



New peptidendrocins and anticancer chartreusin from an endophytic bacterium of *Dendrobium officinale*

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Background: Endophyte has now become a potential source for the discovery of novel natural products, as they participate in biochemical pathways of their hosts and produce analogous or novel bioactive compounds. As an epiphytic plant, *Dendrobium officinale* is one of precious Chinese medicines with various activities. It is well known for containing diverse endophytes, but so far not much is known about their secondary metabolites.

Methods: the plant tissues were cut and cultured on agar plates to isolate and purify the endophytic bacteria from *Dendrobium officinale*. Taxonomical identification of strains was performed by 16s rRNA. At the same time, the crude extracts of the strains were tested for antibacterial and cytotoxic activities to screen out one endophyte, *Streptomyces* sp. SH-1.2-R-15 for further study. After scale-up fermentation, isolation, purification and structure elucidation by using MS, 1D/2D-NMR spectroscopic method, secondary metabolites were identified and submitted for biological activity test.

Results: Fifty-eight endophytic strains representing 9 genera were obtained, with 50% of strains were *Streptomyces*. One of the most active strain, *Streptomyces* sp. 1.2-R-15, was selected for bioassay-guided isolation, which led to the discovery of two new peptide-type compounds 1 and 2, as well as a bioactive chartreusin, and four other known natural products. Their structures were determined by comprehensive spectroscopic techniques. Chartreusin showed potent cytotoxicity against Hep3B2.1-7 (IC₅₀ =18.19 μM) and H1299 (IC₅₀ =19.74 μM) cancer cell lines, and antibacterial activity against *S. aureus* (IC₅₀ =23.25 μM).

Conclusions: This study highlights the endophytic bacteria from medical plant *D. officinale* have potential bioactivity and natural product diversity, thus implicates them as a valuable source for new anticancer and antibiotics agents.

Keywords: *Dendrobium officinale*; endophyte; anticancer; peptide; chartreusin

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Introduction

Endophytic microorganisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic (1,2). Since the report of taxol and taxane produced by a fungal endophyte from the phloem (inner bark) of Pacific yew in 1993 (3), endophyte has served as a widely used model for the discovery of natural products, with participating in biochemical pathways and produce analogous or novel bioactive compounds (1,4-7). Therefore, the prospects of finding new drugs from endophytes for treating newly developing diseases in humans, plants, and animals are great. Compared with higher organisms, microorganisms can be easily maintained under laboratory conditions, ensuring a constant and inexpensive supply of their secondary metabolites. Moreover, many compounds originally believed to be produced by medicinal plants have been found to be produced by host-associated microorganisms (3,6).

Dendrobium officinale, a tonic herb in Chinese materia medica and health food in folk, has been utilized for the treatment of Yin-deficiency diseases for thousands of years, and was popularly consumed as a functional dietary supplement and one of the most expensive tea materials (8). Modern pharmacological researches have demonstrated that *Dendrobium* genus have multiple biological activities, including hepatoprotective, anticancer, antimicrobial, hypoglycemic, antifatigue and gastric ulcer protective effects (9-11). As part of an effort to explore the microbial diversity and corresponding metabolic potential of actinomycetes associated with unique ecological environments (12-18), 75 endophytic bacteria (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>) were pure cultured from different parts of *D. officinale* and submitted for cytotoxic and antibacterial activity screening. After the dereplication by their morphological features and LC-MS screening, 58 total diverse strains were then identified through phylogenetic analysis. The 16S rRNA analysis revealed that the 58 strains represent 9 genera including 4 Actinobacterial genera (Figures 1, <http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>), and 50% of them belonged to *Streptomyces*. According to the bioactivity results (for testing both antibacterial and cytotoxic activities) and LC-HRMS analyses, SH-1.2-R-15 was select as a potentially antibacterial strain for the next scale-up fermentation and secondary metabolites study. Bioassay-guided isolation yielded two new peptides, peptidendrocins A and B [1,2],

together with bioactive chartreusin [3], and four other known compounds (Figure 2). Chartreusin [3] showed moderate cytotoxicity against Hep3B2.1-7, J82 and H1299 cancer cell lines, and antibacterial activity against *S. aureus*. Herein we report the strain isolation, fermentation, isolation, structure elucidation and bioactivity assay of the strains and natural products.

