The configuration of another double bond in compound **3** (between C-2 and C-3) was elucidated by a NOE correlation between H-2 and H-4.

Clonostach acid B (**4**) was obtained as a white amorphous powder. The molecular formula of compound **4** was established as $C_9H_{12}O_3$ by HRESITOFMS data coupled with ^{1}H and ^{13}C NMR results. This analysis also revealed that compound **4** contained one additional double bond when compared to compound **3**. The HMBC correlations from H-4 to C-2, and from H-3 to C-1 revealed that the newly induced double bond was located between C-2 and C-3. The location of the additional double bond in compound **4** was also supported by UV absorption at approximately 300 nm, which is characteristic of octa-2,4,6-trienoic acid. The HMBC correlations from H-1′ to C-4, C-5, and C-6 (Figure S2) revealed that a hydroxymethyl was attached at C-5.

Clonostach acid C (**5**) was obtained as a white amorphous powder. The molecular formula of **5** was established as $C_9H_{14}O_4$ by HRESITOFMS data. The ^{1}H NMR spectrum of **5** consisted of a doublet methyl [δ_H 1.66 (3 H, d, $J = 8.4$ Hz, H-8)], three methylenes [δ_H 1.50 (1 H, m, H-4), 1.69 (1 H, m, H-4), 1.51 (1 H, m, H-3), 1.61 (1 H, m, H-3) and 2.26 (2 H, t, $J = 6.6$ Hz, H-2)], a methine [δ_H 2.90 (1 H, q, $J = 6.6$ Hz, H-5)], and two *trans* coupled olefinic protons [δ_H 5.58 (1 H, dq, $J = 15.0$, 8.4 Hz, H-7), and 5.41 (1 H, dd, $J = 15.0$, 6.6 Hz, H-6)]. Two carbonyl carbons (δ_C 175.9 and 176.8) were observed in the ^{13}C NMR spectrum. The structural elucidation of compound **5** was achieved with COSY and HMBC data (Figure S2). The molecular formula of compound **5** suggested that it contains two carboxylic acids and the HMBC correlations revealed that these carboxylic groups were attached to C-2 and C-5. The relative configuration at C-4 remains uncertain.

Compounds **1**-**5** were assayed for antimicrobial activity utilizing the agar diffusion method. The assay was performed with Gram-positive and Gram-negative bacteria as well as yeast and fungal strains. None of the tested compounds showed inhibitory activity against any of the microbes used in the assay. Furthermore, the potential phytotoxicity of compounds against lettuce seedlings (*Lactuca sativa* L.) was studied. Compound **2** (50 mg L^{-1}) inhibited shoot growth by 23% and root growth by 65%

compared to the untreated controls (Figure S3). In contrast, compounds **1**, **3**, **4**, and **5** did not exhibit phytotoxicity in this study (>100 mg L^{-1}).

3. Experimental

3.1. General experimental procedures

See detailed experimental section in supplementary material.

3.2. Fungal material and fermentation

See detailed experimental section in supplementary material.

3.3. Extraction and purification

The extract was separated by silica gel column chromatography with a stepwise elution of *n*-hexane/EtOAc (100:0-0:100) to give 11 fractions (Frs.1-1 to 1-11). Fr. 1-4 (2.0 g) was further separated on a silica gel column with $CHCl_3$/EtOAc to provide 11 fractions (Frs. 2-1 to 2-11). Fr. 2-3 was chromatographed on silica gel CC using a stepwise gradient of $CHCl_3$–EtOAc to afford **1** (2.1 mg). Frs. 1-5 and 1-6 (3.2 g) were further separated on a silica gel column with $CHCl_3$/EtOAc to provide 11 fractions (Frs. 3-1 to 3-11). Fr. 3-4 was further separated on a silica gel column with $CHCl_3$/EtOAc to provide 11 fractions (Frs. 4-1 to 4-11). Fr. 4-8 (100 mg) was subjected to ODS CC by eluting with H_2O-MeOH (20:80) to afford **2** (7.0 mg) and **4** (2.5 mg). Fr. 3-9 was subjected to ODS CC by eluting with H_2O-MeOH (20:80) to afford **3** (4.1 mg) and **5** (5.0 mg).

(-)-Dihydrovertinolide (**2**): white amorphous powder; UV (MeOH); λ_{max} (log ε) 231 (4.0), 260 (3.7) nm; HRESITOFMS *m/z* 253.1444 $[M+H]^+$, (calcd for $C_{14}H_{21}O_4$, 253.1442); IR (KBr): 3300, 2935, 1720, 1666, 1180, 1064 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.96 (1 H, m, H-5), 2.00 (1 H, m, H-5), 2.26 (1 H, m, H-6), 2.34 (1 H, m, H-6), 2.44 (2 H, t, *J* = 7.8 Hz, H-8), 2.15 (2 H, m, H-9), 5.37 (1 H, m, H-10), 5.41 (1 H, m, H-11), 1.58 (3 H, d, *J* = 6.6 Hz, H-12), 1.64 (3 H, s, H-13), 1.40 (3 H, s, H-14); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 175.6 (C-1), 95.2 (C-2), 177.5 (C-3), 82.6 (C-4), 29.9 (C-5), 35.9 (C-6), 209.9 (C-7), 42.0 (C-8), 26.5 (C-9), 125.4 (C-10), 129.5 (C-11), 16.7 (C-12), 4.6 (C-13), 22.2 (C-14).

