

ORIGINAL ARTICLE

# Indole-based alkaloids from deep-sea bacterium *Shewanella piezotolerans* with antitumor activities

Yao Wang<sup>1</sup>, Xixiang Tang<sup>2</sup>, Zhongzhe Shao<sup>2</sup>, Jinwei Ren<sup>1</sup>, Dong Liu<sup>1</sup>, Peter Proksch<sup>3</sup> and Wenhan Lin<sup>1</sup>

Chromatographic separation of a crude extract obtained from a fermentation broth of a chemically unknown bacterium *Shewanella piezotolerans* WP3 collected in deep-sea yielded three new indole alkaloids namely shewanellines A (1a), B (1b) and C (2), together with 12 known indole alkaloids. The structures were unambiguously elucidated on the basis of 1D and 2D NMR (<sup>1</sup>H, <sup>13</sup>C, COSY, HMBC, HSQC and NOESY) in association with MS and CD data. Compounds 1–4, 7, 9 and 11–14 were selected for the evaluation of their cytotoxic activities against human tumor cell lines HL-60 and BEL-7402, whereas compounds 2, 4 and 9 exhibited significant inhibition toward HL-60.

*The Journal of Antibiotics* (2014) 67, 395–399; doi:10.1038/ja.2014.3; published online 5 February 2014

**Keywords:** bacterium; chromatographic separation; cytotoxic evaluation; indole alkaloid; *Shewanella piezotolerans*; shewanellines A-C; structural elucidation

## INTRODUCTION

Exploration of phylogenetically diverse and unique organisms from rare or extreme ecosystems to obtain novel structural patterns with medicinally relevant bioactivities becomes attractive challenge in natural product chemistry. The deep sea has emerged as a new source for the discovery of lead compounds, which may supply as new drug templates against human diseases such as infectious diseases and cancer. Deep-sea organisms survive under extreme conditions in the absence of light, low levels of oxygen and intensely high pressures,<sup>1</sup> whereas these extreme conditions are regarded to affect the pathways to derive primary and secondary metabolites to produce a greater genetic diversity and structurally unique metabolites. Recent publication showed that the deep sea is extraordinarily rich in biodiversity with respect to microorganisms. Microorganisms derived from deep-sea have been uncovered as a new source for the discovery of drug lead compounds with the development of methods for sampling, identification and successful culturing technologies. However, a limited number of metabolites have been isolated from truly deep-sea microorganisms until now.<sup>2</sup> Genus *Shewanella* is one of the typical deep-sea bacterial genera.<sup>3</sup> Although the ecological and genomic functions of *Shewanella* species are investigated comprehensively, few report related to the secondary metabolites. *Shewanella piezotolerans* WP3 is a psychrotolerant and psychrophilic strain obtained from west Pacific deep-sea sediment at a depth of 1914 m.<sup>4</sup> It is characterized by growing optimally at 15–20 °C and with a broad pressure optimum extending from atmospheric pressure to about 20 MPa, as well as relatively rapid growth and broad range of

physical adaptations.<sup>5</sup> In the course of our study on the chemical diversity derived from the microorganisms derived from deep-sea, the bacterium *Shewanella piezotolerans* WP3 was selected for chemical examination. Herein, we report the isolation, structure elucidation and cytotoxicity of 15 indole alkaloids, including 3 new indole alkaloids to be isolated from the fermentation broth.

## RESULTS AND DISCUSSION

### Structural elucidation of new compounds

Chemical examination of the EtOAc extract of the supernatant of *S. piezotolerans* WP3 was conducted by chromatographic separation, whereas the spectroscopic data analysis resulted in the identification of 3 new compounds (1a/1b and 2) together with 12 known alkaloids (Figure 1).

Shewanellines A and B (1a/1b) were obtained as a pale brown solid. Both compounds showed duplicated NMR spectra with two peaks in chiral-phase HPLC chromatography, assuming a pair of enantiomers. The molecular formula of 1a/1b was determined as C<sub>17</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub> from the HR-ESI-MS data (*m/z* = 277.0983 [M–H]<sup>–</sup>). The <sup>1</sup>H NMR spectrum displayed two aromatic ABCD spin systems for two *ortho*-substituted aromatic rings. In addition, two D<sub>2</sub>O exchangeable protons at δ<sub>H</sub> = 11.09 (br, NH-12) and δ<sub>H</sub> = 10.59 (br, NH-1), and an olefinic proton at δ<sub>H</sub> = 6.93 (br, H-11) correlating to NH-12 weakly in COSY spectrum were observed. The COSY, HSQC and HMBC correlations enabled to establish an indole moiety and a 2-oxo-indole moiety (Figure 2). Their linkage through C-3 (δ<sub>C</sub> = 81.2) and C-10 (δ<sub>C</sub> = 112.7) bond was determined by the HMBC correlation between