Methods

General experimental procedures

NMR spectra were measured on a Bruker Avance AV500 spectrometer (Bruker, Germany). LC-MS was conducted with an Agilent 1290-6120 LC-Quadrupole MSD mass spectrometer (Agilent Technologies, USA). HRESIMS spectra were obtained on a Q-Exactive Plus mass spectrometer (Thermo Scientific, USA). HPLC analyses were performed on an Agilent 1260 HPLC with PDA detector (Agilent Technologies, USA). Preparative HPLC was performed on a Waters 150 LC system by using a Ultimate[®]AQ-C18 column (250 × 21.2 mm, 5 μm, Welch Material, USA). MCI gel High porous polymer (75-150 μm) was purchased from Mitsubishi chemical corporation (Japan). Sephadex LH-20 (25-100 μm) was obtained from GE Healthcare (Sweden). XAD16N resin (20-60 mesh) was obtained from Yuanye (Nanjing, Jiangsu, China). TaKaRa Ex Taq DNA Polymerase (USA) and OMEGA gel extraction kit (200) were purchased from Kaimoer (Nanjing, China). TLC silica gel plates (HSGF₂₅₄) were obtained from Juyou company (Shandong, China). Chemicals were purchased from Acros or Juyou and used without further purification unless otherwise noted.

Strain isolation

Plant samples of *D. officinale* with two different growth ages were collected from Zhejiang Haixing Biotech Company (Wuyi City, Zhejiang Province, China). The root, leaf and stem of plants were separated and cleaned with water then rinsed in 0.1% Tween-20 for 30 s, sequentially immersed in 75% ethanol for 5 min and in 2% sodium hypochlorite for 5 min and rinsed with 10% NaHCO₃ for 10 min to inhibit fungal growth (2). After each treatment, samples were rinsed three times in sterile water. The surface sterilized samples were aseptically dissected into small pieces. 0.5 g of each sample was suspended in 1.0 mL of sterile H₂O, and heated at 75 °C for 1 min to eliminate nonsporulating

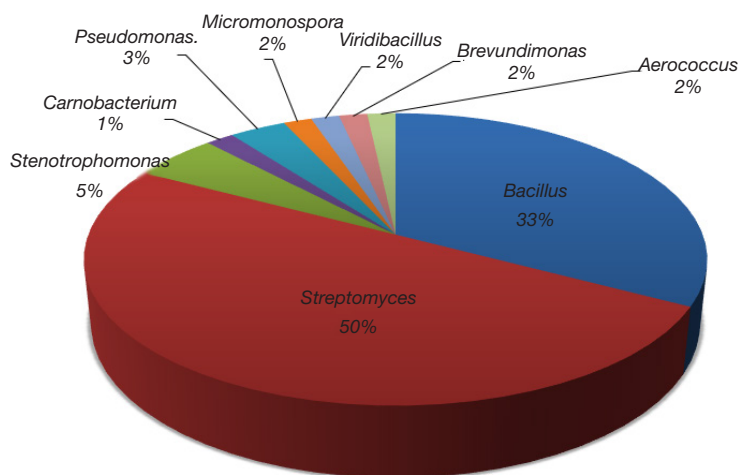


Figure 1 Diversity of cultivated endophytic bacterial genera isolated from *D. officinale*.

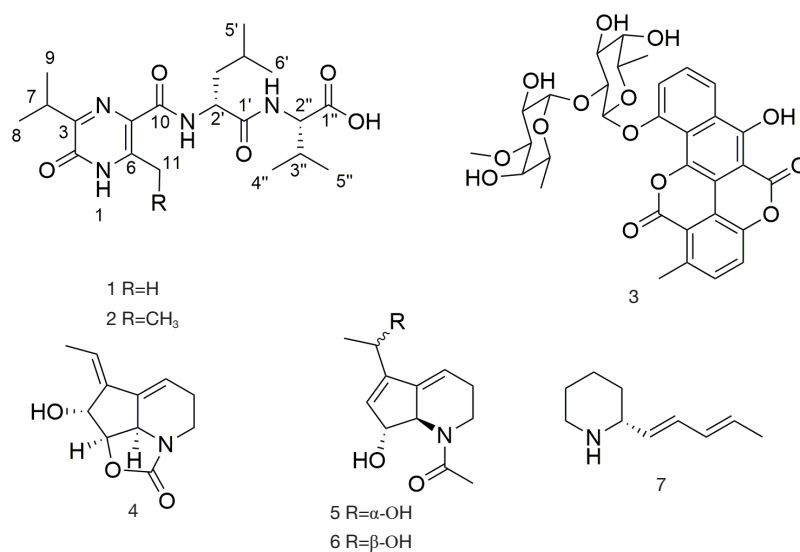


Figure 2 Compounds [1–7] isolated from *Streptomyces* sp. SH-1.2-R-15.

bacteria. A 100 μ L aliquot of supernatant was streaked on oatmeal agar and on ISP4 agar plates supplemented with nalidixic acid (25 μ g/mL) and amphotericin B (25 μ g/mL). A number of sporulating bacterial colonies were observed after 1–2 month of incubation at 28 $^{\circ}$ C, and each colony was subsequently purified on a M2 agar plate (17). Overall, 58 endophytic strains were isolated from 4 different plant samples as evidenced by morphological features (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>) and 16S rRNA analysis (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>).

