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Comparison of the Bioactive Secondary Metabolites from the Scale Insect Pathogens, Anamorph *Paecilomyces cinnamomeus*, and Teleomorph *Torrubiella luteorostrata*

Masahiko Isaka, Somporn Palasarn, Kanokarn Kocharin, Nigel L. Hywel-Jones

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Abstract A scale insect pathogen *Paecilomyces cinnamomeus* BCC 9616 and its teleomorph *Torrubiella luteorostrata* BCC 9617, collected on the same host specimen, were fermented and chemically explored. Both fungi produced paecilodepsipeptide A (**1**) and zeorin (**4**) as major constituents of mycelia extracts. The culture broth extract of BCC 9616 provided a known diketopiperazine, terezine D (**5**), and a new xanthone glycoside, norlichexanthone-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**6**). On the other hand, the broth extract of BCC 9617 contained small amounts of a new naphthopyrone glycoside, rubrofusarin-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**7**) along with **5**. Structures of the new compounds, **6** and **7**, were elucidated by interpretation of NMR and mass spectroscopic data. The overall results demonstrated that the metabolite profiles of the cultured anamorph (BCC 9616) and teleomorph (BCC 9617) originating from the same host specimen resemble each other closely. The ^{1}H-NMR spectroscopic analysis of the culture extracts from other strains of *P. cinnamomeus* and *T. luteorostrata* revealed that zeorin is the most commonly occurring fermentation product of these fungi, whereas paecilodepsipeptide A was the metabolite specific to the particular isolate BCC 9616/BCC 9617.

Keywords *Paecilomyces cinnamomeus*, *Torrubiella luteorostrata*, paecilodepsipeptide A, chemotaxonomy, zeorin

M. Isaka (Corresaponding author)**, S. Palasarn, K. Kocharin, N. L. Hywel-Jones:** National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, 113 Phaholyothin Road, Klong Luang, Pathumthani 12120, Thailand, E-mail: isaka@biotec.or.th

Introduction

The fungus *Torrubiella luteorostrata* is a rarely described scale insect (Homoptera) pathogen within the genus *Torrubiella sensu lato*. Its association with a *Paecilomyces cinnamomeus* anamorph was shown for Thai isolates [1]. The potential of such rare species as producers of bioactive compounds is unknown. As part of the research program on the search for bioactive compounds from insect pathogenic fungi [2], we have recently isolated an antimalarial and antitumor cyclohexadepsipeptide, paecilodepsipeptide A (**1**), its linear minor analogues paecilodepsipeptides B (**2**) and C (**3**), and a known hopane triterpene, zeorin (**4**), from *P. cinnamomeus* BCC 9616 [3]. These compounds were obtained from the mycelium extract of a liquid-media fermentation [3]. In the present study, constituents of the extract from culture broth (filtrate) were investigated, which led to the isolation of a known diketopiperazine, terezine D (**5**) [4], and a new xanthone glycoside, norlichexanthone-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**6**, Fig. 1). Fortunately, we also have the corresponding teleomorph, *T. luteorostrata* BCC 9617, collected together with the anamorph *P. cinnamomeus* BCC 9616 on the same host insect specimen. For comparison of the metabolite profiles, BCC 9617 was also subjected to large-scale fermentation, under the same culture conditions as BCC 9616, and chemical investigation was conducted for broth and mycelium extracts. **1** and **4** were again isolated as the major constituents of the mycelium extract, while the broth extract provided **5** and a small amount of a new naphthopyrone glycoside, rubrofusarin-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**7**). In addition to these studies, metabolite profiles of several other isolates of