<sup>1</sup>State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, PR China; <sup>2</sup>Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen, PR China and <sup>3</sup>Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, Geb.26.23, Düsseldorf, Germany  
Correspondence: Dr W Lin, State Key Laboratory of Natural and Biomimetic Drug, Peking University, Xueyuan Road 38, Beijing 100191, PR China.  
E-mail: whlin@bjmu.edu.cn

Received 22 July 2013; revised 20 December 2013; accepted 6 January 2014; published online 5 February 2014

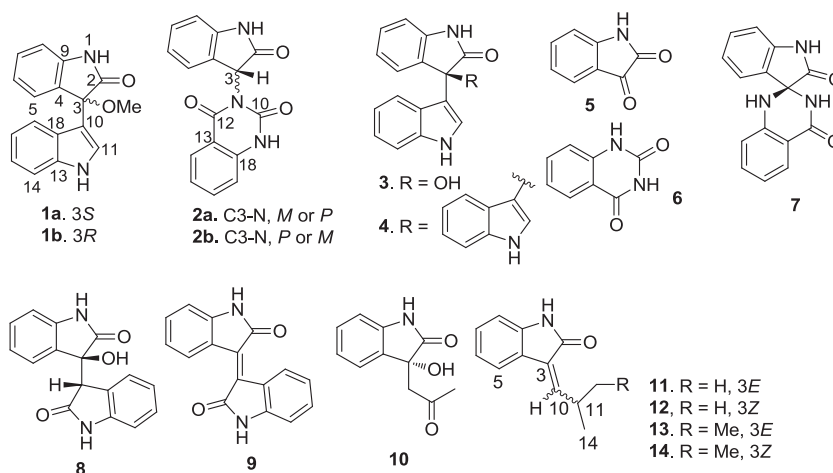


Figure 1 Structures of alkaloids from *S. piezotolerans* WP3.

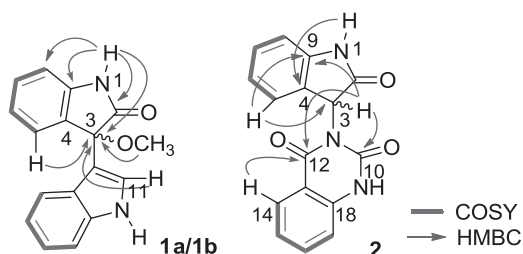


Figure 2 Key HMBC and COSY correlations of **1a/1b** and **2**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

H-11 and C-3. Additional HMBC correlation between the methoxy protons at  $\delta_{\text{H}}=3.13$  (3H, s) and C-3 clarified C-3 to be substituted by a methoxy group. Thus, the structures of **1a/1b** were determined as 3-methoxylated analog of 3-hydroxy-3,3'-biindoline-2-one (**3**).<sup>6</sup> The chiral-phase HPLC detection revealed **1a/1b** to be an enantiomeric mixture, which was further separated on the semi-preparative chiral-phase HPLC to obtain **1a** and **1b**. The specific rotation ( $[\alpha]_{\text{D}}^{25}=+147.5$ ) of **1a** was closely similar to those of known analogs with 3*S* configuration,<sup>7</sup> whereas the specific rotation ( $[\alpha]_{\text{D}}^{25}=-146.2$ ) of **1b** was compatible to those of 3*R* analogs with negative specific rotation.<sup>8</sup> Thus, **1a** was estimated to be 3*S* configuration, whereas **1b** was in agreement with 3*R* configuration.

Shewanelline C (**2**) presented as a pair of inseparable rotational isomers. The NMR spectroscopic data enabled to distinguish their structural features, whereas the proton integrates in <sup>1</sup>H NMR revealed the ratio of 1:0.96. Compound **2** had a molecular formula of C<sub>16</sub>H<sub>11</sub>N<sub>3</sub>O<sub>3</sub> as determined by HR-ESI-MS ( $m/z=294.0873$  [M+H]<sup>+</sup>). The NMR data of **2a** exhibited two ABCD spin systems, which were closely related to those of **1**, whereas 2D NMR data analyses declared the structure of **2** consisting with two moieties. One substructure was identified as 3-substituted indoline-2-one on the basis of the HMBC correlations of the aromatic proton at  $\delta_{\text{H}}=7.11$  (d,  $J=7.2$  Hz, H-5) to C-3 ( $\delta_{\text{C}}=52.6$ ), C-7 ( $\delta_{\text{C}}=128.5$ ) and C-9 ( $\delta_{\text{C}}=142.8$ ), the aromatic proton at 6.86 (d,  $J=7.2$  Hz, H-8) to C-4 ( $\delta_{\text{C}}=125.8$ ) and C-6 ( $\delta_{\text{C}}=121.4$ ), the NH proton at  $\delta_{\text{H}}=10.61$  (s) to C-2 ( $\delta_{\text{C}}=173.9$ ), C-3 and C-4, and H-3 ( $\delta_{\text{H}}=6.30$ , s) to C-2, C-4 and C-9. The remaining NMR data (Table 1) presented an aromatic ABCD spin system attributed to an *ortho*-phenyl group. The HMBC correlations from H-14 ( $\delta_{\text{H}}=8.07$ , d,  $J=7.7$  Hz) to C-12

