Paenibacillin A, a new 2(1H)-pyrazinone ring-containing natural product from the endophytic bacterium Paenibacillus sp. Xy-2

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A new 2(1H)-pyrazinone ring-containing natural product, paenibacillin A (1), together with five known diketopiperazine derivatives 2–6 and two known isoflavones 7–8, was isolated from the culture of an endophytic bacterium Paenibacillus sp. Xy-2. The structure of compound 1 was elucidated by extensive spectral methods, including UV, IR, HR-ESI-MS, 1D and 2D NMR and ECD experiments. Compound 1 exhibited moderate cytotoxicity against HL-60 cell line with IC\textsubscript{50} value of 50.48 \textmu M.

Keywords: Paenibacillus sp; endophytic bacterium; secondary metabolites; cytotoxicity

1. Introduction

Endophytic bacteria are common inhabitants of both the surfaces and the internal tissues of most plants, and do not visibly harm the plants (Hallmann et al. 1997). Actually, they may have diverse effects on the development and physiology of the host plant (Bibi et al. 2012; Venkateswarlu 2013; Jalgaonwala and Mahajan 2014). Endophytic bacteria are important resources of novel and bioactive compounds. Their secondary metabolites including alkaloids, anthraquinones, terpenoids, isocoumarin derivatives, phenols and aliphatic compounds showed antimicrobial, nematicidal activities and cytotoxicities on some tumour cell lines (Piel 2004). Previous research has shown that the ethyl acetate extract of the endophytic bacterium Paenibacillus sp. Xy-2 obtained from the plant of Houttuyniae cordata exhibited relatively strong cytotoxic activity against HL-60 cell line. Up to now, the secondary metabolites of this bacterium have not been studied. Our present investigation on the endophytic bacterium Paenibacillus sp. Xy-2 led to the isolation of eight compounds, including a new 2(1H)-pyrazinone ring-containing natural product, paenibacillin A (1), five diketopiperazine...
derivatives, containing cyclo(Pro-Val) (2), cyclo(Pro-Ile) (3), cyclo(Pro-Leu) (4), cyclo(Pro-Phe) (5) and cyclo(Leu-Hyp) (6), and two isoflavones 4’, 5, 7-trihydroxyisoflavone (7), 4’, 7-dihydroxyisoflavone (8) (Figure 1). Herein, we reported the isolation, structural elucidation and cytotoxic activity of the new compound (1).

2. Results and discussion

Compound 1 was isolated as a white crystal. Its molecular formula was determined as C_{11}H_{18}N_{2}O by HR-ESI-MS at m/z 195.1489 [M + H]^+ (calcd 195.1497), indicating 4 degrees of unsaturation. The UV spectrum showed absorption maxima at 323 and 227 nm. The IR spectrum indicated the presence of carbonyl (1642 cm\(^{-1}\)) and amino functional group (3421 cm\(^{-1}\)). The \(\text{^1H NMR spectrum of 1 showed a broad singlet at } \delta_H 13.20 \text{ (1H, br s) which was assigned to the acylamino proton, one olefinic proton at } \delta_H 7.25 \text{ (1H, s, H-5), two methines at } \delta_H 2.85 \text{ (1H, sept, } J = 6.8 \text{ Hz, H-1''} \text{) and } 3.24 \text{ (1H, sext, } J = 6.8 \text{ Hz, H-1'} \text{), one pair of coupled methylene protons at } \delta_H 1.55 \text{ (1H, dqunt, } J = 13.6 \text{ and } 6.8 \text{ Hz, H}_2-2') \text{ and } 1.82 \text{ (1H, dqunt, } J = 13.6 \text{ and } 6.8 \text{ Hz, H}_2-2'') \text{, and four methyls at } \delta_H 1.35 \text{ (6H, d, } J = 6.8 \text{ Hz, H-2''', 3''}, 1.22 \text{ (3H, d, } J = 6.8 \text{ Hz, H-4'}) \text{ and } 0.90 \text{ (3H, t, } J = 6.8 \text{ Hz, H-3')). The } \text{^{13C NMR spectrum displayed 11 signals containing one carbonyl at } \delta_C 158.2, \text{ three } sp^2 \text{ carbon signals at } \delta_C 161.0, 143.5, 120.5, \text{ seven } sp^3 \text{ carbon signals at } \delta_C 36.9, 30.2, 27.7, 21.2, 21.2, 17.8, 12.2, \text{ which can be also demonstrated by HSQC spectrum.}