Clonostach acid A [**3**, (2*Z*,4*E*)-3-(Hydroxymethyl)-2,4-hexadienoic acid]: white amorphous powder; UV (MeOH); λ_{max} (log ε) 260 (4.3); HRESITOFMS *m/z* 165.0514 $[M+Na]^+$, (calcd for $C_7H_{10}O_3Na$, 165.0528); IR (KBr): 3282, 2942, 1700, 1261, 971 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.82 (3 H, d, *J* = 6.6 Hz, H-6), 6.11 (1 H, dq, *J* = 16.2, 6.6 Hz, H-5), 7.37 (1 H, d, *J* = 16.2 Hz, H-4), 5.88 (1 H, s, H-2), 4.33 (2 H, s, H-1′); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 18.1 (C-6), 126.3 (C-5), 131.7 (C-4), 153.3 (C-3), 112.7 (C-2), 168.9 (C-1), 61.3 (C-1′).

Clonostach acid B [**4**, (2*E*,4*Z*,6*E*)-5-(Hydroxymethyl)-2,4,6-octatrienoic acid]: white amorphous powder; UV (MeOH); λ_{max} (log ε) 300 (4.4); HRESITOFMS *m/z* 167.0710 $[M-H]^-$, (calcd for $C_9H_{11}O_3$, 167.0708); IR (KBr): 3417, 2935, 1700, 1612, 1411, 1276, 1056 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.85 (3 H, d, *J* = 6.6 Hz, H-8), 6.00 (1 H, dq, *J* = 15.6, 6.6 Hz, H-7), 6.62 (1 H, d, *J* = 15.6 Hz, H-6), 6.30 (1 H, d, *J* = 11.4 Hz, H-4), 7.78 (1 H, dd, *J* = 15.0, 11.4 Hz, H-3), 5.88 (1 H, d, *J* = 15.0 Hz, H-2), 4.28 (2 H, s,

H-1′); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 18.0 (C-8), 129.8 (C-7), 124.9 (C-6), 145.4 (C-5), 122.0 (C-4), 139.7 (C-3), 120.8 (C-2), 169.5 (C-1), 62.2 (C-1′).

Clonostach acid C [**5**, 2-[(*E*)-1-Propenyl]adipic acid]: white amorphous powder; HRESITOFMS *m/z* 290.0791 $[M + Na]^+$, (calcd for $C_9H_{14}O_4Na$, 209.0790); IR (KBr): 3300, 2942, 1708, 1415, 1283, 1060 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.66 (3 H, d, $J = 8.4$ Hz, H-8), 5.58 (1 H, dq, $J = 15.0$, 8.4 Hz, H-7), 5.41 (1 H, dd, $J = 15.0$, 6.6 Hz, H-6), 2.90 (1 H, q, $J = 6.6$ Hz, H-5), 1.50 (1 H, m, H-4), 1.69 (1 H, m, H-4), 1.51 (1 H, m, H-3), 1.61 (1 H, m, H-3), 2.26 (2 H, t, $J = 6.6$ Hz, H-2); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 16.7 (C-8), 127.7 (C-7), 128.9 (C-6), 49.0 (C-5), 31.6 (C-4), 22.4 (C-3), 33.2 (C-2), 175.9 (C-1), 176.8 (C-1′).

3.4. General procedures for hydrogenation of the olefin 2 to give compound 2a

See detailed experimental section in supplementary material.

3.5. Bioassays

3.5.1. Antimicrobial activity

Antimicrobial assay was carried out using a published protocol (Shiono et al. 2005).

3.5.2. Phytotoxic assay

Phtotoxicity assay was carried out using a published protocol (Shiono et al. 2005).

4. Conclusion

The addition of apple juice to the rice medium when culturing *C. rosea* B5-2 significantly enhanced secondary metabolite production. As a results, under these conditions, we found four new compounds (**2**-**5**) together with the known compound (-)-vertinolide (**1**). Their structures were established on the basis of NMR spectroscopic data. Compound **2** showed the moderate phytotoxicity on lettuce seeding at a concentration of 50 mgL^{-1}. Recently, Hemphill et al. have applied the OSMAC strategy with *Fusarium tricinctum* grown in the presence of fruit or vegetable juices to obtain new biologically active secondary metabolites (Hemphill et al. 2017). We continue to investigate the secondary metabolites produced by endophytic fungi subjected to the OSMAC approach, which provides an opportunity to explore new natural products.

Acknowledgments

This investigation was financially supported by Directorate General of Scientific Resources, Technology and Higher Education, Ministry of Research, Technology and Higher Education, Indonesia (World Class Professor Grant, Number T/83/D2.3/KK.04.05/2019 for US). The authors thank Dr. Takako Aboshi in Yamagata University for MS experimental supports.

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