( $\delta_{\text{C}}=162.6$ ) and C-18 ( $\delta_{\text{C}}=139.4$ ) and from H-17 ( $\delta_{\text{H}}=7.27$ , d,  $J=8.3$  Hz) to C-13 ( $\delta_{\text{C}}=113.6$ ) in addition to the NOE interaction between H-17 and the second NH ( $\delta_{\text{H}}=11.86$ , s) indicated the presence of a quinazoline-2,4-dione moiety. The linkage of the two partial structures through C-3 and N-11 bond was confirmed by the HMBC correlation from H-3 to C-12 and C-10 ( $\delta_{\text{C}}=148.2$ ) and between H-14 and C-12.

The NMR data of **2b** were closely similar to those of **2a**, except for the downfield shifted C-10 ( $\delta_{\text{C}}=150.6$ ) and C-3 ( $\delta_{\text{C}}=53.5$ ) and the upfield shifted C-12 ( $\delta_{\text{C}}=160.3$ ) in comparison with the corresponding carbons of **2a**. Analyses of 1D and 2D NMR data including the NOE interaction between H-17 ( $\delta_{\text{H}}=7.27$ , d,  $J=8.3$  Hz) and the second NH ( $\delta_{\text{H}}=11.58$ , s) revealed the gross structure of **2b** to be the same as that of **2a**. The chiral-phase HPLC analysis revealed **2a** and **2b** presenting two shoulder peaks, which were inter-exchangeable during separation. Thus, the NMR distinction between **2a** and **2b** was assumed to be derived from the axial chirality of C<sub>3</sub>-N<sub>11</sub> bond. This was partly supported by the NOE interaction between HN-19 and H-5 in **2b**, which was absent in **2a** (Figure 3). Based on the optical rotation computation,<sup>9</sup> the specific rotation of **2** at  $[\alpha]_{\text{D}}^{25}=-125.4$  ( $c=0.12$ , MeOH) was compatible to the value of  $-182$  for 3*R* congener as calculated at the B3LYP/aug-cc-pVDZ // B3LYP/6-31G(d) levels. Thus, the absolute configuration of **2** was estimated to be 3*R*.

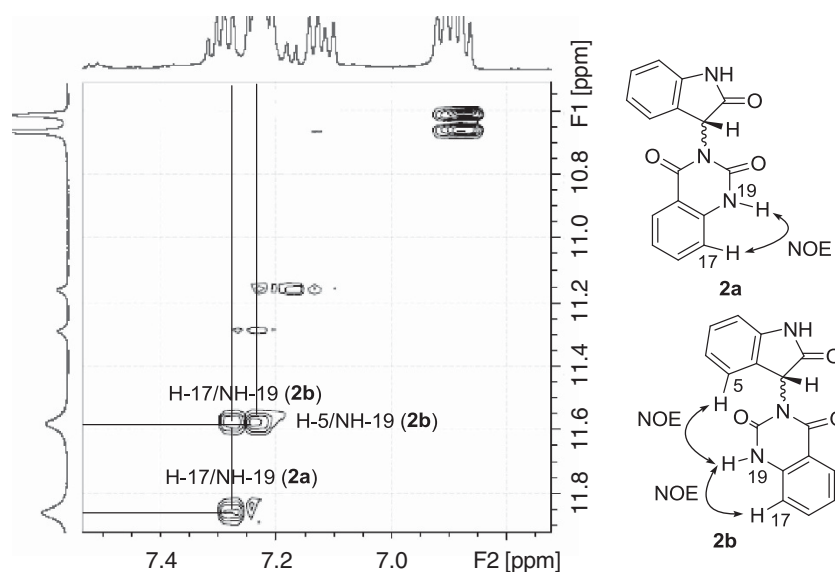
Based on the spectroscopic comparison (Supporting Information), the known compounds were identical to isatin (**5**),<sup>10</sup> (*S*)-3-hydroxy-3,3'-biindoline-2-one (**3**),<sup>6</sup> trisindoline (**4**),<sup>11</sup> quinazoline-2,4-dione (**6**),<sup>12</sup> spiro[indoline-3,2'-quinazoline]-2,4'(3'H)-dione (**7**),<sup>13</sup> isatin (**8**), isindigo (**9**),<sup>14</sup> 3-(2-oxopropyl)-3-hydroxyindolin-2-one (**10**),<sup>15</sup> (*Z*) and (*E*) 3-(2-methylpropylidene)-indol-2-one (**11/12**).<sup>16</sup>