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Phylogenetic analysis

Each strain was inoculated in 100 mL baffled flask with 20 mL TSB broth. After 3 days culture at 28 $^{\circ}$ C with 160 rpm agitation, cells were recovered via centrifugation (12,000 rpm for 15 min at 4 $^{\circ}$ C) and used for genomic DNA isolation using DNA Isolation Kit. The partial 16S rRNA (19) gene fragment was amplified using universal primers (Forward 5'-CAGAGTTTGATCCTGGCT-3';

Reverse 5'-AGGAGGTGATCCAG CCGCA-3') and the desired PCR-amplified product isolated using the OMEGA gel extraction kit (200). 16S rRNA gene sequences were compared with GenBank database using BlastN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for searching the closest match sequence. The sequences of strain 16S rRNA have been deposited in the NCBI nucleotide database (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>).

Strain prioritization

Individual bacterial colonies were fermented in 50 mL of medium Bran (corn flour, 40 g/L; glucose, 10 g/L; gluten powder, 5 g/L; K₂HPO₄·3H₂O, 0.5 g/L; bran, 10 g/L; CaCO₃, 2 g/L; (NH₄)₂SO₄, 1 g/L) using 250 mL baffled Erlenmeyer flasks for six days at 28 °C with 200 rpm agitation. 0.5 g XAD-16 resin was added to the culture 24 h prior to harvesting. Each individual culture was transferred to a 50 mL falcon tube and centrifuged at 5,000 rpm for 15 min. The supernatant was discarded and the mycelia-XAD resin portion was washed twice with 30 mL of distilled water (vortexed, centrifuged and discarded). 15 mL methanol was then added and sonicated for 15 min, followed by centrifuge to generate the crude extract after evaporation of supernatant solvent. Each extract was then subjected to cytotoxic (against Hep3B2.1-7 cancer cell) and antimicrobial (against *E. coli* and *S. aureus*) activities screening. 28 strains showed potent inhibition more than the hit cutoff (inhibition =11.7%) in Hep3B2.1-7 cell viability assay (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). And 21 strains showed inhibition against *S. aureus* more than the hit cutoff (36.2%) (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). Only one strain was active against *E. coli* (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). The bioactivity combined with LC-MS profiles (used for identification of potential novel secondary metabolites) decided strain SH-1.2-R-15 from the root of one year old *D. officinale*, to be the most interesting strain capable of producing novel metabolites, based on SciFinder database comparison, and its potent cytotoxic and antimicrobial activity (23.97% and 72.75% inhibition, respectively).

Fermentation, extraction and isolation

Streptomyces sp. SH-1.2-R-15 was cultivated on M₂-agar

(glucose, 10.0 g/L; malt extract, 10.0 g/L; yeast extract, 4.0 g/L; agar, 15.0 g/L) plates at 28 °C for 7 days. Chunks of agar with the fully-grown strain were used to inoculate five 250 mL Erlenmeyer baffled flasks, each containing 50 mL of medium Bran [corn flour, 40.0 g/L; glucose, 10.0 g/L; gluten powder, 5.0 g/L; K₂HPO₄·3H₂O 0.5 g/L; bran 10.0 g/L; CaCO₃ 2.0 g/L; (NH₄)₂SO₄ 1.0 g/L]. After three days of incubation at 28 °C with 200 rpm agitation, the seed cultures were used to inoculate 80 flasks (250 mL), each containing 100 mL of medium Bran. The fermentation was continued at 28 °C with 200 rpm agitation. After 7 days, the culture broth was combined and centrifuged at 8,000 rpm for 20 min (4 °C). The biomass (mycelium) was extracted with MeOH (3 × 1,000 mL) and the corresponding organics were evaporated *in vacuo* to yield 54.8 g of mycelium crude extract. The supernatant was mixed with 4% (w/v) XAD-16 resin and stirred overnight, followed by filtration. The resin was washed with H₂O (3 × 500 mL) and then extracted with MeOH until the eluent was colorless. The MeOH extract was subsequently evaporated to afford 27.5 g of XAD-crude extract. HPLC and TLC analysis revealed a similar metabolite profile in both mycelial cake and culture extracts. Therefore, the extracts were combined to afford 82.3 g and then fractionated by MCI column (500 g) using gradient elution from 20% to 100% aqueous MeOH to provide nine fractions (A–I). Fraction B (1.51 g) was subjected to Sephadex LH-20 column (80% aqueous MeOH) to obtain two sub-fractions, B1–B2. Sub-fraction B1 (147 mg) was further purified by prep-HPLC (13% aqueous CH₃CN) to yield compounds 5 (2 mg, t_R=15.2 min) and 6 (3 mg, t_R=15.7 min). Fraction D (1.95 g) was subjected to Sephadex LH-20 column (80% aqueous MeOH) to obtain two sub-fractions, D1–D2. Sub-fraction D1 (120 mg) was further purified by prep-HPLC (11% aqueous CH₃CN) to yield compound 4 (29 mg, t_R=16.0 min). Sub-fraction D2 (206 mg) was further purified by prep-HPLC (30–70% aqueous CH₃CN) to yield compounds 1 (1 mg, t_R=25.4 min) and 2 (16 mg, t_R=25.8 min). Fraction F (1.24 g) was purified by prep-HPLC (50–80% aqueous CH₃CN) to yield compound 7 (6 mg, t_R=16.7 min). Fraction H (0.99 g) was crystallized in methanol to yield compound 3 (62 mg, t_R=25.7 min).

