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Induction of new metabolites from the endophytic fungus *Bionectria* sp. through bacterial co-culture

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ABSTRACT

A new alkaloid, 1,2-dihydrophenopyrrozin (**1**), along with five known compounds (**2–6**) was isolated from an axenic culture of the endophytic fungus, *Bionectria* sp., obtained from seeds of the tropical plant *Raphia taedigera*. Co-cultivation of this fungus either with *Bacillus subtilis* or with *Streptomyces lividans* resulted in the production of two new *o*-aminobenzoic acid derivatives, bionectriamines A and B (**7** and **8**) as well as of two additional known compounds (**9** and **10**). None of the latter compounds (**7–10**) were detected in axenic cultures of the fungus or of the bacteria indicating activation of silent biogenetic gene clusters through co-cultivation with bacteria. The structures of the new compounds were unambiguously determined based on detailed NMR and MS spectroscopic analysis and by comparison with the literature. The crystal structure of agathic acid (**6**) is reported here for the first time. Penicolinate A (**4**) exhibited potent cytotoxic activity against the human ovarian cancer cell line A2780 with an IC_{50} value of 4.1 μM.

1. Introduction

Plant endophytic fungi are microorganisms that thrive in the inner tissues of plants usually without causing apparent harm to their hosts. Endophytes are known to produce a wide plethora of new bioactive secondary metabolites [1]. Tropical and temperate forests represent diverse terrestrial ecosystems, featuring taxonomically diverse endophytic fungi [2]. Since the isolation of the block buster anticancer agent paclitaxel from *Taxomyces andreanae*, formerly known only from *Taxus brevifolia*, endophytic fungi have attracted wide attention of natural product chemists. Fungi of the genus *Bionectria* have been reported to produce numerous new bioactive secondary metabolites such as the antibiotically active compounds verticillin G, bionectrins A-C, the tetramic acid derivatives virgineone and virgineone aglycone, or the cytotoxic pullularins E and F [3–6]. Bacteria and fungi co-exist in many ecosystems such as soil, water, or inside the living tissues of higher plants as endophytes [7,8]. An important interaction between fungi and bacteria is competition for limited nutrients, which is known as a major ecological factor that triggers natural product biosynthesis and accumulation in prokaryotes and eukaryotes alike [9,10]. Co-culture of different microbes rather than maintaining axenic cultures is increasingly practiced in microbial natural product research as the interaction of two or more different microbes may enhance the accumulation of constitutively present natural products [11–13], or may trigger the expression of silent biosynthetic pathways thereby yielding new compounds [14,15].

In the present study, investigation of an axenic culture of the endophytic fungus *Bionectria* sp. fermented on rice afforded one new alkaloid, 1,2-dihydrophenopyrrozin (**1**), along with five known compounds (**2–6**). Co-cultivation of this fungus either with *Bacillus subtilis* or with *Streptomyces lividans* resulted in the production of two new amides, bionectriamines A and B (**7** and **8**) in addition to two known compounds (**9** and **10**). The presence of compounds **7–10** in both co-culture extracts (*Bionectria* sp. with *B. subtilis* or with *S. lividans*)

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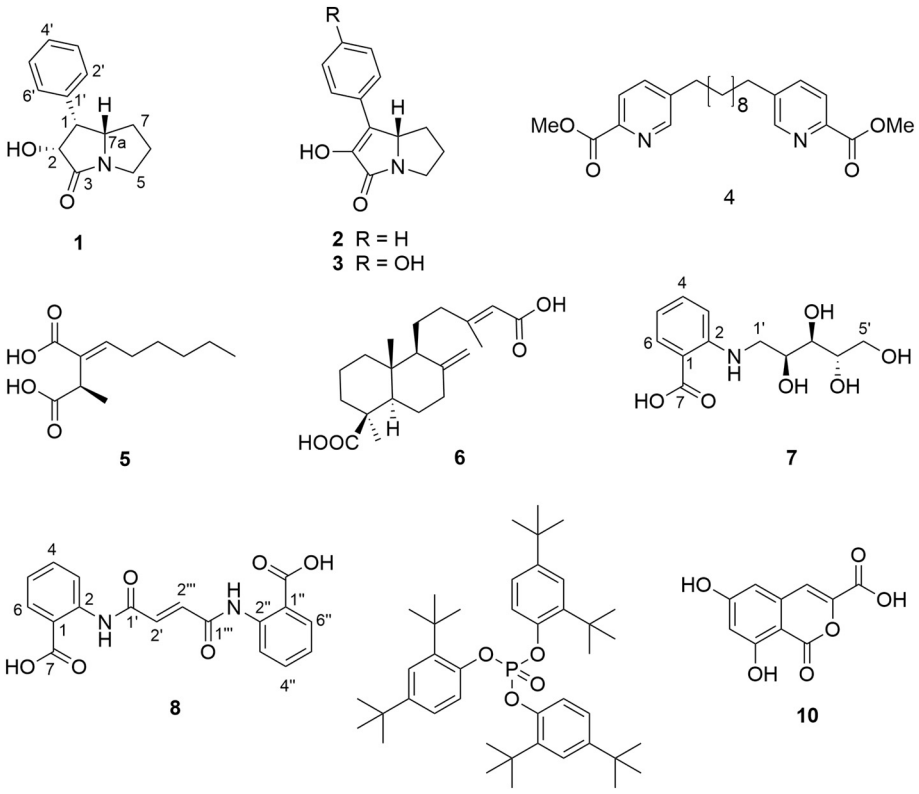

