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HPLC-SPE-NMR Identification of a Novel Metabolite Containing the Benzo[c]oxepin Skeleton from the Endophytic Fungus *Pestalotiopsis virgatula* Culture

Authors

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Key words

- endophytes
- *Pestalotiopsis virgatula*
- Amphisphaeriaceae
- HPLC-SPE-NMR
- NMR hyphenation

Abstract

HPLC-SPE-NMR analysis of a crude extract of fermentation broth of cultured *Pestalotiopsis virgatula* isolate TC-320 from *Terminalia chebula* Retz.

(Combretaceae) disclosed the presence of a simple but unprecedented low-molecular-weight metabolite, 9-hydroxybenzo[c]oxepin-3[1H]-one, subsequently isolated by a targeted purification procedure.

Endophytic fungi are one of the major sources of new secondary metabolites with astonishing chemical diversity [1–4]. By coevolution with their host plants and gene exchange, endophytes have adapted themselves to the biosynthesis of a number of chemicals, including those perceived as useful phytochemicals [5]. While the search for new natural products from endophytic fungi is laborious and requires considerable scale-up of fermentation cultures, the recent progress in hyphenation of NMR spectroscopy with HPLC [6–9] enables the rapid and rigorous identification of extract constituents on a microscale, which may serve as a preparatory step for the possible targeted isolation of selected metabolites [10].

Pestalotiopsis species (family Amphisphaeriaceae) are widely distributed all over the world [1–4, 11–13]; most species are plant pathogens but some are saprobes in soil or in plant debris. *Pestalotiopsis* species continue to yield structurally diverse secondary metabolites at a rapid pace [14–19], including production of important compounds such as taxol [20]. In continuation of previous reports on endophytes from medicinally important plants [21,22], we now describe application of HPLC-SPE-NMR technology for the discovery of an unusual constituent (• Fig. 1) produced by *Pestalotiopsis virgatula* isolate obtained from *Terminalia chebula* of Indian origin.

HPLC investigation of a crude extract of fermentation broth of cultured endophyte disclosed the presence of a very complex mixture with only one predominant peak (• Fig. 2), corresponding to a metabolite with $m/z = 177$ and a UV spectrum with $\lambda_{\max}$ at 280 nm and an inflection at 310 nm.

1D- and 2D-NMR spectra (COSY, NOESY, gHSQC, gHMBC) acquired from this peak in the HPLC-SPE-NMR mode showed the presence of a metabolite with a 1,2,3-trisubstituted benzene ring, a *cis*-double bond, and a methylene group. From the heteronuclear correlations (• Fig. 3) it was evident that the compound also contains a carbonyl group ($\delta = 168.8$) along with three other quaternary carbon atoms ($\delta = 155.0, 138.5$, and 123.0). Based on observation of a practically full network of HMBC (• Fig. 3) and NOESY connectivities, it became obvious that the metabolite must have the structure **1** with a hydroxylated benzo[c]oxepin-3[1H]-one skeleton. The position of the hydroxy group was confirmed, *inter alia*, by an NOE effect between H-5 and H-6 and HMBC interaction between H-1 and C-9, and the presence of the lactone ring was shown by an HMBC interaction between H-1 and C-3. The structural elucidation in the HPLC-SPE-NMR mode was carried out using less than 10 mg of the crude extract, including the material used for HPLC method development. Time-sliced HPLC-SPE-NMR experiments with the remaining sections of the chromatogram (• Fig. 2) failed to afford any useful NMR data, presumably due to the presence of numerous co-eluting constituents in insignificant amounts. Following this identification of **1** in the crude extract by HPLC-SPE-NMR, the compound was isolated by targeted preparative HPLC. The biosynthesis of **1** presumably involves aromatization of a polyketide and hydroxylation of the terminal methyl group. Phenol derivatives with an aliphatic chain at C-3 and an aldehyde or hydroxymethyl substituent at C-2 were previously isolated from *Sor-*

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Fig. 1 Structure of novel metabolite identified in this work.

Fig. 2 HPLC trace (254 nm) of a crude broth extract from cultured *Pestalotiopsis virgatula* endophyte (isolate TC-320); 150 × 4.6 mm i.d. Phenomenex C₁₈(2) Luna column (3 μm), elution rate 0.8 mL/min, 40 °C, acetonitrile gradient in water as shown.

daría macrospora [23,24], from an unidentified fungus probably belonging to *Cladosporium* [25], and from Ascomycetes [26].

Materials and Methods

The fungus (code TC-320) was isolated from inner bark of *Terminalia chebula* Retz. (Combretaceae) collected in Gopalaswamy Hills in Southern India (11°57' N, 75°12' E) and identified as *Pestalotiopsis virgatula* (Kleb.) Steyaert (family Amphispheariaceae) as described in detail elsewhere [22]. The host plant was identified by Mr. S. Kumara, PhytoMyco Research Pvt. Ltd., Nanjangud, India; the voucher specimen (accession number BSI/SC/5/23/07-08/tech-2) was deposited in herbarium MH (Botanical Survey of India, Southern Circle, Tamil Nadu, Agricultural University). The isolate was subjected to fermentation in ten 1000-mL Erlenmeyer flasks containing 500 mL of M1D medium [27] at 23 °C for 21 days under static conditions. Fungal mycelia were separated from the culture filtrate by passing through four layers of cheese-cloth. Each filtrate was extracted with ethyl acetate (2 × 1 L) and the combined extracts were dried by flash evaporation; total yield: 0.31 g of a dark, resinous residue.