Peptidendrocin A [1]: yellow-brown oily substance. NMR data, see Table 1; (+)-ESI-MS: *m/z* 409.2 [M + H]⁺; (–)-ESI-MS: *m/z* 407.2 [M – H][–]; (+)-HR-ESI-MS *m/z* = 409.2444 [M + H]⁺ (calcd. for C₂₀H₃₃N₄O₅, 409.2451), (–)-HR-ESI-MS *m/z* = 407.2294 [M – H][–] (calcd. for C₂₀H₃₁N₄O₅, 407.2294).

Peptidendrocin B [2]: yellow-brown oily substance. NMR data, see Table 1; (+)-ESI-MS: m/z 423.2 $[M + H]^+$; (-)-ESI-MS: m/z 421.2 $[M - H]^-$; (+)-HR-ESI-MS m/z =423.2596 $[M+H]^+$ (calcd. for $C_{21}H_{35}N_4O_5$, 423.2607), (-)-HR-ESI-MS m/z =421.2451 $[M - H]^-$ (calcd. for $C_{21}H_{33}N_4O_5$, 421.2451).

Chartreusin [3] (20,21): pale green powder, HRESIMS m/z 663.1688 $[M + Na]^+$ (calcd. for $C_{32}H_{32}O_{14}Na^+$, 663.1690). 1H NMR (500 MHz, DMSO- d_6) δ 8.09 (1H, d, J =8.3 Hz, H-8), 7.73 (1H, t, J =8.3 Hz, H-9), 7.53 (1H, d, J =8.3 Hz, H-10), 7.70 (1H, d, J =8.3 Hz, H-16), 7.63 (1H, d, J =8.3 Hz, H-17), 2.82 (3H, s, H-19), 5.38 (1H, d, J =7.7 Hz, H-1'), 3.96 (1H, m, H-2'), 3.63 (1H, m, H-3'), 4.58 (1H, d, J =6.6 Hz, 3'-OH), 3.62 (1H, m, H-4'), 4.89 (1H, d, J =3.8 Hz, 4'-OH), 3.94 (1H, m, H-5'), 0.99 (3H, d, J =6.3 Hz, H-6'), 5.43 (1H, d, J =3.7 Hz, H-7'), 3.37 (1H, m, H-8'), 3.60 (1H, brs, 8'-OH), 3.09 (1H, dd, J =10.1, 2.6 Hz, H-9'), 3.60 (1H, m, H-10'), 4.24 (1H, d, J =4.4 Hz, 10'-OH), 4.21 (1H, m, H-11'), 1.21 (3H, d, J =6.3 Hz, H-12'), 3.13 (3H, s, H-13'); ^{13}C NMR (125 MHz, DMSO- d_6) δ 158.8 (C-1), 117.2 (C-2), 120.0 (C-3), 108.7 (C-4), 97.3 (C-5), 155.6 (C-6), 126.3 (C-7), 116.5 (C-8), 128.5 (C-9), 114.5 (C-10), 154.3 (C-11), 118.2 (C-12), 138.6 (C, C-13), 164.0 (C, C-14), 146.5 (C, C-15), 121.0 (C-16), 133.1 (C-17), 138.6 (C-18), 21.9 (C-19), 99.6 (C-1'), 78.0 (C-2'), 72.2 (C-3'), 71.4 (C-4'), 70.4 (C-5'), 16.6 (C-6'), 99.9 (C-7'), 67.0 (C-8'), 79.9 (C-9'), 67.6 (C-10'), 65.9 (C-11'), 16.7 (C-12'), 56.0 (C-13').