Fig. 1. Structures of isolated compounds.

suggested that they are of fungal origin. Compounds **7–10** were not detected in axenic cultures of the fungus or of the bacteria indicating an activation of silent biogenetic fungal gene clusters through fungal-bacterial co-cultivation. Herein, we report the structure elucidation of the new compounds (**1**, **7** and **8**), the X-ray diffraction study of agathic acid (**6**) and the biological activities of the isolated compounds (Fig. 1).

2. Result and discussion

Compound **1** was isolated as colorless powder. The HRESIMS data of **1** revealed the pseudomolecular ion peak at *m/z* 218.1176 $[M + H]^+$, corresponding to the molecular formula $C_{13}H_{15}NO_2$ with seven degrees of unsaturation. The ^{1}H NMR spectrum of **1** exhibited five aromatic methines at δ_H 7.28 (H-3′ and 5′, t, J = 7.2 Hz, 2H), 7.25 (H-4′, t, J = 7.2 Hz, 1H) and 7.03 (H-2′ and 6′, d, J = 7.2 Hz, 2H) while the ^{13}C NMR spectrum of **1** showed four peaks of aromatic carbons at δ_C 137.0 (C-1′), 130.8 (C-2′ and 6′), 129.3 (C-3′ and 5′) and 128.2 (C-4′), suggesting the presence of a mono-substituted benzene ring in **1**. This was confirmed by the COSY correlation between H-2′(6′)/H-3′(5′) and H-3′(5′)/H-4′ as well as the HMBC correlations from H-2′(6′) to C-4′, from H-3′(5′) to C-1′, and from H-4′ to C-2′(6′). Apart from these signals, three methylene groups at δ_C 42.5 (C-5), 26.7 (C-7) and 26.5 (C-6), three aliphatic methines at δ_C 75.5 (C-2), 62.3 (C-7a) and 53.2 (C-1), and a carbonyl carbon at δ_C 175.8 (C-3) were observed in the ^{13}C NMR and DEPT spectra of **1**. The COSY correlations between H-2 (δ_H 4.95)/H-1 (δ_H 3.92), H-1/H-7a (δ_H 4.13), H-7a/H_{ab}-7 (δ_H 1.63 and 0.98), H_{ab}-7/H_{ab}-6 (δ_H 1.93 and 1.77), and H_{ab}-6/H_{ab}-5 (δ_H 3.44 and 3.13) established the partial structure C(2)-C(1)-C(7a)-C(7)-C(6)-C(5). The attachment of the mono-substituted benzene ring at C-1 was confirmed by the HMBC correlations from H-2 and H-7a to C-1′, from H-1 to C-1′ and C-2′(6′), and from H-2′(6′) to C-1. Presence of a hydroxy group at C-2 was suggested based on the chemical shifts of C-2 and H-2. The HMBC correlations from H-1 and H-2 to C-3, combined with the molecular formula and the chemical shifts of C-5 and C-7a, indicated the linkages from C-2 to C-5 and C-7a via an amide bond. Thus, the planar structure of **1** was elucidated as shown (Fig. 2). The NOE correlations between H-1/H-2 and H-1/H-7a suggested these protons to be on the same side of the lactam ring. Based on the close biogenetic relationship between 1,2-dihydrophenopyrrozin (**1**) and the two co-isolated analogues (*S*)-phenopyrrozin (**2**) [16,17] and (*S*)-*p*-hydroxyphenopyrrozin (**3**) [17,18], compound **1** is suggested to possess the same absolute configuration at C-7a as the known compounds. Thus, the absolute configuration of **1** is proposed to be (1*R*, 2*R*, 7a*S*).