HPLC-PDA-MS-SPE-NMR analyses were performed using a system consisting of an Agilent 1100 liquid chromatograph (degasser, quaternary pump, autosampler, and column oven) equipped with a 150 × 4.6 mm i.d. Phenomenex C₁₈(2) Luna column (3 μm) operated at 40 °C and eluted at a flow rate of 0.8 mL/min, a Bruker Esquire LC ion trap mass spectrometer operating in the ESI mode and receiving 5% of the column effluent by means of a splitter, an

Fig. 3 Assignment of ¹³C-NMR chemical shifts of **1** from gHSQC (top) and gHMBC (bottom) spectra acquired from crude broth extract in the HPLC-SPE-NMR mode. Insert shows selected HMBC correlations (from H to C); horizontal lines denote residual one-bond correlations. The gHSQC spectrum (with WET solvent suppression) was acquired with 1 k × 128 data points and 64 transients per increment (total experiment time 5 h 7 min). The gHMBC spectrum was acquired with 2 k × 256 data points and 128 transients per increment (total experiment time 24 h 28 min). The ¹H-NMR spectrum shown as a projection was acquired with NOESYPRESAT solvent suppression, collecting 512 transients consisting of 64 k data points over a sweep-width of 20 ppm (total acquisition time 1 h 24 min).

Agilent 1100 photodiode-array detector, a Knauer K100 Wellchrom pump delivering water (2 mL/min) to the PDA detector effluent, a Spark Holland Prospekt 2 solid-phase extraction device, and a Bruker Avance 600 MHz spectrometer with an inverse ¹H {¹³C} flow-probe (30 μL active volume, 60 μL total volume) kept at 25 °C. The linear gradient elution profile was made of water/

acetonitrile 95:5 + 0.1% formic acid (eluent A) and acetonitrile/water 95:5 + 0.1% formic acid (eluent B) with 0% of B at 0 min, 35% of B at 50 min, and 100% of B at 123 min. GP phase [general-purpose poly(divinylbenzene)-based resin] solid-phase extraction cartridges (10 × 2 mm i.d.; Spark Holland) were used for trapping based on UV absorption levels (254 nm) and time intervals. Four successive separations of crude extract and cumulative SPE trappings were performed using 50 µL injections of crude extract solution (34.5 mg/mL in acetonitrile), and the analytes were eluted to the NMR probe with acetonitrile-*d*₃ using optimized elution and push volumes of the solvent. Standard Bruker library pulse sequences were used for NMR data acquisition (see also **Fig. 3**).

Preparative-scale isolation of **1** was performed with an Agilent 1100 chromatographic system consisting of two preparative pumps, an autosampler, a sample collector, a multiple-wavelength UV detector, and a 250 × 21.2 mm i.d. Phenomenex C₁₈ (2) Luna column (5 µm) operated at ambient temperature and using an elution gradient profile (20 mL/min) similar to that described above. Two injections (900 µL) of a crude extract solution in acetonitrile (76.8 mg/mL) afforded, after repeated purification, 4.5 mg of **1**.

9-Hydroxybenzo[c]oxepin-3[1H]-one (1): ¹H-NMR (CD₃CN, 600 MHz, hyphenation mode): δ = 7.61 (1H, br s, 9-OH), 7.31 (1H, t, *J*_{H-6,H-7} = *J*_{H-7,H-8} = 7.9 Hz, H-7), 7.25 (1H, d, *J*_{H-4,H-5} = 12.1 Hz, H-5), 7.00 (1H, br d, *J*_{H-6,H-7} = 7.9 Hz, H-6), 6.99 (1H, dd, *J*_{H-7,H-8} = 7.9, *J*_{H-6,H-8} ≈ 1.1 Hz, H-8), 6.27 (1H, d, *J*_{H-4,H-5} = 12.1 Hz, H-4), 5.18 (2H, br s, H-1); ¹H-NMR (CDCl₃, 600 MHz, isolated material): δ = 7.30 (1H, t, *J*_{H-6,H-7} = *J*_{H-7,H-8} = 8.0 Hz, H-7), 7.27 (1H, br s, 9-OH), 7.19 (1H, br d, *J*_{H-4,H-5} = 12.1 Hz, H-5), 6.99 (1H, br d, *J*_{H-6,H-7} = 8.0 Hz, H-6), 6.90 (1H, br d, *J*_{H-7,H-8} = 8.0, H-8), 6.37 (1H, d, *J*_{H-4,H-5} = 12.1 Hz, H-4), 5.26 (2H, br s, H-1); ¹³C-NMR (CD₃CN, 150 MHz, from HSQC and HMBC correlations acquired in hyphenation mode): δ = 168.6 (C-3), 154.8 (C-9), 141.3 (C-5), 138.5 (C-11), 131.2 (C-7), 123.6 (C-4), 123.0 (C-10), 122.3 (C-6), 118.0 (C-8), 61.9 (C-1); ¹³C NMR (CDCl₃, 100 MHz, isolated material): δ = 168.2 (C-3), 153.0 (C-9), 140.6 (C-5), 137.6 (C-11), 130.2 (C-7), 123.0 (C-4), 122.2 (C-6), 122.1 (C-10), 117.4 (C-8), 61.1 (C-1); ESI-MS (hyphenation mode): *m/z* = 177.3 [M + H]⁺; HR-ESI-TOF-MS (isolated material): *m/z* = 199.0366 [M + Na]⁺, C₁₀H₈O₃Na⁺ requires 199.0366 (Δ*m* = 0.0 ppm).

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