Compounds **13** and **14** were a pair of inseparable mixture with the ratio of 10:1. However, the NMR spectra are able to distinct both compounds. The <sup>1</sup>H MMR spectrum of **13** exhibited an aromatic ABCD spin system at  $\delta_{\text{H}}=7.59$  (1H, d,  $J=7.4$  Hz, H-5), 6.97 (1H, dd,  $J=7.5$ , 7.4 Hz, H-6), 7.21 (1H, dd,  $J=7.7$ , 7.5 Hz, H-7) and 6.85 (1H, d,  $J=7.7$  Hz, H-8), an olefinic proton at  $\delta_{\text{H}}=6.58$  (1H, d,  $J=10.4$  Hz, H-10), a methine at  $\delta_{\text{H}}=3.04$  (1H, m, H-11), a methylene at  $\delta_{\text{H}}=1.54$  (1H, m, H-12a) and 1.48 (1H, m, H-12b), and two methyl at  $\delta_{\text{H}}=1.12$  (3H, d,  $J=6.6$  Hz, H-14) and 0.88 (3H, t,  $J=7.5$  Hz, H-13), in addition to a NH proton at  $\delta_{\text{H}}=10.49$  (1H, s, NH-1). The COSY correlations from H-11 to H<sub>3</sub>-14 and H<sub>2</sub>-12 and between H<sub>2</sub>-12 and H<sub>3</sub>-13 established a butyl group, which was linked to an olefinic carbon C-10 ( $\delta_{\text{C}}=146.1$ ) on the basis of the COSY

**Table 1**  $^1\text{H}$  NMR data (500 MHz,  $\text{DMSO-}d_6$ ) for compounds **1a/1b**, and **2a/2b**,  $\delta$  in p.p.m.,  $J$  in Hz

Position	<b>1a/1b</b>	<b>2a</b>	<b>2b</b>
NH-1	10.59, s	10.61, s	10.66, s
NH-19		11.58, br	11.86, br
2		175.9, qC	173.9, qC
3		81.3, qC	52.6, CH
4		128.4, qC	125.8, qC
5	7.32, d (8.1)	125.3, CH	122.9, CH
6	7.04, m <sup>a</sup>	121.9, CH	121.4, CH
7	7.33, m <sup>a</sup>	129.8, CH	128.5, CH
8	6.94, m <sup>a</sup>	110.0, CH	109.6, CH
9		142.4, qC	142.8, qC
10		112.7, qC	148.2, qC
11	6.93, m <sup>a</sup>	124.3, CH	
12	11.09, br s		162.6, qC
13		136.8, qC	113.6, qC
14	7.34, m <sup>a</sup>	111.6, CH	127.9, CH
15	7.06, m <sup>a</sup>	121.3, CH	123.0, CH
16	6.92, m <sup>a</sup>	118.7, CH	135.7, CH
17	7.55, d (8.0)	120.9, CH	115.5, CH
18		125.1, qC	139.4, qC
C3-OMe	3.13, s	51.8, CH <sub>3</sub>	

<sup>a</sup>Overlapped signals assigned by  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC and HSQC spectra without designating multiplicity.

**Figure 3** The NOE differentiation between **2a** and **2b**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

correlation between H-11 and H-10. The presence of a 3-substituted indoline-2-one was evident from the ABCD spin system for an *ortho*-phenyl group, in association with the HMBC interactions from NH-1 to C-2 ( $\delta_{\text{C}} = 168.2$ ), C-3 ( $\delta_{\text{C}} = 127.2$ ), C-4 ( $\delta_{\text{C}} = 121.8$ ), C-8 ( $\delta_{\text{C}} = 109.8$ ) and C-9 ( $\delta_{\text{C}} = 142.2$ ), and from H-5 to C-3 and C-8. The linkage of C-10 to C-3 through an olefinic bond was based on additional HMBC interactions from H-10 to C-2 and C-3. The NOE interaction between H-11 and H-5 was indicative of *3E* geometry.

Analyses of 1D and 2D NMR data and comparison of the NMR data revealed **14** possessing the same gross structure as **13**. The distinction was attributed to the NOE interaction between H-5 ( $\delta_{\text{H}} = 7.50$ , d,  $J = 7.4$  Hz) and H-10 ( $\delta_{\text{H}} = 6.79$ ) instead of the interaction between H-5 and H-11, confirming **14** to be a *3Z* isomer.

Literature survey informed that **13** was documented by a patent cooperation treaty (PCT) without detailed structure and lacking chemical and physical properties,<sup>17</sup> whereas the NMR data of **14** differed from those reported for (3'-methylbutylidene)-2-indolinone.<sup>18</sup> Thus, compounds **13** and **14** were regarded as new natural products.

Compounds **3**, **7**, **8**, **10** and **11** were isolated for nature for the first time. Application of 2D NMR data, the previously unreported  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **11–14** were assigned.

#### Cytotoxic results

Compounds **1–4**, **7**, **9** and **11–14** were selected to test for the cytotoxicities toward the human promyelocytic leukemia cell line (HL-60) and human hepatocellular carcinoma cell line (Bel-7402).