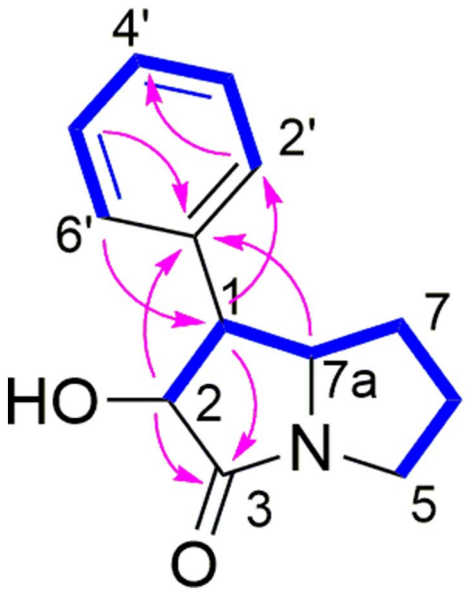

Fig. 2. COSY and key HMBC correlations of **1**.

In addition to the new natural product 1,2-dihydrophenopyrrozin (**1**), five known compounds including (*S*)-phenopyrrozin (**2**) [16,17], (*S*)-*p*-hydroxyphenopyrrozin (**3**) [17,18], penicolinate A (**4**) [18], (*R*)-piliformic acid (**5**) [19,20] and agathic acid (**6**) [21,22] were also isolated from the axenic culture of the endophytic fungus *Pestalotiopsis* sp.

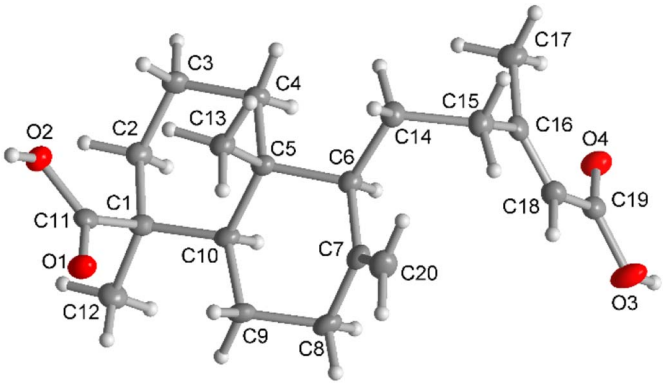

Fig. 3. Molecular structure of **6** by single-crystal X-ray diffraction analysis.

Herein we report the molecular structure of agathic acid (**6**) by single-crystal X-ray diffraction analysis (Fig. 3) for the first time using Cu-Kα radiation with an absolute structure (Flack) parameter of 0.07(10) [23]. Crystallographic data of compound **6** has been deposited to the Cambridge Crystallographic Data Center (CCDC 1519094).

Bionectria sp. was further subjected to mixed fermentation on rice medium with either *Bacillus subtilis* or with *Streptomyces lividans*. The HPLC chromatograms of the two co-cultivation experiments were almost identical and differed remarkably from those of the axenic fungal culture. Two new *o*-aminobenzoic acid derivatives, bionectriamines A and B (**7** and **8**) as well as two known compounds tris(2,4-di-tert-butylphenyl) phosphate (**9**) [24] and 6,8-dihydroxyisocoumarin-3-carboxylic acid (**10**) [25] were isolated from the co-culture extracts. Compounds **1–3**, **5** and **6** which had been isolated from the axenic culture of *Bionectria* sp. were not detected in the HPLC chromatograms of the co-culture extracts indicating a dramatic shift of the natural product pattern during co-cultivation of *Bionectria* sp. either with *B. subtilis* or with *S. lividans*.

