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Maximizing Chemical Diversity of Fungal Metabolites: Biogenetically Related Heptaketides of the Endolichenic Fungus *Corynespora* sp.¹

E. M. Kithsiri Wijeratne[†], Bharat P. Bashyal[†], Malkanthi K. Gunatilaka[§], A. Elizabeth Arnold[§], and A. A. Leslie Gunatilaka^{*†}

[†]SW Center for Natural Products Research and Commercialization, Office of Arid Lands Studies, College of Agriculture and Life Sciences, University of Arizona, 250 E. Valencia Road, Tucson, Arizona 85706-6800

[§]Division of Plant Pathology and Microbiology, School of Plant Sciences, College of Agriculture and Life Sciences, University of Arizona, Tucson, Arizona 85721-0036

Abstract

In an attempt to explore the biosynthetic potential of the endolichenic fungus, *Corynespora* sp. BA-10763, its metabolite profiles under several culture conditions were investigated. When cultured in potato dextrose agar, it produced three new heptaketides, 9-*O*-methylscytalol A (**1**), 7-desmethylherbarin (**2**), and 8-hydroxyherbarin (**3**) together with biogenetically related metabolites, scytalol A (**4**), 8-*O*-methylfusarubin (**5**), scorpinone (**6**), and 8-*O*-methylbostrycoidin (**7**) that are new to this organism, and herbarin (**8**) a metabolite previously encountered in this fungal strain. The use of malt extract agar as the culture medium led to the isolation of **6**, **8**, 1-hydroxydehydroherbarin (**9**), and 1-methoxydehydroherbarin (**10**), that was found to be an artifact formed during the extraction of the culture medium with methanol. The structures of all new compounds were determined by interpretation of their spectroscopic data and chemical interconversions.

Endosymbiotic microorganisms represent a largely untapped resource of small-molecule natural products.² This is especially true for endolichenic fungi³ as to date only two reports have described their secondary metabolites.^{4,5} This combined with the potential to produce a variety of new metabolites from a single strain by systematic alteration of its cultivation parameters,⁶ use of elicitors or enzyme inhibitors to induce or inhibit certain biosynthetic and/or signal transduction pathways,⁷ and the feeding of small-molecule alternative precursors,⁸ provide new opportunities to maximize chemical diversity of their metabolites. The approach involving alteration of culture conditions has been used to release the chemical diversity of a number of soil-borne fungi and actinomycetes,⁹ and a fungus of marine origin.¹⁰ Recent success in maximizing chemical diversity of metabolites of plant-associated fungal strains¹¹ prompted us to investigate the effect of different culture conditions on the production of metabolites by the endolichenic fungus, *Corynespora* sp. BA-10763, isolated from the cavern beard lichen, *Usnea cavernosa* (Parmeliaceae).⁴ The genus *Corynespora* contains over 100 described species of primarily plant-associated fungi with host species including phylogenetically diverse monocotyledonous and dicotyledonous angiosperms as well as a small number of non-flowering vascular plants.¹² Known to grow asymptotically under some conditions and in some hosts, several host-specific strains of *Corynespora* spp. have been explored as agents for biological control of invasive plants.¹³

*To whom correspondence should be addressed. Tel: (520) 741-1691. Fax: (520) 741-0113. leslieg@ag.arizona.edu.

The type species for the genus, *C. cassicola*, is a necrotrophic fungus that has been reported from more than 70 host plants including economically important crops such as rubber, cotton, and soybean.¹⁴ Only a few reports have appeared on the chemistry of *Corynespora* sp. An endophytic strain, *C. cassicola* L36, isolated from *Lindenbergia philippensis* has been reported to contain two novel depsidones, corynesidones A and B, and a diaryl ether, corynether.¹⁵ Chemical investigation of an EtOAc extract of *Corynespora* sp. BA-10763 derived from the liquid culture medium, potato dextrose broth (PDB), has led to the isolation and characterization of herbarin (**8**), 1-hydroxydehydroherbarin (**9**), and corynesporol (**11**).⁴ In an attempt to explore the biosynthetic potential of this endolichenic fungus, we have investigated the effect of different culture conditions on the production of metabolites and in this paper we report the isolation and structure elucidation of seven additional, but biosynthetically related heptaketides **1**–**7** of which **1**–**3** are new natural products. 1-Methoxydehydroherbarin (**10**) encountered in the extract derived from a malt extract agar (MEA) culture was shown to be an artifact formed from **9**.