**Table 2** IC<sub>50</sub> values of **2**, **4** and **9** toward HL-60 and Bel-7402 (24 h)<sup>a</sup>

Compound tested	IC <sub>50</sub> (μg ml <sup>-1</sup> )	
	HL-60	Bel7402
<b>4</b>	5.70	6.85
<b>2</b>	5.91	10.03
<b>9</b>	6.24	27.4

<sup>a</sup>Compounds **1**, **3**, **7** and **11–14** were inactive at the range of concentration analyzed (0.1–20 μg ml<sup>-1</sup>).

Primary assay of the compounds with the concentration of 25 μg ml<sup>-1</sup> revealed compounds **2**, **4** and **9** showing potent inhibition against HL-60, whereas their IC<sub>50</sub> values were presented in Table 2. It is noted that compounds **2** and **4** also exhibited significant inhibition against Bel-7402, whereas **9** showed weak inhibition against Bel-7402.

## METHODS

### General experiments

Optical rotations were recorded on a Rudolph Research Analytical AUTOPOL IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). 1D and 2D NMR spectra (chemical shifts in p.p.m., coupling constants in Hz) were recorded on Bruker Avance DRX 500 NMR spectrometer (Bruker, Billerica, MA, USA) using DMSO-*d*<sub>6</sub> as solvent. NMR spectra were referenced to the solvent signal. ESI mass spectra were obtained on a ThermoFinnigan LTQ Orbitrap-XL mass spectrometer (Thermo Finnigan, Waltham, MA, USA). HR-ESI-MS spectra were determined on a ThermoFinnigan LTQ-Orbitrap FT-ESI-MS. For HPLC analysis, 20 μl samples were injected into a Shimadzu LC-20AD HPLC system equipped with a SPD-M20A detector (Shimadzu, Kyoto, Japan), employing a linear gradient from 0.1% phosphoric acid to MeOH (HPLC grade) at 40 min. Routine detection was at 254 nm. A Shim-pack VP-ODS column (150 × 4.6 mm, Shimadzu) was used as the separation column, and the temperature of the column oven was set at 25 °C. Semi-preparative HPLC was performed on Alliance Series III pump, Model 201 UV detector, and Grace Allsphere ODS-2 5 μm column (250 × 10 mm) (Alltech, Lexington, KY, USA). TLC was performed on TLC plates precoated with Si 60 F<sub>254</sub> (Merck, Darmstadt, Germany). The compounds were detected from their UV absorbance and by spraying the TLC plates with anisaldehyde reagent.

### Taxonomic studies

*Shewanella piezotolerans* WP3 was isolated from west Pacific deep-sea sediment (142° 30' 08" E, 8° 00' 11" N) at a depth of 1914 m. The species was identified by Dr Xiao<sup>3</sup> through 16S rDNA detection.

### Fermentation

The modified marine 2216E broth (5 g l<sup>-1</sup> tryptone, 1 g l<sup>-1</sup> yeast extract, 0.1 g l<sup>-1</sup> FePO<sub>4</sub>, 34 g l<sup>-1</sup> NaCl) was used for cultivation and fermentation. *Shewanella piezotolerans* WP3 was inoculated into a 1000-ml Erlenmeyer flask containing 400 ml 2216E medium and cultured at 20 °C for 24 h at 200 r.p.m. Then, 3000 ml of the resultant seed culture was pumped into a 100-l fermentor (Model D-100, B.Braun Biotech, Melsungen, Germany) with a working volume of 70 l for 48 h with a 0.6 v/v/m air flow rate at the same conditions.

### Extraction and isolation

A 70 l of culture broth of *S. piezotolerans* WP3 was separated by centrifugation into the mycelia and supernatant. The supernatant was exhaustively extracted with EtOAc, and the total extract was concentrated in vacuum to yield a brown gum (10.84 g). The EtOAc extract was separated by MPLC with gradient elution using octadecylsilane (ODS) as stationary phase and MeOH/H<sub>2</sub>O as eluent to afford five fractions F1–F5. Fraction F2 (348.6 mg) was separated upon semi-preparative HPLC with a mobile phase of MeOH:H<sub>2</sub>O (7:3) to yield a mixture of **11/12** (3.5 mg), a mixture of **13/14** (2.5 mg), **4** (2.8 mg) and

**9** (1.9 mg). Fraction F1 (431 mg) was followed by the same separation protocol as that for F2 with a mobile phase of MeOH:H<sub>2</sub>O (1:1) to obtain **7** (4.8 mg), **2** (4.6 mg), **3** (9.6 mg) and a mixture of **1a/1b** (2.3 mg). Fraction F3 (200 mg) was separated upon ODS column eluted with MeOH:H<sub>2</sub>O (4:6) to afford **5** (6.8 mg), **6** (6.3 mg), **10** (1.4 mg) and **8** (3.1 mg). Compounds **1a/1b** were further separated by chiral phase semi-preparative HPLC (chiralcel OD-RH, 0.46 × 15 cm) with MeOH-H<sub>2</sub>O (1:3) containing 0.04% trifluoroacetic acid (TFA) as a mobile phase to obtain **1a** (Rt 32 min, 0.7 mg) and **1b** (Rt 35 min, 0.5 mg).