(+)-*Streptazolin* [4] (22,23): pale yellow oil, HRESIMS m/z 208.0971 $[M + H]^+$ (calcd. for $C_{11}H_{14}NO_3^+$, 208.0974). 1H NMR (500 MHz, DMSO- d_6) δ 1.91 (3 H, d, J =7.3 Hz, H-1), 2.21-2.50 (2 H, m, H-9), 3.40-3.47 (2 H, m, H-10), 4.31 (1 H, d, J =6.5, H-6), 4.59 (1 H, s, H-5), 4.61 (1 H, d, J =6.6, H-4), 6.06 (1 H, m), 6.10 (1 H, q, J =7.2 Hz); ^{13}C NMR (125 MHz, DMSO- d_6) δ 15.0 (C-1), 22.5 (C-9), 40.1 (C-10), 59.0 (C-6), 73.6 (C-4), 81.9 (C-5), 118.3 (C-8), 122.5 (C-2), 139.5 (C-3), 143.7 (C-7), 159.2 (C-11).

Streptazolin A [5] (24): pale yellow oil, HRESIMS m/z 206.1177 $[M - H_2O + H]^+$ (calcd. for $C_{12}H_{16}NO_2^+$, 206.1181). 1H NMR (500 MHz, CD $_3$ OD) δ 1.37 (3H, d, J =6.5 Hz, H-1), 4.50 (1H, q, J =6.5 Hz, H-2), 5.84 (1H, brs, H-4), 4.56 (1H, m, H-5), 4.31 (1H, brs, H-6), 6.05 (1H, m, H-8), 2.30 (2H, m, H-9), 3.16 (1H, td, J =12.2, 3.2 Hz, H-10a), 3.82 (1H, dt, J =12.2, 3.2 Hz, H-10b), 2.19 (3H, s, H-12); ^{13}C NMR (125 MHz, CD $_3$ OD) δ 22.7 (C-1), 64.9 (C-2), 148.7 (C-3), 130.6 (C-4), 81.3 (C-5), 68.6 (C-6), 140.9 (C-7), 118.0 (C-8), 26.1 (C-9), 46.4 (C-10), 175.7 (C-11), 22.9 (C-12).

Streptazolin B [6] (24): pale yellow oil, HRESIMS m/z 206.1173 $[M - H_2O + H]^+$ (calcd. for $C_{12}H_{16}NO_2^+$,

206.1181). 1H NMR (500 MHz, CD $_3$ OD) δ 1.37 (3H, d, J =6.5 Hz, H-1), 4.55 (1H, q, J =6.5 Hz, H-2), 5.89 (1H, brs, H-4), 4.57 (1H, m, H-5), 4.31 (1H, brs, H-6), 5.98 (1H, m, H-8), 2.30 (2H, m, H-9), 3.16 (1H, td, J =12.2, 3.2 Hz, H-10a), 3.84 (1H, dt, J =12.2, 3.2 Hz, H-10b), 2.20 (3H, s, H-12); ^{13}C NMR (125 MHz, CD $_3$ OD) δ 23.0 (C-1), 65.0 (C-2), 149.4 (C-3), 129.2 (C-4), 81.2 (C-5), 68.6 (C-6), 141.1 (C-7), 117.4 (C-8), 26.1 (C-9), 46.4 (C-10), 175.6 (C-11), 22.9 (C-12).

(*R*)-(*E,E*)-2-(1,3-pentadienyl) piperidine [7] (25): pale yellow oil, HRESIMS m/z 152.1432 $[M + H]^+$ (calcd. for $C_{10}H_{18}N^+$, 152.1439). 1H -NMR (DMSO- d_6 , 500 MHz): δ 6.31(1H, dd, J =15.4, 10.5 Hz, H-8), 6.09(1H, dd, J =15.0, 10.6 Hz, H-9), 5.83(1H, m, H-10), 5.52(1H, dd, J =15.4, 7.2 Hz, H-7), 3.66 (1H, brs, H-2), 2.89, 3.23 (2H, m, H-6), 1.72, 1.52 (2H, m, H-5), 1.82, 1.50 (2H, m, H-3), 1.49, 1.77 (2H, m, H-4), 1.76 (3H, d, J =6.5 Hz, H-11); ^{13}C -NMR (DMSO- d_6 , 125 MHz): δ 134.8 (C-8), 132.5 (C-10), 130.7 (C-9), 126.4 (C-7), 57.1 (C-2), 44.0 (C-6), 29.1 (C-3), 21.9 (C-4), 22.0 (C-5), 18.4 (C-11).