Results and Discussion

Fractionation of the EtOAc extract of a potato dextrose agar (PDA) culture of *Corynespora* sp. BA-10763 involving gel permeation and silica gel column chromatography followed by normal and reversed-phase preparative TLC, yielded metabolites **1**–**8**. Compound **1** was obtained as an off-white solid that analyzed for C₁₆H₂₀O₆ by a combination of HRFABMS, ¹³C NMR, and HSQC data, and indicated the molecule to have seven degrees of unsaturation. Its IR spectrum had absorption bands at 3411 and 1658 cm⁻¹ indicating the presence of OH and α, β-unsaturated CO groups. The ¹H NMR spectrum of **1** indicated the presence of two *meta*-coupled aromatic protons (δ 6.87 and 6.37), a proton attached to an oxygenated carbon (δ 4.41), two geminally coupled protons attached to an oxygenated carbon (δ 4.12 and 3.96), two OCH₃ groups (δ 3.89 and 3.84), four protons attached to sp³ carbons (δ 2.39–2.28, m), and a CH₃ group attached to a quaternary carbon (δ 1.46). The ¹³C NMR spectrum of **1**, when analyzed in combination with the HSQC data, showed the presence of a conjugated ketone carbonyl (δ 194.2), six aromatic carbons of which two are oxygenated (δ 164.6 and 162.2) and two are protonated (δ 102.2 and 98.1), one dioxygenated quaternary carbon (δ 95.1), one oxymethine carbon (δ 73.0), two OCH₃ carbons (δ 58.8 and 55.5), two methylene carbons (δ 59.9 and 38.3), of which one is oxygenated, two methine carbons (δ 47.5 and 40.8), and a methyl carbon (δ 29.9). The spectroscopic data of this compound is similar to that of **4**, a metabolite occurring in the same extract (see later) and identified as scytalol A (**4**). Scytalol A (**4**), a modulator of melanin biosynthesis, has previously been reported from *Scytalidium* sp. 36–93.¹⁶ Methylation of **4** with Me₂SO₄/K₂CO₃/acetone afforded the monomethyl derivative with spectroscopic characteristics identical with those of **1** confirming its structure as 9-*O*-methylscytalol A.

The molecular formula of **2** was determined as C₁₅H₁₄O₆ from its HRFABMS and ¹³C NMR data and indicated the molecule to have nine degrees of unsaturation. Its UV spectrum exhibited maxima at 445 and 427 nm characteristic of a pyranonaphthoquinone,¹⁷ and its IR spectrum showed absorption bands at 3480 and 1656 cm⁻¹ indicating the presence of OH and quinone CO functions. The ¹H NMR data of **2** indicated it to be structurally related to herbarin (**8**)⁴ except for the absence of one of the two *O*-methyl groups in **2**. Methylation of **2** with CH₃I/K₂CO₃/acetone afforded its monomethyl derivative, identified as **8** indicating **2** to contain a phenolic OH group either at C–7 or C–9. Its ability to undergo methylation under mild conditions (CH₃I/K₂CO₃/acetone/25 °C) showed the OH in **2** to be located at C–7. Thus, **2** was identified as 7-desmethylherbarin.