### Spectroscopic data

Shewanelline A (**1a**): amorphous solid; [α]<sub>D</sub><sup>25</sup> = +147.5 (*c* = 0.10, MeOH); UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 215, 261 nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESI-MS *m/z* = 301.1 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* = 277.0983 [M–H]<sup>–</sup> (277.0977 calcd for C<sub>17</sub>H<sub>13</sub>O<sub>2</sub>N<sub>2</sub>).

Shewanelline B (**1b**): amorphous solid; [α]<sub>D</sub><sup>25</sup> = –146.2 (*c* = 0.09, MeOH); UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 215, 261 nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESI-MS *m/z* = 301.1 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* = 277.0983 [M–H]<sup>–</sup> (277.0977 calcd for C<sub>17</sub>H<sub>13</sub>O<sub>2</sub>N<sub>2</sub>).

Shewanelline C (**2**): amorphous solid; the mixture rate (1:0.96); [α]<sub>D</sub><sup>25</sup> = –125.4 (*c* = 0.12, MeOH); UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 199, 220, 311 nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESI-MS *m/z* = 294.1 [M + H]<sup>+</sup>; HR-ESI-MS *m/z* = 294.0873 [M + H]<sup>+</sup> (294.0879 calcd for C<sub>16</sub>H<sub>12</sub>O<sub>3</sub>N<sub>3</sub>).

Isatan (**8**): amorphous solid; UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 200, 247, 256, 289 nm; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ = 10.36 (1H, s, NH-1), 10.21 (1H, s, NH-12), 7.50 (1H, d, *J* = 7.5 Hz, H-17), 7.25 (1H, dd, *J* = 7.6, 7.5 Hz, H-15), 7.12 (1H, dd, *J* = 7.6, 7.6 Hz, H-7), 7.00 (1H, dd, *J* = 7.5, 7.5 Hz, H-16), 6.76 (1H, d, *J* = 7.6 Hz, H-14), 6.74 (1H, d, *J* = 7.6 Hz, H-8), 6.64 (1H, dd, *J* = 7.6, 7.2 Hz, H-6), 6.15 (1H, d, *J* = 7.2 Hz, H-5), 3.99 (1H, s, C3-OH), 1.15 (6H, d, *J* = 6.6 Hz, H-12 and H-13); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 125 MHz) δ = 177.0 (qC, C-2), 174.2 (qC, C-11), 143.3 (qC, C-13), 142.7 (qC, C-9), 129.6 (CH, C-7), 128.4 (CH, C-15), 128.3 (CH, C-4), 126.2 (CH, C-17), 125.8 (qC, C-18), 123.6 (CH, C-5), 121.1 (CH, C-6), 121.0 (CH, C-16), 109.6 (CH, C-8), 109.0 (CH, C-14), 75.5 (qC, C-3), 51.3 (CH, C-10); ESI-MS *m/z* = 210.1 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* = 188.1070 [M + H]<sup>+</sup> (188.1075 calcd for C<sub>12</sub>H<sub>14</sub>ON).

Compounds **11/12**: amorphous solid; the mixture rate (10:1); UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 200, 247, 256, 289 nm; for compound **11**, <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ = 10.48 (1H, s, NH-1), 7.56 (1H, d, *J* = 7.6 Hz, H-5), 7.21 (1H, dd, *J* = 7.8, 7.7 Hz, H-7), 6.97 (1H, dd, *J* = 7.7, 7.6 Hz, H-6), 6.85 (1H, d, *J* = 7.8 Hz, H-8), 6.61 (1H, d, *J* = 10.0 Hz, H-10), 3.24 (1H, m, H-11), 1.15 (6H, d, *J* = 6.6 Hz, H-12 and H-13); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 125 MHz) δ = 168.3 (qC, C-2), 146.9 (CH, C-10), 142.1 (qC, C-9), 129.0 (CH, C-7), 126.2 (qC, C-3), 123.6 (CH, C-5), 121.6 (qC, C-4), 121.4 (CH, C-6), 109.8 (CH, C-8), 27.6 (CH, C-11), 21.6 (CH<sub>3</sub>, C-12 and C-13); ESI-MS *m/z* = 210.1 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* = 188.1070 [M + H]<sup>+</sup> (188.1075 calcd for C<sub>12</sub>H<sub>14</sub>ON). For compound **12**, <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ = 10.48 (1H, s, NH-1), 7.49 (1H, d, *J* = 7.5 Hz, H-5), 7.16 (1H, dd, *J* = 7.7, 7.6 Hz, H-7), 6.92 (1H, dd, *J* = 7.6, 7.5 Hz, H-6), 6.82 (1H, d, *J* = 10.5 Hz, H-10), 6.78 (1H, d, *J* = 7.7 Hz, H-8), 4.00 (1H, m, H-11), 1.07 (6H, d, *J* = 6.7 Hz, H-12 and H-13); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 125 MHz) δ = 168.0 (qC, C-2), 148.3 (CH, C-10), 140.4 (qC, C-9), 128.6 (CH, C-7), 126.4 (qC, C-3), 123.5 (qC, C-4), 121.0 (CH, C-6), 119.5 (CH, C-5), 109.3 (CH, C-8), 25.4 (CH, C-11), 22.1 (CH<sub>3</sub>, C-12 and C-13); ESI-MS *m/z* = 210.1 [M + Na]<sup>+</sup>; HR-ESI-MS *m/z* = 188.1070 [M + H]<sup>+</sup> (188.1075 calcd for C<sub>12</sub>H<sub>14</sub>ON).