The molecular formula of compound **7** was determined as $C_{12}H_{17}NO_6$ by HRESIMS, indicating five degrees of unsaturation. The ^{1}H NMR spectrum of **7** (Table 2) exhibited four aromatic protons at δ_H 7.89 (dd, J = 8.0, 1.6 Hz, H-6), 7.36 (ddd, J = 8.4, 8.0, 1.6 Hz, H-4), 6.84 (dd, J = 8.4, 0.8 Hz, H-3) and 6.60 (ddd, J = 8.0, 8.0, 0.8 Hz, H-5), suggesting the presence of a 1,2-substituted benzene ring, which was confirmed by the COSY correlations between H-3/H-4, H-4/H-5 and H-5/H-6 as well as the HMBC correlations from H-3 to C-1 (δ_C 111.7) and C-5 (δ_C 115.2), from H-4 to C-2 (δ_C 152.0) and C-6 (δ_C 133.0), from H-5 to C-1 and C-3 (δ_C 111.9), and from H-6 to C-2 and C-4 (δ_C 135.2) (Fig. 4). Moreover, the HMBC correlation from H-6 to C-7 (δ_C 171.5) indicated a carboxyl group to be attached at the C-1 position. According to the HSQC spectrum, seven proton signals at δ_H 4.10 (ddd, J = 7.7, 5.6, 1.7 Hz, H-2′), 3.81 (dd, J = 11.2, 3.5 Hz, H_a-5′), 3.71 (ddd, J = 8.2, 5.9, 3.5 Hz, H-4′), 3.63 (dd, J = 11.2, 5.9 Hz, H_b-5′), 3.52 (dd, J = 8.2, 1.7 Hz, H-3′), 3.43 (dd, J = 13.1, 5.6 Hz, H_a-1′) and 3.36 (dd, J = 13.1, 7.7 Hz, H_b-1′) were assigned to four oxygenated carbons at δ_C 72.8 (C-3′), 72.6 (C-4′), 69.5 (C-2′) and 64.7 (C-5′) and one nitrogenated carbon at δ_C 46.4 (C-1′), which were attributed to a 1-amino-1-deoxyhexose unit, as further supported by the COSY correlations between H_{ab}-1′/H-2′, H-2′/H-3′, H-3′/H-4′, H-4′/H_{ab}-5′. An amine linkage between C-2 and C-1′ was confirmed by key HMBC correlations from H_{ab}-1′ to C-2. The absolute configuration of **1** was determined as

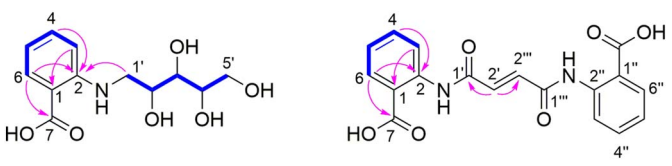

Fig. 4. COSY and key HMBC correlations of **7** and **8**.

2′*S*, 3′*R*, 4′*S* by comparison of the coupling constants and optical rotation of **1** with those of the previously published compound, 1- [(2-carboxyphenyl) amino] - 1- deoxy-D- ribitol [26]. Thus, the structure of **7** was elucidated as a new *o*-aminobenzoic acid derivative as shown, for which the trivial name bionectriamine A is proposed.

Bionectriamine B (**8**) possesses the molecular formula $C_{18}H_{14}N_2O_6$ as deduced from HRESIMS. Compared with **7**, the ^{1}H NMR spectrum of **8** (Table 3) showed similar characteristic signals indicative of an ortho-disubstituted benzene ring at δ_H 8.43 (dd, J = 8.3, 1.1 Hz, H-3), 8.00 (dd, J = 7.9, 1.6 Hz, H-6), 7.63 (ddd, J = 8.3, 7.9, 1.6 Hz, H-4), and 7.24 (ddd, J = 7.9, 7.9, 1.1 Hz, H-5). However, the signals of the 1-amino-1-deoxyhexose unit of **7** were replaced by a singlet olefinic proton at δ_H 7.09 (s, H-2′ and 2‴) in **8**. The HMBC correlations from H-2′ to C-1′ (δ_C 161.6) and C-2‴ (δ_C 134.3) together with the molecular formula concluded compound **8** to be a dimer of two *o*-aminobenzoic acid moieties that are linked through a 2-butenedioic acid residue (Fig. 4). The configuration of the C-2′/C-2‴ double bond was suggested to be *E* by comparison of the chemical shifts of the 2-butenediamide subunit in **8** with those of (*Z*)- and (*E*)-2-butenediamide [27,28].

Compounds **7** and **8** contain an anthranilic acid moiety which is a well known bacterial metabolite. However, the presence of similar anthranilate derivatives and of 1-*O*-carboxyphenylamino-1-deoxyribulose in basidiomycetes and yeasts [29,30], is in favour of a fungal origin of compounds **7** and **8**. Compound **9** features a phosphate group which is rare in nature. Interestingly, this compound was previously isolated from the plant *Vitex negundo* [24]. The isolation of this compound from the mixed fungal-bacterial co-culture in this study raises the question whether compound **9** is perhaps produced by an endophytic fungus residing in the plant *Vitex negundo*. Compound **10** and its analogues were previously isolated from spore-derived mycobionts of the crustose lichen *Graphis vestitoides* [25]. The presence of compounds **7–10** in both two co-culture extracts regardless of the bacteria used suggest that they are of fungal origin.