Metabolite **3**, obtained as an orange solid, was determined to have the molecular formula $C_{16}H_{16}O_7$ by a combination of its HRFABMS, ^{13}C NMR, and HSQC data and indicated the molecule to have nine degrees of unsaturation. Its UV spectrum showed characteristic maxima for a pyranonaphthoquinone,¹⁷ and its IR spectrum had absorption bands at 3433 and 1654 cm^{-1} indicating the presence of OH and quinone CO functions. The 1H NMR spectrum of **3** indicated the presence of an aromatic proton (δ 7.40), two protons attached to an oxygenated carbon (δ 4.64), two OCH_3 groups (δ 3.98 and 3.85), two geminally coupled protons (δ 2.77 and 2.48), and a CH_3 group attached to a quaternary carbon (δ 1.54). The ^{13}C NMR spectrum of **3** when analyzed in combination with HSQC data showed the presence of two quinone carbonyls (δ 182.9 and 182.1), eight aromatic carbons of which three are oxygenated (δ 151.1, 147.0, and 144.8) and one is protonated (δ 105.9), one dioxygenated carbon (δ 94.1), two OCH_3 carbons (δ 61.4 and 58.0), two methylene carbons of which one is oxygenated (δ 56.5), and a methyl carbon (δ 28.8). These data showed close resemblance to those of herbarin (**8**)⁴ except for the presence of an additional phenolic OH in **3** at C-6 or C-8. In the HMBC spectrum of **3** (Figure 1) the aromatic proton at δ 7.40 showed a correlation with the carbonyl carbon at δ_C 182.9 (C-5) placing this proton at C-6. It was thus identified as 8-hydroxyherbarin (**3**).

The remaining metabolites isolated were identified as scytalol A (**4**),¹⁶ 8-*O*-methylfusarubrin (**5**),¹⁸ scorpinone (**6**),¹⁹ 8-*O*-methylbostrycoidin (**7**),¹⁸ and herbarin (**8**)^{4,17} by comparison of their experimental and reported physical (mp, UV, MS, and NMR) data.

Separation of an EtOAc extract obtained from the same organism, cultured in 2% malt extract agar (MEA) for 14 days, employing gel permeation and silica gel column chromatography followed by reversed-phase preparative TLC, yielded scorpinone (**6**),¹⁹ herbarin (**8**),^{4,17} 1-hydroxydehydroherbarin (**9**),⁴ and **10**. The molecular formula of **10** was determined as $C_{17}H_{16}O_6$ from its HRMS data and indicated the molecule to have 10 degrees of unsaturation. Its UV spectrum exhibited maxima at 446 and 422 nm characteristic of a pyranonaphthoquinone,¹⁷ and the 1H NMR spectrum showed the presence of two *meta*-coupled aromatic protons (δ 7.26 and 6.71, $J = 2.5\text{ Hz}$), two 1H singlets (δ 6.62 and 6.04), three OCH_3 groups (δ 3.94, 3.93 and 3.57) and a CH_3 group on an olefinic carbon (δ 2.11). These data closely resembled those for 1-hydroxydehydroherbarin (**9**);⁴ the major difference being due to the presence of an additional OCH_3 group in **10**. The chemical shift (δ 3.57) of the third OCH_3 group showed it to be attached to an sp^3 carbon and was therefore placed at C-1. Methylation (Me_2SO_4/K_2CO_3 /acetone) of **9** afforded **10** confirming the structure of **10** as 1-methoxydehydroherbarin. The presence of **9** and **10** in the same extract and the use of MeOH for extraction of the fungal culture suggested a possible artifactual origin of **10** from **9**. Although **9** failed to yield **10** on stirring with MeOH overnight, when MeOH was replaced with *n*-BuOH in the extraction process, **10** was found to be absent in the resulting extract, suggesting that this compound is an artifact formed from **9** during the extraction of culture medium with MeOH. However, it is noteworthy that 1-methoxy-9-demethyldehydroherbarin (ascomycone A) has recently been reported as a genuine metabolite of an unidentified saprophytic ascomycete.²⁰

The ability of the endolichenic fungus, *Corynespora* sp. BA-10763, to produce new secondary metabolites when grown in different culture media provides additional support for the notion that manipulation of culture conditions of endosymbiotic fungi is a promising approach for the expression of certain silent biosynthetic pathways.^{6,9-11} Interestingly, all the isolable compounds encountered were of heptaketide origin and biogenetically related to each other,²¹ with differences only in hydroxylation, *O*-methylation, and substitution of a nitrogen atom for an oxygen atom in the *O*-heterocyclic ring. In addition, the yield of herbarin (**8**) was found to be significantly higher when this fungus was cultured in potato dextrose agar (PDA) (8.3% of extract weight) compared with potato dextrose broth (PDB)