Cell culture and 96-well cell viability assay

Human hepatocellular carcinoma cell line Hep3B2.1-7 (ATCC HB-8064), bladder cancer cell J82, and lung cancer cell H1299 were obtained from Cell Bank of Chinese Academy of Sciences (www.cellbank.org.cn), and cultured according to the manufacturer's suggested protocol. Cancer cells were cultured in flasks in MEM (Part no. 10-010-CVR, Corning) supplemented with 10% fetal bovine serum (Part no. 10270-106, Thermo Fisher), 1 \times Anti-Anti (part no. 15240-062, Thermo Fisher) at 37 $^{\circ}C$, 5% CO $_2$, and 95% relative humidity. Prior to plating, cells were grown to 80% confluence in MEM complete growth media. After washing once with phosphate-buffered saline (PBS), cells were detached by TrypLE Express (part no. 12604021, Thermo Fisher) and centrifuged at 300g for 5 min. Cells were suspended and counted. Two thousand cells in 80 μ L of culture media were seeded in 96-well plates (part no. 3603, Corning). After incubation of the assay plates overnight (about 15 h), cells were treated with test samples *i.e.*, crude extracts or compounds (20 μ L, final concentration of 50 or 25 μ g/mL depends on the solubility of crude extracts) or vehicle (0.25% DMSO). Cell viability was assessed in triplicate after 72 h of incubation using CellTiterGlo reagent according to manufacturer's instructions. The Tecan Spark microplate reader (Tecan Group Ltd., Swiss) was used to quantitate luminescence signal.

CellTiter-Glo Luminescent Cell Viability Assay is a robust, sensitive assay, widely-used in evaluation of drug activity on cell viability based on quantification of ATP levels in culture (26,27).

Compound activity was normalized on a per-plate basis using the following equation (26).

$$\% \text{Inhibition} = 100 \times \frac{(\text{RLU Low control} - \text{RLU Test})}{(\text{RLU Low control} - \text{RLU High control})}$$

Test Well refers to those wells with cells treated with test compounds. High Control is defined as wells containing medium only (100% inhibition), and Low Control wells contain cells treated with DMSO only (0% inhibition).

IC₅₀ values of top hits were determined by fitting the concentration–response curve (CRC) data with a four-parameter variable-slope method in GraphPad Prism (GraphPad Software, USA).

Bacterial culture and 96-well turbidity assay

Standard strain of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were obtained from CICC (China Center of Industrial Culture Collection, China). Bacterial pharmacology of reference controls was verified based on the antibiotic minimum inhibitory concentrations (MICs) determined by the standard broth dilution method according to M7-A7 Clinical and Laboratory Standards Institute (CLSI) guideline. The MIC was defined as the lowest drug concentration that prevented visible bacterial growth following overnight incubation. The MIC determined here using the described absorbance readout method (28) matched the determined MIC according to the CLSI guideline. Bacteria were inoculated in LB Broth media and incubated overnight at 37 °C. Overnight bacterial cultures were quantified via spectrophotometer, diluted to 10⁶ CFU/mL (OD₆₀₀) and dispensed to 96-well black, clear-bottom assay plates (120 µL/well). This was followed by addition of test samples *i.e.*, crude extracts or compounds (final concentration 200 or 100 µg/mL depends on the solubility of crude extracts) and controls (high control of 100 µg/mL Ampicillin and low control of DMSO only) prepared in 30 µL media (final 0.75% DMSO). The plates were incubated for 15 h at 37 °C. After incubation, absorbance at 600 nm was measured using a Tecan Spark microplate reader. Compound activity was calculated on a per-plate basis similar to the cell viability test.

Results

Four different *D. officinale* plants with different growth ages (one and three years old), collected from Zhejiang Haixing Biotech (Wuyi City, Zhejiang Province, China), were selected for endophyte culture. After surface sterilization (2), a total of 75 endophytic strains were isolated from the secondary generation plates, and then fermented in bran medium. The cytotoxic (against Hep3B2.1-7 cancer cell) and antimicrobial (against *E. coli* and *S. aureus*) activities of 75 crude extracts were then tested. 28 strains of them showed potent inhibition more than the hit cutoff (inhibition = 11.7%) in Hep3B2.1-7 cell viability assay (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). And 21 strains showed inhibition against *S. aureus* more than the hit cutoff (36.2%) (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). Only one strain was active against *E. coli* (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>). According to the bioactivity and LC-MS profiles (used for identification of potential novel secondary metabolites), strain SH-1.2-R-15 was selected for further scale-up fermentation and active metabolites analysis. The crude extract of strain SH-1.2-R-15, which was isolated from the root of one-year old *D. officinale*, presented both a cytotoxic and antibacterial activity (23.97% and 72.75% inhibition, respectively).