In the HMBC experiment of 1, correlation of H-1'' (\(\delta 2.85\)) with C-2'' (\(\delta 21.2\)), C-3'' (\(\delta 21.2\)) and C-6 (\(\delta 143.5\)) were observed. Similarly, methine proton H-1' (\(\delta 3.24\)) showed HMBC correlation with C-2 (\(\delta 158.2\)), C-3 (\(\delta 161.0\)), C-2' (\(\delta 27.7\)), C-3' (\(\delta 12.2\)) and C-4' (\(\delta 17.8\)); methylene proton H-2' (\(\delta 1.55, 1.82\)) showed HMBC correlation with C-1' (\(\delta 36.9\)), C-3' (\(\delta 12.2\)) and C-4' (\(\delta 17.8\)); methyl protons H-3' (\(\delta 0.90\)) and H-4' (\(\delta 1.22\)) showed HMBC correlation with C-1' (\(\delta 36.9\)), C-2' (\(\delta 27.7\)). This finding clearly indicated the presence of isopropyl and sec-butyl group which should be attached to C-6 and C-3 positions of 2(1H)-pyrazinone ring, respectively. In addition, HMBC correlation of H-5 (\(\delta 7.25\)) with C-2 (\(\delta 158.2\)), C-3 (\(\delta 161.0\)) and C-6 (\(\delta 143.5\)) confirmed the 2(1H)-pyrazinone skeleton in compound 1.

The stereochemistry of compound 1 at C-1' was proposed according to ECD (Figure 2). The ECD spectrum of 1 showed a positive cotton effect at 254 nm (\(\Delta\epsilon = 3.24\)) and two negative cotton effects at 224 and 317 nm (\(\Delta\epsilon = -11.44 \text{ and } -5.16, \text{ respectively})\), which matched well
with the experimental one. It allowed us to unambiguously determine the structure of 1 with the absolute configuration of 1'R. Consequently, the structure of compound 1 was established as paenibacillin A (1).

It was found that the natural products containing 2(1H)-pyrazinone ring such as compound 1 were rare in the secondary metabolites of bacteria, and almost all of them have been isolated from fungus and actinomycetes (Okada et al. 1996; Motohashi et al. 2011; Wyatt et al. 2012).

The cytotoxic activity of compound 1 against HL-60 (human promyelocytic leukaemia cells) cell line was tested. Compound 1 exhibited moderate inhibitory effect with IC_{50} value of 50.48 μM, compared with 5-fluorouracil (5-FU; IC_{50} = 2.80 μM).

In addition, the structures of seven known compounds 2–8 (Figure 1) were characterised by comparison of their NMR and MS spectra with those reported previously as cyclo(Pro-Val) (2) (Kwak et al. 2014), cyclo(Pro-Ile) (3) (Dong et al. 2014), cyclo(Pro-Leu) (4) (Wang, Shaaban, et al. 2014), cyclo(Pro-Phe) (5) (Wang, Zhao, et al. 2014), cyclo(Leu-Hyp) (6) (Li et al. 2008), 4',5,7-trihydroxyisoflavone (7) (Lee et al. 2005), and 4',7-dihydroxyisoflavone (8) (Basset et al. 2012). To the best of our knowledge, isoflavones were the phenolic secondary metabolites found mostly in legumes (Yu and McGonigle 2005), but were rare in micro-organisms. Until now, it is the first report on isoflavones found in endophytic bacteria.

3. Experimental
3.1. General experimental procedures
The optical rotation was measured using a Perkin-Elmer 241 polarimeter (Perkin Elmer, Manhattan, North America) with a 1 cm cell at 25°C. UV spectrum was recorded on a Shimadzu UV-1601 (Kyoto, Japan). IR spectrum was obtained on a Bruker IFS-55 infrared spectrophotometer (Bruker TFS-55, Aargau, Switzerland) with KBr pellets. CD spectrum was measured on a JASCO CD-2095 Chiral Detector (Bio-logic Co., Claix, France). The HR-ESI-MS data were detected on a Bruker microTOF-Q mass spectrometer (Bruker Co., Karlsruhe, Germany). The NMR spectral data were recorded on Bruker ARX-400 (400 MHz for ^1^H and 100 MHz for ^13^C) and Bruker AV-600 (600 MHz for HSQC and HMBC) (Bruker Co., Billerica, MA, USA) with TMS as the internal standard. TLC analyses were carried out using precoated silica gel GF254 plates (Qingdao Marine Chemical Plant, Qingdao, China). Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Plant, Qingdao, China) and Sephadex LH-20 (Pharmacia, Piscataway, NJ, USA).
3.2. Isolation and identification of the bacterial strain

The endophytic bacterium Xy-2 was originally obtained from fresh, healthy plant of *H. cordata*, which was collected in April 2012 in Shenyang, China. It was obtained using the standard protocol for the isolation of endophytic microbes from plant materials. This strain was identified as *Paenibacillus* sp. on the basis of *in vitro* colony growth and micromorphology. The strain was also identified using DNA amplification and sequencing of the ITS. The sequence data has been deposited at GenBank (Accession no. KP715166). A voucher strain has been stored at one of the author’s laboratory (Jiao Bai).

3.3. Fermentation and extraction

The *Paenibacillus* sp. Xy-2 growing on LB medium at 28°C for 5 days was inoculated in the liquid medium and cultured at 28°C for 5 days under shaking conditions at 180 rpm. The liquid medium was composed of starch of 1%, yeast extract of 0.4% and tryptone of 0.2% which were dissolved in water. After 5 days, the fermented broth (130 L) was centrifuged to be separated into the supernatant and the mycelia. Finally, the supernatant was concentrated to 10 L and successively extracted with ethyl acetate, and then the crude extract (19.6 g) was obtained.