(1.7% of extract weight). It is also noteworthy that 2-azaanthraquinones, scorpinone (**6**), and 8-*O*-methylbostrycoidin (**7**), are produced in solid media (PDA and MEA), but not in the liquid medium (PDB) used. This is not surprising as previous studies have shown the production of bostrycoidin by *Fusarium solani* to be sensitive to the carbon to nitrogen ratio and pH of the culture conditions.²² We have previously reported the cancer cell migration inhibitory activity of dehydroherbarin obtained from herbarin (**8**) at a non-cytotoxic concentration of 5 μ M.⁴ When tested in this assay and cancer cell proliferation inhibition (cytotoxicity) assay using the MTT method,²³ compounds **1** – **10** showed no significant activity at 5 μ M.

Experimental Section

General Experimental Procedures

Melting points were determined with an Electrothermal melting point apparatus. Optical rotations were measured with a JASCO Dip-370 digital polarimeter using CHCl₃ or MeOH as solvent. UV spectra were recorded on a Shimadzu UV-1601 UV-VIS spectrophotometer. IR spectra were recorded on a Shimadzu FTIR-8300 spectrometer using samples prepared in KBr discs. ¹H and 2D NMR spectra were recorded in CDCl₃ or acetone-*d*₆ with a Bruker DRX-500 instrument at 500 MHz for ¹H NMR and 125 MHz for ¹³C NMR using residual CHCl₃ or acetone resonances as internal references. Low resolution and high resolution MS were recorded on Shimadzu LCMS-QP8000A and JEOL HX110A spectrometers, respectively. LRP-2 Whatman (Catalog # 4776-001) was used for reversed-phase (RP) column chromatography. RP-TLC separations were performed on Merck RP-18 F₂₅₄S pre-coated aluminium sheets.

Culturing and Isolation of Metabolites

For secondary metabolite isolation, the fungus was cultured in 20 T-flasks (800 mL), each containing 135 mL of PDA coated on five sides of the flasks, maximizing the surface area for fungal growth (total surface area/flask ca. 460 cm²). After incubation for 28 days at 28 °C, MeOH (200 mL/T-flask) was added and the flasks shaken overnight at 25 °C, and the resulting extract was filtered through Whatman No. 1 filter paper and a layer of Celite 545. The filtrate was concentrated to ca. 25% of its original volume by evaporation under reduced pressure and extracted with EtOAc (3 × 500 mL). The combined organic extracts were evaporated under reduced pressure to afford the EtOAc extract as a dark brown solid (1.5 g). The majority (1.49 g) of this was subjected to gel-permeation chromatography over Sephadex LH-20 (50.0 g) made up in hexanes and eluted with hexanes followed by hexanes–CH₂Cl₂ (2:1), hexanes–CH₂Cl₂ (1:1), hexanes–CH₂Cl₂ (1:2), CH₂Cl₂, CH₂Cl₂–acetone (3:2), CH₂Cl₂–acetone (1:4), CH₂Cl₂–MeOH (1:1), and finally with MeOH. Fifteen fractions (60 mL each) were collected and combined, based on their TLC profiles, to obtain six combined fractions [F₁ (21.8 mg), F₂ (22.5 mg), F₃–F₅ (86.1 mg), F₆–F₉ (231.1 mg), F₁₀–F₁₃ (692.6 mg) and F₁₄–F₁₅ (361.3 mg)]. A portion (13.0 mg) of combined fraction (F₆–F₉) was separated by preparative TLC [eluant: hexanes–EtOAc (1:9)] to give **6** (5.8 mg, *R*_f 0.5) and **7** (1.6 mg, *R*_f 0.3). Combined fraction F₁₀–F₁₃ (300 mg) was chromatographed over a column of silica gel (10 g) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH. One hundred and ten fractions (7.5 mL each) were collected and fractions having similar TLC patterns were combined to give ten fractions [A (4.3 mg), B (5.9 mg), C (3.7 mg), D (196.9 mg), E (20.4 mg), F (2.4 mg), G (15.2 mg), H (7.4 mg), I (2.0 mg) and J (6.2 mg)]. Fraction D (195 mg) was subjected to column chromatography over LRP-2 (10.0 g) made up in MeOH–H₂O (3:2) and eluted with MeOH–H₂O (3:2) followed by MeOH. The first few fractions from this column were combined to give **8** (33.7 mg) and the rest of the fractions were combined and subjected to repeated column chromatography over LRP-2 using CH₃CN–H₂O (3:7) as eluant to give additional quantities