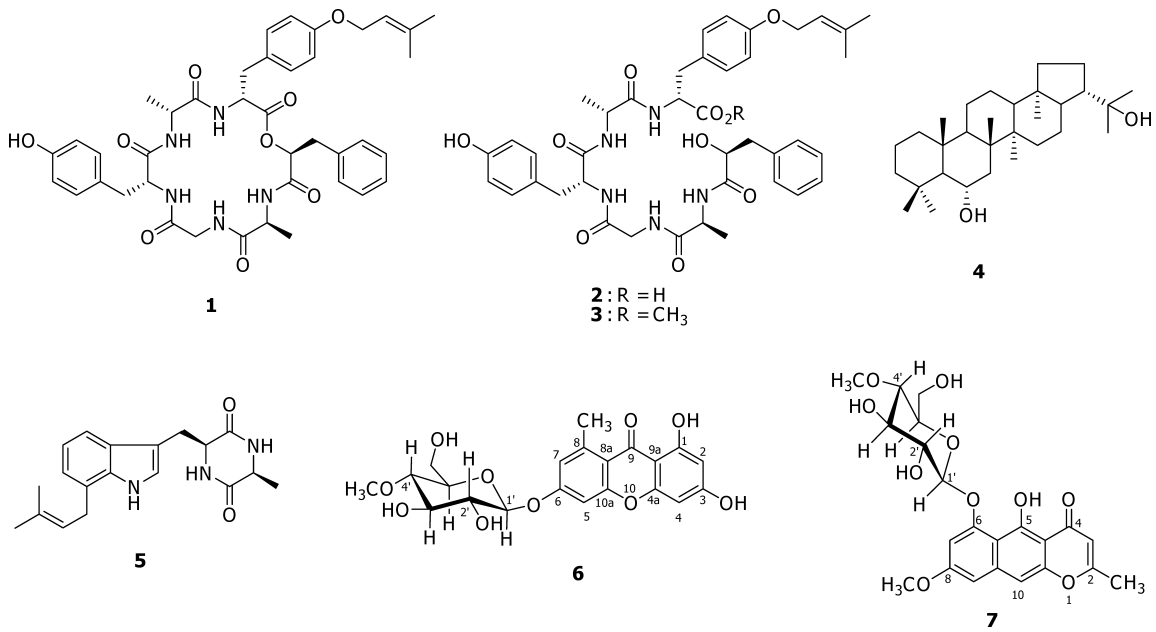

Fig. 1 Structures of the compounds isolated from *Paecilomyces cinnamomeus* BCC 9616 and/or *Torrubiella luteorostrata* BCC 9617.

P. cinnamomeus and *T. luteorostrata* were also briefly examined simply by analysis of ^{1}H-NMR spectra of the extracts.

Results and Discussion

The broth extract of *P. cinnamomeus* BCC 9616 was subjected to Sephadex LH-20 and silica gel column chromatography to furnish **5** (10 mg) and **6** (3.0 mg). **1** and **4** [5, 6] were detected in the broth extract, but with much less quantity than mycelium extract. The NMR, MS and IR spectroscopic data for **5** were identical to those of terezine D, which was previously isolated from the coprophilous fungus *Sporormiella teretispora* (Pleosporales, Sporormiaceae) [4].

The molecular formula of **6** was established as $C_{21}H_{22}O_{10}$ using HRESI-MS in combination with the ^{13}C-NMR data. The ^{1}H- and ^{13}C-NMR data (DMSO-d_6) suggested that **6** was an aromatic polyketide attached with a sugar. The aglycone was composed of a conjugated ketone resonated at δ_C 181.8, seven quaternary sp^2 carbons (δ_C 166.8, 163.5, 161.7, 158.9, 157.2, 143.0, 113.3, and 102.4), two sets of *meta*-coupled methines, δ_H 6.99 (δ_C 101.7)/δ_H 6.84 (δ_C 116.9) and δ_H 6.26 (δ_C 94.0)/δ_H 6.11 (δ_C 98.8), and a methyl group (δ_H 2.77, s; δ_C 23.4) attached to an aromatic ring. In addition, ^{1}H resonance of a chelated phenolic proton was observed at δ_H 13.27. Interpretation of the HMBC correlations (Fig. 2) revealed that the aglycone moiety was norlichexanthone (8-methyl-1,3,6-trihydroxy-

