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Anthraquinone Metabolites from Endophytic *Nigrospora sp* BM-2 and Optimization of Fermentation Medium for High Bostrycin Production

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Bioassay-guided separation led to identification of bostrycin[1], which was a potentially promising anti-tumor drug candidate, together with other four anthraquinone [2-5] derivatives from liquid culture of endophytic *Nigrospora* sp. BM-2 isolated from *Saccharum arundinaceum* Retz. In order to obtain high bostrycin production, three single-factor experiments and a $L_9(3^4)$ orthogonal matrix experimental design have been employed in the cultivation of Nigrospora sp. BM-2. The optimal fermentation medium were glucose 20.0 g/L, yeast extract 8.0 g/L, $MgSO_4$ 0.05 g/L, KH_2PO_4 0.01g/L, NaCl 0.5 g/L. After 9 days of incubation under shake flask conditions at 120 rpm and 28 °C in optimal media, the highest amount of bostrycin was 86.25 ± 7.23 mg/L in culture broth. Furthermore, enzymatic inhibitor as regulate factor in the biosynthesis of bostrycin was attempted to give adverse effects.

Key words: Endophytic *Nigrospora sp.*, Anthraquinone, Bostrycin, Optimization, Medium.

Plant-derived fungi have proven to be rich source of structurally novel and biologically active secondary metabolites that have become interesting and significant resources for drug discovery[1]. Many literatures revealed natural products from endophytic fungus of Chinese Medicinal Plants possessed similarly pharmacological activities provided by plants[3-7]. Furthermore, many host plant active constituents such as taxol[2], vincristine[3] and camptothecine[4] were isolated from plant endophytic fungus. As a strategy for discovery of new anti-cancer or anti-inflammatory compounds derived from endophytic fungus of Chinese Medicinal Plant, it was focused that the crude metabolites of an edophytic fungus *Nigrospora* sp. BM-2 isolated from *Saccharum arundinaceum* Retz. revealed significant anti-cancer activity. Bioassay-guided separation led to the exhibition of five anthraquinone derivatives (compound 1-5) , which were previously isolated from other *Nigrospora* sp.

Bostrycin (compound 1) had tetrahydroanthraquinone skeleton and was first isolated from *Bostryconema alpestre*. Subsequently[8], it was separated in other fungus including *Nigrospora* oryzae[9], *Arthrinium phaeospermum*[10], *Alternaria eichhorniae*[11] and *Aspergillus sp*[12]. In pharmacological studies, bostrycin was discovered to have considerable cytotoxicity toward breast cancer cells and human lung carcinoma A549 cells[13, 14]. Also, it has been reported to induce apoptosis of breast cancer cells through Akt/FOXO pathway[15] and inhibit proliferation of human lung

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carcinoma A549 cells via downregulation of the P13K/Akt pathway[16]. Chen et al. synthesized a series of new derivatives of marine-derived bostrycin, the in vitro cytotoxic activities of all compounds were evaluated against MCF-7, MDA-MB-435, A549, HepG2, HCT-116 and MCF-10A cells, and the structure-activity relationship (SAR) of bostrycin derivatives was also discussed[17]. The favorable bioactivities of bostrycin, as well as its in vivo antitumor efficacy, provided us with reasonable optimism that bostrycin could become a promising Akt inhibitor and anticancer drug candidate. However, few papers on optimal fermentation medium for enhance bostrycin production were investigated. Herein, we report the isolation and structural characterization of five anthraquinone analogues. Especially, our interst in bostrycin was focused on the optimization of fermentation medium for high bostrycin production. In this work, a series of metabolic operations in *Nigrospora* sp. BM-2 including selections of salts, carbon and nitrogen sources, and enzymatic inhibitors involved in side metabolic pathways were performed in detail. As expected, the bostrycin concentration was remarkably increased by improved medium compared to PDB medium.

MATERIALS AND METHODS

Microorganisms

The endophyte fungus *Nigrospora* sp. BM2 was isolated from a piece of fresh tissue from the inner part of a medicinal plant leaf of Saccharum arundinaceum Retz., collected from the Yichang, Hubei province in April 2011. The fungus was deposited as *Nigrospora* sp. BM2 (GenBank accession numbers JN687964) at Hubei Key Laboratory of Natural Products Research and Development, College of Chemistry and Life Sciences, China Three Gorges University, Yichang, P. R. China.