of **6** (23.8 mg) and **8** (90.2 mg). Fraction G was further purified by preparative TLC [eluant: *i*-PrOH–CH₂Cl₂ (6:94)] to give **5** (11.8 mg, *R_f* 0.5). A portion (360 mg) of combined fraction (F₁₄–F₁₅) from the first column was chromatographed over a column of silica gel (25 g) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH. One hundred and seventeen fractions (7.0 mL each) were collected and fractions having similar TLC patterns were combined to give 10 fractions [K (6.3 mg), L (44.0 mg), M (80.7 mg), N (29.2 mg), O (15.6 mg), P (21.0 mg), Q (2.2 mg), R (5.4 mg), S (58.5 mg), and T (14.2 mg)]. Fraction O (15.0 mg) was separated on reversed-phase preparative TLC [eluant: CH₃CN–H₂O (2:3)] to give **3** (6.7 mg, *R_f* 0.4). Purification of fraction P (20.0 mg) by silica gel preparative TLC [eluant: *i*-PrOH–CH₂Cl₂ (5:95)] followed by reversed-phase preparative TLC [eluant: CH₃CN–H₂O (3:7)] afforded **2** (1.6 mg, *R_f* 0.5) and **4** (4.5 mg, *R_f* 0.4). A portion (30 mg) of fraction S was purified by reversed-phase preparative TLC [eluant: CH₃CN–H₂O (1:4)] to yield **1** (20.0 mg, *R_f* 0.5).

9-O-Methylscytalol A (1)—off white solid (CH₂Cl₂/hexanes); mp 172–174 °C; [α]_D²⁵ + 92 (*c* 1.1, MeOH); UV (EtOH) $\lambda_{\max}$ (log ϵ) 309 (3.64), 276 (4.11), 231 (4.10) nm; IR $\nu_{\max}$ 3411, 1658, 1598, 1569, 1456, 1326, 1218, 1203, 1161, 1051, 1020 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 6.87 (1H, dd, *J* = 2.2 and 0.8 Hz, H-6), 6.37 (1H, d, *J* = 2.2 Hz, H-8), 4.41 (1H, brd, *J* = 7.3 Hz, H-5), 4.12 (1H, dd, *J* = 11.7 and 4.3 Hz, H-1a), 3.96 (1H, dd, *J* = 11.7 and 10.1 Hz, H-1b), 3.85 (3H, s, OCH₃), 3.84 (3H, s, OCH₃), 2.39–2.28 (4H, m, H₂-4, H-4a and H-10a), 1.46 (3H, s, CH₃-3); ¹³C NMR (125 MHz, CDCl₃) δ 194.2 (C, C-10), 164.6 (C, C-7), 162.2 (C, C-9), 150.9 (C, C-5a), 114.1 (C, C-9a), 102.2 (CH, C-6), 98.1 (CH, C-8), 95.1 (C, C-3), 73.0 (CH, C-5), 59.9 (CH₂, C-1), 55.8 (CH₃, OCH₃), 55.5 (CH₃, OCH₃), 47.5 (CH, C-10a), 40.8 (CH, C-4a), 38.3 (CH₂, C-4), 29.9 (CH₃, Me); HRFABMS *m/z* 309.1331 [M + 1]⁺ (calcd for C₁₆H₂₁O₆, 309.1333).