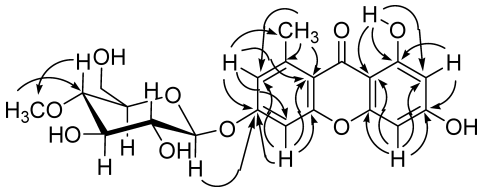

Fig. 2 Selected HMBC correlations for norlichexanthone-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**6**).

xanthone) [7]. The sugar moiety was assigned as 4′-*O*-methyl-*β*-glucopyranose. Thus, the vicinal coupling constants of $J_{1',2'}$=7.8 Hz, $J_{2',3'}$=9.1 Hz, and $J_{3',4'}=J_{4',5'}$=9.3 Hz, clearly indicated that H-1 to H-5 were all placed on axial positions. Intense HMBC correlation from methoxy protons (δ_H 3.47, 3H, s) to C-4′ and the correlation from H-4′ to the methoxy carbon (δ_C 60.1) demonstrated that the 4′-hydroxyl group was methylated. The linkage of the sugar to the xanthone was evident from the HMBC correlation from the anomeric proton (H-1′, δ_H 5.14) to C-6 (δ_C 161.7). The NOESY correlation found between H-5 and H-1′ further supported the sugar junction. On the basis of these data, **6** was designated as norlichexanthone-6-*O*-*β*-(4-*O*-methylglucopyranoside). Because of the sample shortage, acid hydrolysis of **6** was not conducted.

The teleomorph, *T. luteorostrata* BCC 9617 was cultured under the same scale (5.0 liters) and conditions as *P. cinnamomeus* BCC 9616. The mycelium extract was mainly composed of **4** and **1**, similar to the constituent of the BCC 9616 mycelium extract. A small amount of **2**

(10 mg) was obtained, while **3** was not purified due to the very low concentration in the complex mixture. These were probably isolation artefacts produced by hydrolysis or methanolysis of **1** during the column chromatography or in the methanolic extraction of mycelia. Upon examination of further purification of **1** by trituration in MeOH, we also observed partial transformations to **2** and **3**.

The extract from BCC 9617 broth was subjected to chromatographic fractionations using Sephadex LH20, silica gel and preparative HPLC (ODS column) to furnish **5** (5.6 mg) and a new naphthopyrone glycoside **7** (3.6 mg). The molecular formula of **7**, $C_{22}H_{24}O_{10}$, was determined by HRESI-MS. The ^{1}H- and ^{13}C-NMR data suggested that **7** was an aromatic polyketide glycoside, but the aglycone moiety was very different from **6**. Analysis of NMR spectroscopic data revealed the naphthopyrone structure of the aglycone to be identical with rubrofusarin (Fig. 3). The sugar moiety of **7** was also elucidated on the basis of NMR data, and it was identical to that of **6**. The linkage of the sugar to the aglycone was indicated by the HMBC correlation from H-1′ to C-6, which was also supported by the NOESY cross-peaks between H-7 and H-1′. A closely related compound, rubrofusarin-6-*O*-*β*-D-glucopyranoside, was previously isolated from the plants *Cassia pudibunda* [8] and *C. quinquangulata* [9]. **7** is its 4′-*O*-methyl derivative.

Taken together, it was concluded that the major chemical constituents of the anamorph, *P. cinnamomeus* BCC 9616, and those of the teleomorph, *T. luteorostrata* BCC 9617 are the same, although some differences were found in minor components. Since the differences between BCC 9616 and BCC 9617 is related to the life-cycle (asexual in the former, sexual in the latter), it is not unreasonable to assume that the profiles of the cell cultures by liquid-media fermentation should be identical. However, it is considered worth reporting here, because such comparisons have rarely been performed. Importantly, it also suggests that these metabolites are not dependent on the sexual form of the life-cycle but are involved in more general aspects of the function of the pathogen.