Fermentation and metabolites isolation of *Nigrospora* sp. BM2

The fungal strain *Nigrospora* sp. BM2 was grown in 500 ml Erlenmeyer flasks containing 200 ml of potato dextrose broth (PDB), and then incubated on a rotary shaker (120 rpm) at 28°C for 14 days. After filtration, the culture broth (totaling 40.0 L) was extracted three times with equal volumes of ethyl acetate (EtOAc). The EtOAc layer was evaporated to dryness (4.48 g), and mixed with 10 g silica gel, dried at 50°C, and then loaded on a silica gel column (40 × 1,000 mm) containing 240 g silica gel (200-300 mesh). The column was eluted with petroleumether : acetone (1 : 0, 9 : 1, 4 : 1, 2 : 1, 1 : 1, and 1 : 2, v/v), Six fractions were collected and labeled Fr. 1, Fr. 2, Fr. 3, Fr. 4, Fr. 5 and Fr. 6. The Fr. 2 (560 mg) was purified by CC over SephadexLH-20 using an equal-ratio mixture of methanol and chloroform as eluent to give compound 3 (45 mg). The Fr. 3 (326 mg) was subjected to subfraction on reversed-phase silica gel C-18 eluting with a gradient of MeCN-H_2O and further purification was carried out with preparative HPLC on a ODS semipreparative C18 column (COSMOSIL 5μm, 10 × 250 mm, 2 mL/min) eluted with 45% MeCN/H_2O to obtain compound 2 (16 mg).According to preparative protocol of Fr. 3, Fr. 4 (1.12 g) gave compound 1 (58 mg) and Fr. 5 (600 mg) afforded compound 4 (24 mg) and 5 (26 mg).

A scaled-up fermentation (4.0 L) of *Nigrospora* sp. BM-2 treated with 250 μM 2-iodoacetamid for 12 days was carried out, and the culture medium was extracted with EtOAc. Separation of the extract (600 mg) by silica gel and Sephadex LH-20 column chromatography gave compound 6 (10 mg).

Spectral measurements

^1H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker UltrashiedTM Plus spectrometer. Chemical shifts are expressed in δ (ppm) referring to the TMS peak and coupling constants J in Hz. The electrospray ionization mass spectra were obtained on an API 4000 LC-MS/MS system. Semipreparative HPLC was performed on an Dionex Ultimate 3000 system using a semipreparative C18 (COSMOSIL 5μm, 10 × 250 mm) column coupled with an diode-array detection, at a flow rate of 2.0 mL/min and a gradient between MeCM/H_2O (1 : 5) and MeCN/H_2O (8 : 2). TLC was performed on precoated silica gel GF254 (Merck) plates (PTLC). Silica gel (200-300 mesh) for column chromatography was produced by Qingdao Marine Chemical Factory, Qingdao, China. SephadexLH-20 was produced by Pharmacia Biotech, Sweden.

Fermentation and extraction of bostrycin

The endophyte fungus *Nigrospora* sp. BM2 grown in 500 ml Erlenmeyer flasks containing 200 ml of medium at 28 °C shaking for 9 days. After

filtration, the broth was extracted three times with an equal volume of EtOAc, and the combined EtOAc layer was evaporated to dryness under reduced pressure. The mycelia were treated with ultrasound-assisted extraction of EtOAc three times, filtered, concentrated. The combined residues were dissolved in 25 mL methanol.

HPLC analysis of bostrycin and mycelia growth of *Nigrospora* sp. BM2

Extractive metabolites of *Nigrospora* sp. BM2 were analyzed by HPLC and diode-array detection (HPLC-DAD) according to Fiedler[18]. Wavelength monitoring was performed at 226 nm. A total of 20 µl of the sample was analyzed by HPLC with a Cosmosil C18 column (4.6 × 250 mm, 5 µm) at 28°C. The samples were separated by linear gradient elution using solvent acetonitrile/water (1 : 5, v/v) in 15min at 1.0 mL/min. A standard curve generated with standard compound of bostrycin was used to determine specimen concentrations. Mycelia growth was determined by measuring the DCW (dry cell weight). DCW was measured after drying the wet mycelia at 105°C to constant weight.