3.4. Isolation and purification

The EtOAc crude extract (19.6 g) was subjected to silica gel column (240 g), eluted with CH$_2$Cl$_2$ with increasing amounts of CH$_3$OH to give 15 fractions (Fr. 1–15). Fraction 4 (2.8 g) was subjected to silica gel column eluted by CH$_2$Cl$_2$—CH$_3$OH (100:0 to 0:100) to give five fractions (subfr. 4.1–4.5). Subfraction 4.1 (357.2 mg) was subjected to PTLC and then purified with reverse phase semi-preparative HPLC (54% MeOH) to yield compound 1 (22.4 mg). Subfraction 4.3 (478.8 mg) was subjected to Sephadex LH-20 CC (40 g) eluted by methanol to give four subfractions (subfr. 4.3.1–4.3.4). The subfraction 4.3.2 (1.5 g) was subjected to Sephadex LH-20 CC (40 g) eluted by methanol to give six subfractions (subfr. 5.0–5.5). Subfraction 5.3 (1.5 g) was subjected to Sephadex LH-20 CC (40 g) eluted by methanol to give four subfractions (subfr. 5.3.1–5.3.4). The subfraction 5.3.4 (80.3 mg) was purified with reverse-phase semi-preparative HPLC (54% MeOH) to give compounds 7 (2.4 mg) and 8 (4.4 mg).

**Paenibacillin A (1):** white crystal (CH$_3$OH), [α]$_D$ + 1.99° (chcl$_3$ 0.13), uv (ch$_3$oh), $\lambda_{max}$ 323 (3.55), 227 (3.52) nm; IR (KBr) $\nu_{max}$ 3421 and 1642 cm$^-1$; CD (CH$_3$OH, $\Delta\epsilon$) $\lambda_{max}$ 254 (+3.24), 224 (−11.44), 317 (−5.16) nm; HR-ESI-MS $m/z$ 195.1489 [M + H]$^+$ (calcd 195.1497), $^1$H NMR (400 MHz, CDCl$_3$): $\delta_H$ 13.20 (1H, br s, −CONH), 7.25 (1H, s, H-5), 3.24 (1H, sext, $J = 6.8$ Hz, H$_b$-20), 2.85 (1H, sept, $J = 6.8$ Hz, H-10'), 2.85 (1H, sept, $J = 6.8$ Hz, H-10'), 1.82 (1H, dquint, $J = 13.6$ and 6.8 Hz, H$_a$-20), 1.55 (1H, dquint, $J = 13.6$ and 6.8 Hz, H$_a$-20), 1.35 (6H, d, $J = 6.8$ Hz, H$_a$-20), 1.22 (3H, d, $J = 6.8$ Hz, H-4'), 0.90 (3H, t, $J = 6.8$ Hz, H-3'), $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta_C$ 161.0 (C-3), 158.2 (C-2), 143.5 (C-6), 120.5 (C-5), 36.9 (C-10'), 30.2 (C-10'), 27.7 (C-2'), 21.2 (C-2', 3'), 17.8 (C-4'), 12.2 (C-3').

3.5. Cytotoxicity assay

RPMI-1640 medium (Gibco, New York, NY, USA) contained 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mmol glutamine and 10% heat-inactivated foetal bovine serum (Gibco). Human leukaemia HL-60 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in the above-mentioned medium at a density of $5 \times 10^4$ cells/mL at 37 under an atmosphere of 5% CO$_2$. Cell growth inhibition assay was performed as reported previously (Jing et al. 1999).
The compounds were dissolved in DMSO, and the amount of DMSO was controlled at lower than 0.1% in the final concentration. Cells were incubated with various drug concentrations for 3 days. The number of cells was determined by haemocytometer, and its viability was determined using trypan blue staining. The growth inhibitory ability of the compound was calculated and expressed using the IC₅₀ value (half-inhibitory concentration). 5-FU (80 mmol/L) and 0.1% DMSO were used as a positive control and a negative control, respectively.

4. Conclusion
The chromatographic separation of the crude EtOAc extracts of the endophytic bacterium *Paenibacillus* sp. Xy-2 yielded eight compounds. Their structures were determined by using spectroscopic methods as a new 2(1H)-pyrazinone ring-containing natural product, paenibacillin A (1), five diketopiperazine derivatives, including cyclo(Pro-Val) (2), cyclo(Pro-Ile) (3), cyclo(Pro-Leu) (4), cyclo(Pro-Phe) (5) and cyclo(Leu-Hyp) (6), and two isoflavones 4',5,7-trihydroxyisoflavone (7), 4',7-dihydroxyisoflavone (8). It was found that the natural products containing 2(1H)-pyrazinone ring such as compound 1 were rare in the secondary metabolites of bacteria, and almost all of them have been isolated from fungus and actinomycetes. In addition, compound 1 exhibited moderate cytotoxicity against HL-60 cell line with IC₅₀ value of 50.48 μM.

**Supplementary material**
Supplementary material relating to this article is available online, alongside Table S1 and Figures S1–S8.

**Disclosure statement**
No potential conflict of interest was reported by the authors.

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