Given the evidence of the similarity of the metabolite profiles of *P. cinnamomeus*/*T. luteorostrata* within the same isolate, we compared the metabolites of different isolates. Several isolates of *P. cinnamomeus* (4 strains; BCC 7826, BCC 14491, BCC 18640, BCC 18642) and *T. luteorostrata* (5 strains; BCC 1419, BCC 7630, BCC 7841, BCC 12904, BCC 14522), collected at various locations in Thailand, were cultured using 250 ml of liquid medium for each strain, and the extracts from mycelium and broth were analyzed by ^{1}H-NMR (400 MHz). None of these extracts contained **1**. In contrast, **4** was detected in all mycelia

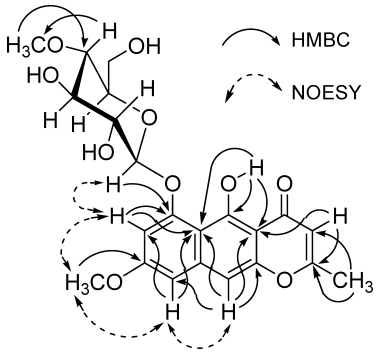

Fig. 3 Selected HMBC and NOESY cross-peaks for rubrofusarin-6-*O*-*β*-(4-*O*-methylglucopyranoside) (**7**).

extracts, with only one non-production case in *T. luteorostrata* BCC 14522. These results indicate that **1** is specific to BCC 9616/BCC 9617, whereas zeorin was the commonly-occurring metabolite of *P. cinnamomeus*/*T. luteorostrata* throughout Thailand. It should also be reported that similar NMR studies for other species of *Paecilomyces* (21 strains) and *Torrubiella* (12 strains) did not show any example of zeorin production. Zeorin (6*α*,22-dihydroxyhopane) has been isolated from lichens [10, 11], liverworts [12～14], and higher plants [15～18], but, it is not a commonly occurring fungal metabolite. In our chemical studies on the fungal metabolites, we noticed very frequent occurrence of zeorin in species of *Hypocrella* and their anamorph *Aschersonia* (unpublished result), both of which are also exclusive scale-insect pathogens. Although belonging to the same family, Clavicipitaceae, the species *P. cinnamomeus*/*T. luteorostrata* are taxonomically (morphology, phylogenetics) rather far from the genera *Hypocrella*/*Aschersonia* having affinities with the common insect pathogen *Metarhizium*. The specificity of zeorin found in *P. cinnamomeus*/*T. luteorostrata* compared with other members of the genus *Paecilomyces sensu lato* [19, 20] indicates that the metabolite could be used as a chemotaxonomic marker. In this context, we are currently planning the correlation with phylogenetic data.

Experimental

General

Optical rotations were measured with a JASCO P-1030 digital polarimeter. UV spectra were recorded on a Varian CARY 1E UV-visible spectrophotometer. FT-IR spectra were recorded on a Bruker VECTOR 22 spectrometer. NMR spectra were recorded on a Bruker AV500D spectrometer. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

Fungal Material

P. cinnamomeus (BCC 9616) was isolated by collecting conidia on a small block (*ca.* 0.5 mm) of fresh Potato Dextrose Agar (PDA) and transferring to a sterile petri plate of PDA. *T. luteorostrata* (BCC 9617) ascospores from the same specimen were isolated by allowing the ascospores to discharge over a sterile petri plate of PDA. The host was a scale insect (Homoptera - Aleyrodidae) collected in Khao Yai National Park, Nakorn Nayok province, Thailand on July 18, 2001 from the underside of a dicotyledonous leaf. These isolates were deposited in the BIOTEC Culture Collection (BCC) as BCC 9616 and BCC 9617, respectively, on August 3, 2001.