7-Desmethylherbarin (2)—orange solid (acetone/hexanes); mp 176–178 °C; UV (EtOH) $\lambda_{\max}$ (log ϵ) 445 (4.01), 427 (3.95), 334 (4.26), 280 (4.94) nm; IR $\nu_{\max}$ 3480, 1656, 1580, 1567, 1490, 1325, 1230, 1170 cm⁻¹; ¹H NMR (500 MHz, acetone-*d*₆) δ 7.12 (1H, d, *J* = 2.2 Hz, H-6), 6.78 (1H, d, *J* = 2.2 Hz, H-8), 4.54 (2H, m, H-1a and H-1b), 3.87 (3H, s, OCH₃), 2.66 (1H, dt, *J* = 18.3, 1.8 Hz, H-4a), 2.41 (1H, dt, *J* = 18.3, 3.3 Hz, H-4b), 1.50 (3H, s, CH₃-3); ¹³C NMR (125 MHz, CDCl₃) δ 184.7 (C, C-10), 181.1 (C, C-5), 163.4 (C, C-7 and C-9), 143.9 (C, C-5a), 137.5 (C, C-4a), 136.8 (C, C-10a), 113.1 (C, C-9a), 107.4 (CH, C-6), 105.1 (CH, C-8), 94.4 (C, C-3), 58.7 (CH₂, C-1), 56.3 (CH₃, OCH₃), 32.9 (CH₂, C-4), 28.9 (CH₃, CH₃-3); HRFABMS *m/z* 289.0714 [M - 1]⁻ (calcd for C₁₅H₁₃O₆, 289.0718).

Methylation of 7-Desmethylherbarin (2)

To a solution of **2** (0.5 mg) in acetone (0.5 mL) were added CH₃I (0.05 mL) and K₂CO₃ (2.0 mg) and stirred at 25 °C for 3 h. Reaction mixture was filtered and evaporated under reduced pressure. The product (0.5 mg) formed was shown to be identical (TLC, LC-MS and ¹H NMR) with herbarin (**8**).^{4,17}

8-Hydroxyherbarin (3)—orange solid (CH₂Cl₂/hexanes); mp 163–165 °C; UV (EtOH) $\lambda_{\max}$ (log ϵ) 375 (3.80), 276 (4.71), 213 (4.69) nm; IR $\nu_{\max}$ 3433, 1654, 1589, 1564, 1492, 1330, 1226, 1166 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.40 (1H, s, H-6), 4.64 (2H, m, H₂-1), 3.98 (3H, s, OCH₃), 3.85 (3H, s, OCH₃), 2.77 (1H, ddd, *J* = 18.7, 2.4, 1.6 Hz, H-4a), 2.48 (1H, dt, *J* = 18.7, 3.5 Hz, H-4b), 1.54 (3H, s, CH₃-3); ¹³C NMR (125 MHz, CDCl₃) δ 182.9 (C, C-5), 182.1 (C, C-10), 151.1 (C, C-7), 147.0 (C, C-9), 144.8 (C, C-8), 141.7 (C, C-10a), 137.9 (C, C-4a), 125.6 (C, C-5a), 118.7 (C, C-9a), 105.9 (CH, C-6), 94.1 (C, C-3), 61.4 (CH₃, OCH₃-9), 58.0 (CH₃, OCH₃-7), 56.5 (CH₂, C-1), 31.9 (CH₂, C-4), 28.8 (CH₃, CH₃-3); HRFABMS *m/z* 319.0819 [M - 1]⁻ (calcd for C₁₆H₁₅O₇, 319.0823).

Scytalol A (4)—white solid (CH₂Cl₂/hexanes); mp 166–168 °C (Lit.¹⁶ 165–169 °C); [α]_D²⁵ + 86 (c 1.0, MeOH) [Lit.¹⁶ + 89 (c 0.9, MeOH)]; ¹H NMR and MS data were consistent with reported data.¹⁶

Methylation of Scytalol A (4)

To a solution of **4** (2 mg) in acetone (0.2 mL) were added Me₂SO₄ (10 μ L) and K₂CO₃ (10 mg) and stirred at 80 °C. After 2 h reaction mixture was filtered, filtrate evaporated under reduced pressure and the product purified by preparative TLC (8% *i*-PrOH in CH₂Cl₂) to afford a product (1.35 mg) which was found to be identical (TLC, [α]_D, LC-MS and ¹H NMR) with 9-*O*-methylscytalol A (**1**) obtained above.