Compounds **13/14**: amorphous solid; the mixture rate (10:1); [α]<sub>D</sub><sup>25</sup> + 6.0 (*c* = 0.20, MeOH) for a mixture with the ratio of 10:1; UV (MeOH/H<sub>2</sub>O) λ<sub>max</sub> = 199, 248, 256, 289 nm. For compound **13**, <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ = 10.49 (1H, s, NH-1), 7.59 (1H, d, *J* = 7.4 Hz, H-5), 7.21 (1H, dd, *J* = 7.7, 7.5 Hz, H-7), 6.97 (1H, dd, *J* = 7.5, 7.4 Hz, H-6), 6.85 (1H, d, *J* = 7.7 Hz, H-8), 6.58 (1H, d, *J* = 10.4 Hz, H-10), 3.04 (1H, m, H-11), 1.54 (1H, m, H-12a), 1.48 (1H, m, H-12b), 1.12 (3H, d, *J* = 6.6 Hz, H-14), 0.88 (3H, t, *J* = 7.5 Hz, H-13); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 125 MHz) δ = 168.2 (qC, C-2), 146.1 (CH, C-10), 142.2 (qC, C-9), 129.0 (CH, C-7), 127.2 (qC, C-3), 121.8 (qC, C-4), 121.4 (CH, C-6), 123.5 (CH, C-5), 109.8 (CH, C-8), 34.4 (CH, C-11), 29.2 (CH<sub>2</sub>, C-12), 19.3 (CH<sub>3</sub>, C-14), 11.8 (CH<sub>3</sub>, C-13); (+) ESI-

MS  $m/z = 224.0$   $[M + Na]^+$ ; HR-ESI-MS  $m/z = 202.1226$   $[M + H]^+$  (202.1232 calcd for  $C_{13}H_{16}ON$ ). For compound **14**,  $^1H$  NMR (DMSO- $d_6$ , 500 MHz)  $\delta = 10.46$  (1H, s, NH-1), 7.50 (1H, d,  $J = 7.4$  Hz, H-5), 7.16 (1H, dd,  $J = 8.1, 7.5$  Hz, H-7), 6.92 (1H, dd,  $J = 8.1, 7.4$  Hz, H-6), 6.78 (1H, d,  $J = 7.5$  Hz, H-8), 6.79 (1H, d,  $J = 10.2$  Hz, H-10), 3.83 (1H, m, H-11), 1.37 (2H, m, H-12), 1.04 (3H, d,  $J = 6.7$  Hz, H-14), 0.84 (3H, t,  $J = 7.4$  Hz, H-13);  $^{13}C$  NMR (DMSO- $d_6$ , 125 MHz)  $\delta = 168.1$  (qC, C-2), 147.4 (CH, C-10), 128.6 (CH, C-7), 121.0 (CH, C-6), 119.6 (CH, C-5), 109.3 (CH, C-8), 32.0 (CH, C-11), 29.3 (CH<sub>2</sub>, C-12), 19.8 (CH<sub>3</sub>, C-14), 12.1 (CH<sub>3</sub>, C-13); ESI-MS  $m/z = 224.0$   $[M + Na]^+$ ; HR-ESI-MS  $m/z = 202.1226$   $[M + H]^+$  (202.1232 calcd for  $C_{13}H_{16}ON$ ).

### Tumor cells culture

Human promyelocytic leukemia cell line HL60 and human hepatocellular cell line BEL-7402 were purchased from China Center for Type Culture Collection. HL60 cells were cultured in 25 cm<sup>2</sup> tissue culture flasks at 37 °C under a humidified 5% CO<sub>2</sub> atmosphere in RPMI medium 1640 (Thermo, Waltham, MA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U ml<sup>-1</sup>) and streptomycin (0.1 mg ml<sup>-1</sup>). BEL-7402 cells were cultured in 10 cm plates in minimal essential medium at the same condition.

### Cytotoxicity assay

The cytotoxicity assay was performed with the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the kit instructions. Briefly, the 96-well tumor cell plates were incubated in the incubator for 48 h after adding different concentration of compound into the culture medium. Then, CCK-8 solution (10  $\mu$ l) was added into each well of the 96-well plate, while the plates were incubated for 24 h in the incubator and the absorbance at 450 nm was measured using a micro-plate reader. Each experiment was tested in quadruplicate, and the data were shown as mean  $\pm$  s.e.m. The inhibition ratio was calculated by the following equation: Inhibition ratio =  $[(A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}}] \times 100$ . The IC<sub>50</sub> was taken as the concentration at which it caused 50% inhibition of cell proliferation (50% reduction in the absorbance value in the treated cells, in respect to the control).