Optimization of fermentation medium for bostrycin

Bostrycin production and biomass yields were concerned on carbon and nitrogen sources and mineral elements. The best culture conditions for high bostrycin production were optimized by orthogonal matrix method. Furthermore, enzyme inhibitors involved in side metabolic pathways were also attempted.

RESULTS AND DISCUSSION

The EtOAc extract from the fungal fermentation broth of *Nigrospora* sp. BM-2 exhibited 90% and 75% inhibitory activity toward Caski and HepG2 cancer cell lines respectively. Bioassay-guided separation led to identification of bostrycin as a potentially promising anti-tumor drug candidate together with other four anthraquinone derivatives. Structurally characterizing of these secondary metabolites was performed by NMR and MS spectrums.

Compound 1: Bostrycin, red crystals, molecular formula $C_{16}H_{16}O_8$; mp: 252-254°C; ESI-MS (m/z): 335 [M-H]- ; ^{1}H NMR (400MHz, DMSO-d6): δ 13.39 (1H, s, OH-10), 12.63 (1H, s, OH-9), 6.47 (1H, s, H-6), 5.24 (1H, d, 5.2 Hz, OH-4), 4.94 (1H, d, 4.5 Hz, OH-3), 4.74 (1H, t, 4.5 Hz, H-4), 4.49 (1H, s, OH-2), 3.91 (3H, s, OCH_3-7), 3.52 (1H, t, 4.4 Hz, H-3), 2.73 (1H, d, 18.2 Hz, H-1), 2.66 (1H, d, 18.2 Hz, H-1), 1.22 (3H, s, CH3-2); ^{13}C NMR (100MHz, DMSO-d6): δ 184.1 (C-5), 177.3 (C-8), 160.2 (C-10), 160.0 (C-7), 159.8 (C-9), 139.2 (C-4a), 136.6 (C-9a), 109.63 and 109.60 (C-8a and C-6), 107.3 (C-10a), 76.2 (C-3), 69.1 (C-2), 68.0 (C-4), 56.7 (OCH_3-6), 34.6 (C-1), 25.4 (CH_3-2). The ^{1}H NMR and ^{13}C NMR data are consistent with the literature[14, 17] reported.

Compound 2: 1, 4, 6-trihydroxy-2-methoxy-7-methylanthracene-9, 10-dione, red crystals, molecular formula $C_{16}H_{12}O_6$; mp: 209-211 °C; ESI-MS (m/z): 299 [M-H]- ; ^{1}H NMR (400MHz, DMSO-d6): δ 13.49 (1H, s, OH-1), 13.45 (1H, s, OH-

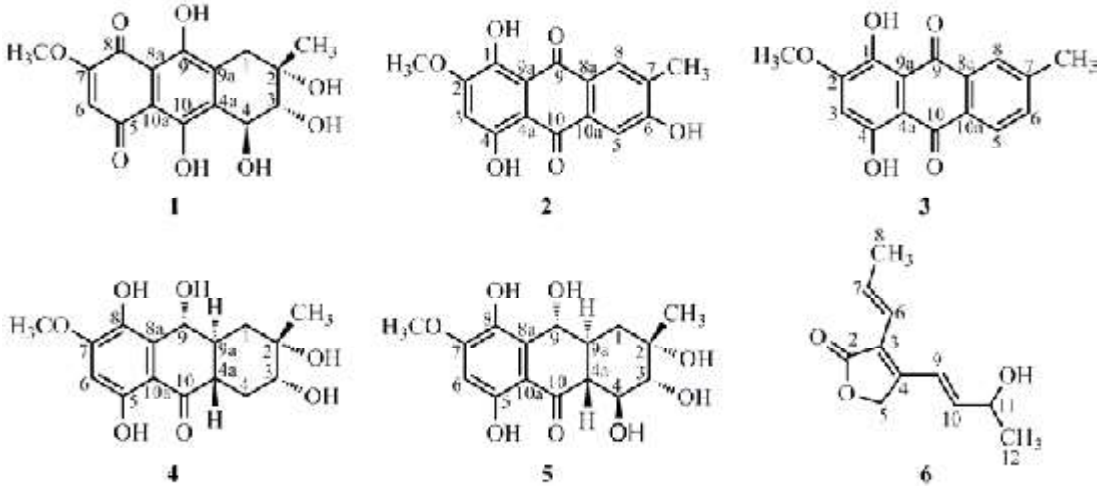

Fig. 1. Structure of anthraquinone derivatives (1-5) and a furanone (6)