Fermentation of BCC 9616 and Isolation

P. cinnamomeus BCC 9616 was maintained on PDA at 25°C, the agar was cut into plugs (1×1 cm) and inoculated into 2×250-ml Erlenmeyer flasks containing 25 ml of potato dextrose broth (PDB; potato starch 4.0 g and dextrose 20 g in 1 liter of distilled water). After incubation at 25°C for 8 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1-liter Erlenmeyer flask containing 250 ml of the same liquid medium (PDB), and incubated at 25°C for 8 days on a rotary shaker (200 rpm). Each 25 ml portion of the secondary cultures (in 2 flasks) was transferred into 20×1-liter Erlenmeyer flasks each containing 250 ml of a liquid medium (composition, sucrose 30 g, malt extract 20 g, bacto-peptone 2.0 g, yeast extract 1.0 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and KH_2PO_4 0.5 g in 1 liter of distilled water), and final fermentation was carried out at 25°C for 24 days under static conditions. The cultures were filtered to separate mycelium (residue) and filtrate. The filtrate was extracted with EtOAc (2×3.5 liters), and the organic layer was evaporated to dryness to leave a pale brown gum (broth extract; 321 mg). The mycelium was macerated in MeOH (1 liter, 2 days) and filtered. The filtrate was defatted with hexane (700 ml) and the MeOH phase was evaporated to dryness. The residue was diluted with EtOAc (700 ml), washed with H_2O (200 ml), concentrated under reduced pressure to leave a pale brown gum (mycelium extract, 8.20 g). Isolation of **1** (174 mg) and zeorin (major constituent) from the mycelium extract was previously reported [3]. The broth extract (321 mg) was passed through a column on Sephadex LH20 (2.8×60 cm) and eluted with MeOH. The fractions, Fr-4 and Fr-5, eluted after zeorin (Fr-2) and depsipeptides (Fr-2, 3), were combined (100 mg) and it was repeatedly subjected to silica gel column chromatography (aqueous NH_4OH/MeOH/CH_2Cl_2; step gradient elution) to furnish **5** (10 mg) and **6** (3.0 mg).

6: colorless amorphous solid; $[\alpha]_D^{25}$ −27 (*c* 0.38, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.06), 241 (4.06), 253 (sh, 3.84), 307 (3.78), 348 (sh, 3.29) nm; IR (KBr) ν_{max} 3480～3200, 1655, 1620, 1281, 1091, 828 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 13.27 (1H, s, 1-O*H*), 6.99 (1H, d, *J*=2.2 Hz, H-5), 6.84 (1H, br s, H-7), 6.26 (1H, d, *J*=1.4 Hz, H-4), 6.11 (1H, d, *J*=1.5 Hz, H-2), 5.14 (1H, d, *J*=7.8 Hz, H-1′), 3.65 (1H, br d, *J*=10.4 Hz, Ha-6′), 3.53 (1H, m, H-5′), 3.52 (1H, m, Hb-6′), 3.47 (3H, s, 4′-OC*H*$_3$), 3.45 (1H, t, *J*=9.1 Hz, H-3′), 3.29 (1H, dd, *J*=8.6, 8.3 Hz, H-2′), 3.06 (1H, t, *J*=9.3 Hz, H-4′), 2.77 (3H, s, 8-*CH*$_3$); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 181.8 (s, C-9), 166.8 (s, C-3), 163.5 (s, C-1), 161.7 (s, C-6), 158.9 (s, C-10a), 157.2 (s, C-4a), 143.0 (s, C-8), 116.9 (d, C-7), 113.3 (s, C-8a), 102.4 (s, C-9a), 101.7 (d, C-5), 99.9 (d, C-1′), 98.8 (d, C-2), 94.0 (d, C-4), 79.4 (d, C-4′), 76.6 (d, C-3′), 76.2 (d, C-5′), 73.8 (d, C-2′), 60.7 (t, C-6′), 60.1 (q, 4′-O*C*H$_3$), 23.4 (q, 8-*C*H$_3$); HRMS (ESI-TOF) *m*/*z* 435.1289 $[M+H]^+$ (calcd for $C_{21}H_{23}O_{10}$, 435.1291).