8-O-Methylfusarubin (5)—red solid; mp 139–140 °C (Lit.^{18a} 138–139 °C); ¹H NMR and MS data were consistent with reported data.¹⁸

Scorpinone (6)—yellow solid (CH₂Cl₂/hexanes); mp 294–296 °C (Lit.¹⁹ 295 °C); ¹H NMR, ¹³C NMR and MS data were consistent with reported data.¹⁹

8-O-Methylbostrycoidin (7)—maroon solid (CH₂Cl₂/hexanes); mp 213–215 °C (Lit.^{18a} 215–216 °C); ¹H NMR, ¹³C NMR and MS data were consistent with reported data.¹⁸

Herbarin (8)—yellow solid (CH₂Cl₂/hexanes); mp 191–193 °C (Lit.⁴ 190–192 °C); ¹H NMR and MS data were consistent with reported data.^{4,17}

Isolation of Metabolites of *Corynespora* sp. BA-10763 Cultured in 2% MEA

The fungal strain was cultured in 20 T-flasks (800 mL), each containing 135 mL of MEA coated on five sides of the flasks (total surface area/flask ca. 460 cm²). After incubation for 14 days at 28 °C, cultures were processed as for PDA (see above) to afford the EtOAc extract as a dark brown solid (144 mg). The majority (143 mg) of this extract was subjected to column chromatography over silica gel (10 g) made up in CH₂Cl₂ and eluted with CH₂Cl₂ containing increasing amounts of MeOH followed by 100% MeOH. Fifty six fractions (7 mL each) were collected and fractions having similar TLC patterns were combined to give 12 major (F₁—F₁₂) fractions. Fraction 2 (F₂) was purified by reversed-phase preparative TLC [eluant: CH₃CN–H₂O (3:2)] to give **10** (1.4 mg, *R*_f 0.6). The major fraction (F₆) was separated by reversed-phase preparative TLC [eluant: CH₃CN–H₂O (3:2)] to give an additional quantity (1.8 mg) of **10** (*R*_f 0.4) together with **6** (1.6 mg, *R*_f 0.3), **8** (6.2 mg, *R*_f 0.8), and **9** (17.8 mg, *R*_f 0.7). Fraction 7 (F₇) was purified by reversed-phase preparative TLC [eluant: CH₃CN–H₂O (3:2)] to give an additional amount of **9** (16.5 mg).

1-Hydroxydehydroherbarin (9)—orange amorphous solid; ¹H NMR and MS data were consistent with reported data.⁴

1-Methoxydehydroherbarin (10)—orange solid (CH₂Cl₂/hexanes); mp 166–168 °C; UV (EtOH) $\lambda_{\max}$ (log ϵ) 446 (1.19), 422 (4.10), 336 (4.28), 276 (4.85) nm; IR $\nu_{\max}$ 1682, 1586, 1320, 1162, 1063 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.26 (1H, d, *J* = 2.5 Hz, H-6), 6.71 (1H, d, *J* = 2.5 Hz, H-8), 6.62 (1H, s, H-4), 6.04 (1H, s, H-1), 3.94 (3H, s, OMe), 3.93 (3H, s, OMe), 3.57 (3H, s, OMe), 2.11 (3H, s, H-3); HRFABMS *m/z* 317.1023 [M+1]⁺ (calcd for C₁₇H₁₇O₆ 317.1020).

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Figure 1.
Selected HMBC correlations for **3**.

1 R = CH₃
4 R = H

2 R₁ = R₂ = R₃ = H
3 R₁ = H, R₂ = CH₃, R₃ = OH
5 R₁ = OH, R₂ = CH₃, R₃ = H
8 R₁ = R₃ = H, R₂ = CH₃

6 R = H
7 R = OH

9 R = H
10 R = CH₃

11