4), 11.23 (1H, s, OH-6), 8.01 (1H, s, H-8), 7.60 (1H, s, H-3), 6.91 (1H, s, H-5), 3.97 (3H, s, OCH_3-2), 2.30 (3H, s, CH_3-7); ^{13}C NMR (100MHz, DMSO-d6): δ 186.6 (C-9), 184.7 (C-10), 162.6 (C-6), 160.4 (C-4), 158.1 (C-2), 149.7 (C-1), 134.3 (C-8a), 132.5 (C-7), 130.2 (C-8), 124.9 (C-10a), 112.3 (C-9a), 111.5 (C-5), 107.2 (C-3), 106.2 (C-4a), 57.3 (OCH_3-2), 16.6 (CH_3-7). The ^1H NMR and ^{13}C NMR data are consistent with the literature[19].

Compound 3: Austrocortirubin, red crystals, molecular formula $C_{16}H_{12}O_5$; mp: 208-209°C; ESI-MS (m/z): 283 [M-H]- ; ^1H NMR (400MHz, DMSO-d6): δ 13.53 (1H, s, OH-4), 13.22 (1H, s, OH-1), 8.17 (1H, d, 8.0 Hz, H-5), 8.09 (1H, s, H-8), 7.79 (1H, d, 8.0 Hz, H-6), 7.00 (1H, s, H-3), 3.98 (3H, s, O CH_3-2), 2.53 (3H, s, CH_3-7). ^{13}C NMR (100MHz, DMSO-d6): δ 187.1 (C-9), 184.2 (C-10), 160.1 (C-4), 157.5 (C-2), 149.5 (C-1), 145.4 (C-7), 135.9 (C-6), 132.7 (C-10α), 131.1 (C-8α), 126.9 (C-8), 126.7 (C-5), 112.2 (C-9α), 107.5 (C-3), 105.5 (C-4α), 56.9 (OCH_3-2), 21.4 (CH_3-7). The 1H NMR and ^{13}C NMR data are consistent with the literature[19] reported.

Compound 4: 9α-hydroxydihydrodesoxybostrycin, red crystals, molecular formula $C_{16}H_{20}O_7$; ESI-MS (m/z): 323 [M-H]- ; ^1H NMR (400MHz, Methanol-d4): δ 6.46 (1H, s, H-6), 4.77 (1H, d, 10.2, Hz, H-9), 3.91 (1H, s, OCH_3-7), 3.48 (1H, dd, 12.0, 4.4 Hz, H-3), 2.43-2.36(2H, m, H-4 and H-1), 2.32 (1H, td, 12.0, 4.2 Hz, H-4a), 2.24-2.13 (1H, m, H-9a), 1.68 (1H, q, 12.0 Hz, H-4), 1.38 (1H, d, 12.8 Hz, H-1), 1.33 (3H, s, CH_3-2); ^{13}C NMR (100 MHz, Methanol-d4): δ 202.3 (C-10), 158.3 (C-5), 155.5 (C-7), 137.3 (C-8), 127.3 (C-8a), 107.8 (C-10a), 98.7 (C-6), 74.0 (C-3), 72.3 (C-9), 70.1 (C-2), 55.2 (OCH_3-7), 46.2 (C-4a), 40.8 (C-1), 28.7 (C-4), 25.6 (CH_3-2). The 1H NMR and ^{13}C NMR data are consistent with the literature[20] reported.