Scale-up fermentation (8 L) of *Streptomyces* sp. SH-1.2-R-15, followed by extraction, fractionation, and chromatographic purification (<http://fp.amegroups.cn/cms/cd8427b7620a53c4c22dc506052aacb/atm.2020.03.227-1.pdf>) yielded two new peptide-type compounds, peptidendrocins A (1, yield: 0.13 mg/L) and B (2, yield: 2.00 mg/L), together with five known compounds: chartreusin (20,21) (3, yield: 7.75 mg/L), (+)-streptazolin (22,23) (4, yield: 3.62 mg/L), strepachazolin A (24) (5, yield: 0.25 mg/L), strepachazolin B (24) (6, yield: 0.38 mg/L), (R)-(E), E)-2-(1,3-pentadienyl)-piperidine (25) (7, yield: 0.75 mg/L).

Structure elucidation

The molecular formula of compound 1 was established as C₂₀H₃₂N₄O₅ based on the HRESIMS data ([M + H]⁺, *m/z* 409.2444). The peptide structure of 1 was evident from the ¹H and ¹³C NMR data recorded in CDCl₃ (Table 1), such as the typical NMR chemical shifts for amide and α-methine resonances (δ_C 176.5 and 172.5, δ_{C/H} 57.4/4.50

Table 1 ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data of 1 and 2 in CDCl_3

No.	1		2	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
2	157.5, C		157.3, C	
3	159.6, C		159.4, C	
5	122.9, C		121.7, C	
6	140.3, C		145.6, C	
7	30.3, CH	3.29, m	30.1, CH	3.30, m
8	20.0, CH_3	1.18, d (6.9)	19.8, CH_3	1.19, m
9	19.9, CH_3	1.18, d (6.9)	19.8, CH_3	1.19, m
10	165.0, C		164.5, C	
11	17.8, CH_3	2.64, s	24.0, CH_2	3.15, m 3.01, m
12			13.5, CH_3	1.21, t (7.5)
1'	172.5, C		172.3, C	
2'	51.5, CH	4.72, m	51.4, CH	4.75, m
3'	40.6, CH_2	1.78, m 1.65, m	40.9, CH_2	1.77, m 1.63, m
4'	25.2, CH	1.69, m	25.1, CH	1.67, m
5'	22.9, CH_3	0.97, d (6.5)	22.8, CH_3	0.96, d (6.3)
6'	22.6, CH_3	0.95, d (6.5)	22.3, CH_3	0.96, d (6.3)
2'-NH		7.99, d (8.5)		8.10, d (8.5)
1''	176.5, C		175.1, C	
2''	57.4, CH	4.50, dd (8.7, 4.7)	57.0, CH	4.50, dd (8.7, 4.5)
3''	31.2, CH	2.20, m	31.0, CH	2.20, m
4''	17.9, CH_3	0.93, d (6.0)	17.4, CH_3	0.91, d (6.7)
5''	19.3, CH_3	0.96, d (6.0)	18.8, CH_3	0.93, d (6.7)
2''-NH		7.22, d (8.5)		7.12, d (8.7)

and 51.5/4.72, respectively). The COSY coupling system of 2'-NH (δ_{H} 7.99)/H-2' (δ_{H} 4.72)/H₂-3' (δ_{H} 1.64)/H-4' (δ_{H} 1.69)/H₃-5' (δ_{H} 0.97)/H₃-6' (δ_{H} 0.95), together with two HMBC correlations of both H-2' and H-3' to a carbonyl (C-1', δ_{C} 172.5) indicated the presence of a leucine residue. Similarly, a valine unit was assigned on the basis of interpretation of 2D NMR data. Long-range cross peak from amide proton 2''-NH (δ_{H} 7.22) to the carbonyl C-1' established the Leu-Val unit as shown in *Figures 2,3*. The remaining isopropyl apparent from COSY spectrum, an

additional methyl (δ_{H} 2.64) and the key HMBC correlations from 2'-NH to two carbons (δ_{C} 165.0 and 122.9) established the pyrazinone moiety of 1, thus confirmed the fragment A (*Figure 3*) as identical with JBIR-57 reported (29). The relative configuration of 1 was also determined by NOESY experiment and comparing the NMR data with literatures. In a similar manner, HRESI-MS and 1D/2D NMR (*Table 1*) revealed 2 as a C-6 ethyl substituted analogue of 1 with only difference of one CH_2 (*Figure 2*). On basis of this cumulative analysis, 1 and 2 were identified as new pyrazinone peptides