Fermentation of BCC 9617 and Isolation

T. luteorostrata BCC 9617 was cultured under the same conditions and scale with those for BCC 9616 described above. Filtration and extractions afforded broth extract (1.12 g) and mycelial extract (8.20 g). The broth extract was fractionated by passing through Sephadex LH20 column and eluted with MeOH. The second fraction (120 mg) was repeatedly fractionated by silica gel column chromatography (aqueous NH_4OH/MeOH/CH_2Cl_2; step gradient elution) and preparative HPLC using a reversed-phase column (NovaPak HRC_{18}, 25×100 mm, 6 μm) and elution with MeCN/H_2O=30 : 70 (8 ml/minute) to furnish **7** (3.6 mg, t_R 5.0 minutes) and **5** (5.6 mg, t_R 6.5 minutes). The mycelial extract (8.20 g) was subjected to column chromatography on silica gel (6×20 cm, step gradient elution, MeOH/CH_2Cl_2) to obtain seven fractions; Fr-1 (1.24 g), Fr-2 (548 mg), Fr-3 (683 mg), Fr-4 (1.03 g), Fr-5 (616 mg), Fr-6 (118 mg), and Fr-7 (1.05 g). Fr-1 and Fr-2 were combined and further fractionated by CC on silica gel (4×20 cm, step gradient elution, MeOH/CH_2Cl_2) to furnish pure zeorin (237 mg) and a mixture mainly composed of zeorin (1.42 g). Fr-4 was further purified by CC on silica gel (3.8×20 cm, step gradient elution with MeOH/CH_2Cl_2) to furnish **1** (889 mg). A small amount of **2** (10.1 mg) was obtained from Fr-6 by silica gel column chromatography (2.8×20 cm, step gradient elution with MeOH/CH_2Cl_2).

7: yellow powder; $[\alpha]_D^{27}$ −88 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.32), 252 (sh, 4.26), 276 (4.49), 399 (3.65) nm; IR (KBr) ν_{max} 3445, 1657, 1626, 1581, 1111, 1049 cm^{-1}; ^{1}H-NMR (500 MHz, acetone-d_6) δ 15.34 (1H, s, 5-O*H*), 7.17 (1H, s, H-10), 6.96 (1H, d, *J*=2.3 Hz, H-9), 6.89 (1H, d, *J*=2.3 Hz, H-7), 6.12 (1H. q, *J*=0.5 Hz,

H-3), 4.96 (1H, d, J=7.5 Hz, H-1′), 4.43 (1H, d, J=4.0 Hz, 3′-O*H*), 4.32 (1H, d, J=2.2 Hz, 2′-O*H*), 3.91 (3H, s, 8-OCH_3), 3.92 (1H, m, Ha-6′), 3.89 (1H, m, 6′-O*H*), 3.74 (1H, m, Hb-6′), 3.68 (1H, dt, J=3.8, 9.8 Hz, H-3′), 3.62 (1H, ddd, J=9.5, 7.5, 2.1 Hz, H-2′), 3.58 (1H, m, H-5′), 3.57 (3H, s, 4′-OCH_3), 3.26 (1H, ddd, J=9.6, 8.7 Hz, H-4′), 2.42 (3H, br s, 2-CH_3); ^{13}C-NMR (125 MHz, acetone-d_6) δ 184.4 (s, C-4), 168.9 (s, C-2), 162.1 (s, C-5), 161.5 (s, C-8), 158.5 (C-6), 153.1 (s, C-10a), 140.8 (s, C-9a), 108.5 (s, C-5a), 106.7 (d, C-3), 104.3 (s, C-4a), 103.33 and 103.26 (d×2, C-7 and C-1′), 101.3 (d, C-10), 100.5 (d, C-9), 79.3 (d, C-4′), 76.6 and 76.4 (d×2, C-3′ and C-5′), 74.5 (d, C-2′), 61.4 (t, C-6′), 59.7 (q, 4′-OCH_3), 55.1 (q, 8-OCH_3), 19.7 (q, 2-CH_3); HRMS (ESI-TOF) m/z 449.1452 $[M+H]^+$ (calcd for $C_{22}H_{25}O_{10}$, 449.1447).

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