Compound 5: 9β-hydroxyhalorosellinia A, red crystals, molecular formula $C_{16}H_{20}O_8$; ESI-MS (m/z): 339 [M-H]- ; 1H NMR (400MHz, Methanol-d4): δ 6.50 (1H, s, H-6), 4.84 (1H, d, 9.2 Hz, H-9), 4.10 (1H, t, 9.2 Hz, H-4), 3.94 (3H, s, OCH_3-7), 3.30 (1H, d, 9.2 Hz, H-3), 3.20 (1H, d, 9.2Hz, H-1), 2.46-2.26 (3H, m, H-4a, H-9a and H-1), 1.46 (1H, dd, 13.5, 11.3 Hz, H-1), 1.35 (3H, s, CH_3-2); ^{13}C NMR (100MHz, Methanol-d4): δ 203.7 (C-10), 158.5 (C-5), 156.1 (C-7), 137.4 (C-8), 127.4 (C-8a), 108.0 (C-10a), 98.8 (C-6), 78.0 (C-3), 72.0 (C-9), 70.70 (C-4), 70.68 (C-2), 55.3 (OCH_3-7), 51.9 (C-4a), 39.7 (C-9a), 39.3 (C-1), 25.8 (CH_3-2). The ^1H NMR and ^{13}C NMR data are consistent with the literature[20] reported.

To increase bostrycin production, culture conditions of fungus *Nigrospora* sp. BM-2 were optimized with respect to the initial carbon sources, nitrogen sources, and mineral elements. The effect of adding various carbon sources on the cell biomass and bostrycin production are shown in Fig.S1. Glucose as marked selectivity was used for further experiments on adding concentration. The addition of 20 g/L glucose gave highest 60.51±10.73 mg/L bostrycin, identical to 1.24±0.01 g/L cell biomass (Fig.S2). The similar experiments were performed on screening of nitrogen sources. The results in Fig.S3 and S4 indicate that 8 g/L yeast extract afforded highest 60.62±9.84 mg bostrycin and 6.21±0.97 g/L cell biomass. In the following experiments, the effects of four types of mineral elements on bostrycin production and mycelia growth of *Nigrospora* sp. BM2 were also investigated by preliminary designed L9(34) orthogonal table as shown table S1. Finally, the optimized medium comprised 20 g glucose, 8 g yeast extract, 0.05 g $MgSO_4$, 0.01 g KH_2PO_4, and 0.5 g NaCl in 1 liter of tap water, natural pH. Under this cultivation conditions at 28°C with reciprocal shaking at 120 rpm, the highest production of 86.25±7.23 mg/L bostrycin was obtained at 9 days, which was higher improvement than 29.82 mg/L in PDB medium (Fig.S5).

According to literatures, epigenetic modifying agents have been introduced as effective tools for inducing post-transcriptional gene silencing to shield side metabolic pathways and enhance major metabolite[21]. So, DNA methyltransferase inhibitors (5-azacytidine) and polyketide synthase inhibitors (2-iodoacetamid) were employed on cultivation to disturb biosynthetic pathway of secondary metabolites in fungi *Nigrospora* sp. BM-2 and enhance production of bostrycin. Disappointedly, the results in Fig.S6 and S7 showed that two enzymatic inhibitors played adverse roles to reduce the production of bostrycin and an inductive metabolite (6) was identified as Pestalafuranones B.

Compound 6: Pestalafuranones B, White powders, molecular fomula $C_{11}H_{14}O_3$; ESI-MS (m/z): 195.1 [M+H]+ ; ^1H NMR (400 MHz, $CDCl_3$): δ 6.95 (1H, dq, 15.7, 6.8 Hz, H-7), 6.77 (1H, d, 16.0 Hz, H-6), 6.26 (1H, dd, 15.7, 1.6 Hz, H-9), 6.03 (1H, dd,

16.1, 5.3 Hz, H-10), 4.86 (2H, s, H-5), 4.53 (1H, m, 6.4 Hz, H-11), 1.90 (3H, d, 6.8 Hz, H-8), 1.37 (3H, d, 6.4 Hz, H-12); 13C NMR (100 MHz, $CDCl_3$): δ 173.1 (C-2), 149.4 (C-4), 140.9 (C-10), 134.0 (C-7), 122.3 (C-3), 118.41 (C-6), 118.36 (C-9), 68.4 (C-5), 68.0 (C-11), 23.2 (C-12), 19.4 (C-8).The ^{1}HNMR and ^{13}C NMR data are consistent with the literature[22] reported .

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