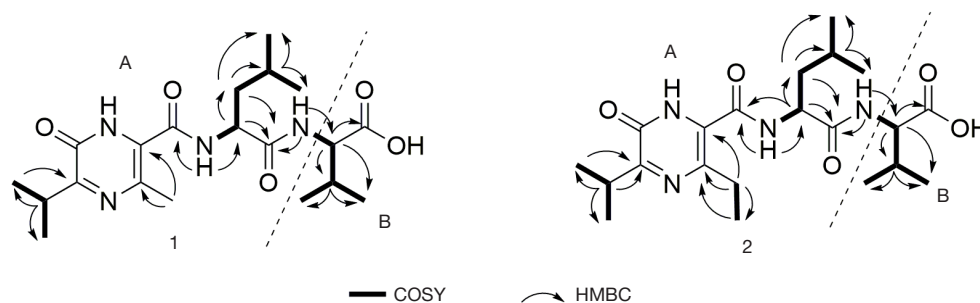


Figure 3 Key correlations observed in 2D NMR spectra of 1 and 2.

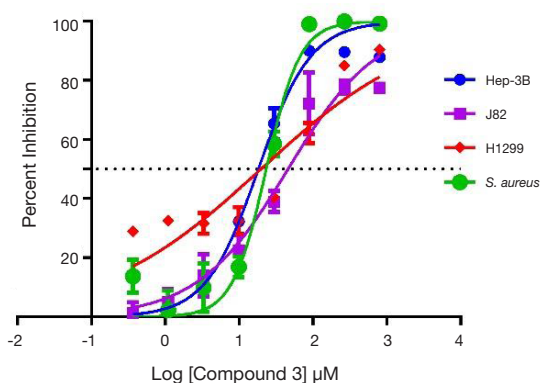


Figure 4 Cytotoxicity and antibacterial activities of compound 3.

and thereby designated peptidendrocins A and B to reflect the producing strain's point of origin.

Five previously reported compounds, chartreusin [3] (20,21), (+)-streptazolin [4] (22-23), strepchazolins A [5] and B [6] (24), (*R*)-(*E,E*)-2-(1,3-penta-dienyl)-piperidine [7] (25) were also identified as fermentation products of *Streptomyces* sp. SH-1.2-R-15 via comparison to previously reported MS and NMR spectroscopic data.

Inspired by the putative antibacterial and anticancer activities reflected from the crude extract, we tested all isolated compounds 1–7 in a similar cytotoxicity (against human hepatoma cancer cell Hep3B2.1-7, bladder cancer cell J82 and lung cancer cell H1299) and antimicrobial assay (against *E. coli* and *S. aureus*). While compound 3 displayed potent cytotoxicity (IC_{50} = 18.19 μ M against Hep3B2.1-7, IC_{50} = 19.74 μ M against H1299 and IC_{50} = 45.86 μ M against J82) and antibacterial activity against *S. aureus* with IC_{50} of 23.25 μ M (Figure 4), the new isolates 1–2 and other known compounds were inactive (IC_{50} s >100 μ M) in the parallel assays.

The results of activity evaluation for isolated compounds suggested that the primary product, chartreusin, is

responsible for the antibacterial and anticancer activities of *Streptomyces* sp. SH-1.2-R-15. Thus, it will be worthy to prepare more active chartreusin for structure modification to improve the activity, or even for animal study.

Conclusions

In summary, as an effort to explore the microbial diversity and corresponding metabolic potential of endophyte, 58 endophytic bacterial strains, which included 29 *Streptomyces* sp., were obtained from a dietetic medicinal plant *D. officinale*. Fifty-eight strains represented 9 diverse genera, including 4 Actinobacterial genera. The scale-up fermentation followed by bioassay-guided isolation of selected *Streptomyces* sp. SH-1.2-R-15, led to the discovery of two new peptidendrocins A and B [1 and 2], and chartreusin [3] with moderate cytotoxicity against Hep3B2.1-7 (IC_{50} = 18.19 μ M) and H1299 (IC_{50} = 19.74 μ M) cancer cell lines, and antibacterial activity against *S. aureus* (IC_{50} = 23.25 μ M), as well as four known secondary metabolites. Endophytes have now become a potential source for the discovery of novel natural products, this study highlights the endophytic bacteria from *D. officinale* with potential antimicrobial/cytotoxic activity and natural product diversity, and implicates them as a valuable source for new antibiotics and anticancer agents.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm.2020.03.227>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All activity screening protocols were approved by Hangzhou Normal University, Hangzhou, China.

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