### ACKNOWLEDGEMENTS

This work was supported by grants from the NSFC (No.30930109), COMRA (DY125-15-T-01) and SOA (2010319123366025-4), and MOST 863 Project (2011AA090701, 2010DFA31610).

- 1 Thornburg, C. C., Zabriskie, T. M. & McPhail, K. L. Deep-sea hydrothermal vents: potential hot spots for natural products discovery? *J. Nat. Prod.* **73**, 489–499 (2010).
- 2 Skropeta, D. Deep-sea natural products. *Nat. Prod. Rep.* **25**, 1131–1166 (2008).
- 3 Xiao, X., Wang, P., Zeng, X., Bartlett, D. H. & Wang, F. *Shewanella psychrophila* sp. nov. and *Shewanella piezotolerans* sp. nov., isolated from west Pacific deep-sea sediment. *Int. J. Syst. Evol. Microbiol.* **57**, 60–65 (2007).
- 4 Wang, F., Wang, P., Chen, M. & Xiao, X. Isolation of extremophiles with the detection and retrieval of *Shewanella* strains in deep-sea sediments from the west Pacific. *Extremophiles* **8**, 165–168 (2004).
- 5 Wang, F. *et al.* Environmental adaptation: genomic analysis of the piezotolerant and psychrotolerant deep-sea iron reducing bacterium *Shewanella piezotolerans* WP3. *PLoS One* **3**, e1937 (2008).
- 6 Deng, J. *et al.* Facile creation of 3-indolyl-3-hydroxy-2-oxindoles by an organocatalytic enantioselective Friedel–Crafts reaction of indoles with isatins. *Adv. Synth. Catal.* **352**, 833–838 (2010).
- 7 Chauhan, P. & Chimni, S. S. Asymmetric addition of indoles to isatins catalysed by bifunctional modified cinchona alkaloid catalysts. *Chem. Eur. J.* **16**, 7709–7713 (2010).
- 8 Hanhan, N. V., Sahin, A. H., Chang, T. W., Fettinger, J. C. & Franz, A. K. Catalytic asymmetric synthesis of substituted 3-hydroxy-2-oxindoles. *Angew. Chem. Int. Ed.* **49**, 744–747 (2010).
- 9 Ribe, S., Kondru, R. S., Beratan, D. N. & Wipf, P. Optical rotation computation, total synthesis, and stereochemistry assignment of the marine natural product pitamide A. *J. Am. Chem. Soc.* **122**, 4608–4617 (2000).
- 10 Smith, K., El-Hiti, G. A. & Hawes, A. C. Carbonylation of doubly lithiated N'-aryl-N,N-dimethylureas: a novel approach to isatins via intramolecular trapping of acyllithiums. *Synthesis (Mass)*. **13**, 2047–2052 (2003).
- 11 Kobayashi, M. *et al.* Marine natural products. XXXIV. trisindoline, a new antibiotic indole trimer, produced by a bacterium of *Vibrio* sp. separated from the marine sponge *Hyrtios altum*. *Chem. Pharm. Bull.* **42**, 2449–2451 (1994).
- 12 Mizuno, T. & Ishino, Y. Highly efficient synthesis of 1H-quinazoline-2,4-diones using carbon dioxide in the presence of catalytic amount of DBU. *Tetrahedron* **58**, 3155–3158 (2002).
- 13 Mohammadi, A. A., Dabiri, M. & Qaraat, H. A regioselective three-component reaction for synthesis of novel 1'H-spiro[isindoline-1,2'-quinazoline]-3,4'(3'H)-dione derivatives. *Tetrahedron* **65**, 3804–3808 (2009).
- 14 Hoessel, R. *et al.* Idirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat. Cell Biol.* **1**, 60–67 (1999).
- 15 Kawasaki, T. *et al.* Synthesis of 3-hydroxyindolin-2-one alkaloids, (+)-donaxaridine and (+)-convolutamydines A and E, through enolization–Claisen rearrangement of 2-allyloxyindolin-3-ones. *Tetrahedron* **60**, 3493–3503 (2004).
- 16 Tacconi, G. *et al.* 3-alkylidene-1,3-dihydroindol-2-ones: influence of configuration on the transmission of the inductive effect to the carbonyl group. *J. Chem. Soc. Perkin Trans.* **2**, 150–154 (1976).
- 17 Huang, Z., Lui, D., Han, X., Zhang, Z. & Wang, J. Small molecule inhibitors of Bcl-2 proteins. WO Patent 2000004901 (2000).
- 18 Lu, L. *et al.* Studies on the constituents of *Cimicifuga foetida* collected in Guizhou province and their cytotoxic activities. *Chem. Pharm. Bull.* **60**, 571–577 (2012).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)