The cytotoxicity of compounds **1–10** was evaluated against the human ovarian cancer cell line A2780 using the MTT assay. Only penicolinate A (**4**) exhibited potent cytotoxic activity with an IC_{50} value of 4.1 μM whereas the remaining compounds proved to be inactive at the range of doses analyzed.

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured with a JASCO p-1020 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer. HRESIMS spectra were recorded on a UHR-QTOF maxis 4G (Bruker Daltonics) mass spectrometer. HPLC analysis was performed using a Dionex P580 system linked to a photodiode array detector (UVD340s). The analytical column (125 × 4 mm) was prefilled with Eurospher-10 C18 (Knauer, Germany). Semi-preparative RP-HPLC was carried out using a Merck Hitachi system (Pump L7100 and UV detector L7400) and a Eurospher 100–10 C18 column (300 × 8 mm, L × ID).

3.2. Fungal material and identification

The fungus was isolated from fresh seeds of *R. teadigera* collected in January 2015 in Haut Plateaux region, Cameroon. The plant was identified by Prof. Sonke, a botanist at the Department of Biology, Higher Teacher Training College, University of Yaounde-1. Fresh seeds of the plant were rinsed twice with disinfected water, surface sterilized with 70% ethanol for 1 min, and cut into small pieces (around 1 cm × 1 cm × 1 cm) using a sterile blade. These pieces were put on malt agar plates (15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8), and then incubated at room temperature for several days. To exclude the presence of surface-adhered fungi, an imprint of unsterilized seeds on biomalt agar was

performed for comparison. The purified fungus was later transferred to solid rice medium for fermentation. The identification of the fungus was done using a molecular protocol [31]. Sequence data were submitted to GenBank with the accession number KY211868. A voucher strain (No. RAS-2) is kept in the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Germany.

3.3. Fermentation of strains

The fermentation of *Bionectria* sp. was performed in Erlenmeyer flasks (1 L each × 15 flasks) on solid rice medium (autoclaving 100 g rice and 110 mL water at 121 °C for 20 min) at 20 °C under static conditions for 28 days. Co-cultivation experiments of *Bionectria* sp. with *B. subtilis* 168 trpC2 or with *S. lividans* 168 TK24 were carried out following the standard procedure as previously described [32].

3.3.1. Co-cultivation experiment of Bionectria sp. with B. subtilis 168 trpC2

Ten Erlenmeyer flasks (1 L each, two flasks for axenic *B. subtilis*, two for axenic *Bionectria* sp. and six for co-culture) containing 60 mL of distilled water and 50 g of commercially available milk rice (Milch-Reis, ORYZA) were autoclaved before inoculation. An overnight culture of *B. subtilis* grown in lysogeny broth (LB) was used to inoculate pre-warmed LB medium (1:20), which was then incubated at 37 °C with shaking at 200 rpm to mid exponential growth phase (optical density at 600 nm of 0.2–0.4). To the rice medium was added 10 mL of the bacterial culture followed by incubation at 37 °C. After four days, four pieces (1 cm × 1 cm) of *Bionectria* sp. grown on malt agar were added to the flasks that had been preincubated with *B. subtilis*. Fungal and bacterial controls were grown axenically on solid rice medium. Co-cultures and axenic cultures of *Bionectria* sp. and *B. subtilis* were kept at 21 °C under static conditions until they reached their stationary phase of growth (3 weeks for controls of *Bionectria* sp. and *B. subtilis*, 4 weeks for co-cultures). The growth of the cultures was stopped by adding 300 mL of EtOAc to each flask, followed by shaking at 150 rpm for 8 h. The flasks were then kept overnight and the solution was filtered the next day using a Büchner funnel. The solvent was evaporated under vacuum, and the residue was dissolved in 50 mL of MeOH, from which 20 μL was injected into the analytical HPLC column.

3.3.2. Co-cultivation experiment of Bionectria sp. with S. lividans 168 TK24

Ten Erlenmeyer flasks (1 L each, two flasks for axenic *S. lividans*, two for axenic *Bionectria* sp. and six for co-culture) containing 60 mL of yeast malt (YM) medium and 50 g of commercially available milk rice (Milch-Reis, ORYZA) were autoclaved before inoculation. An overnight culture of *S. lividans* was used to inoculate prewarmed YM medium (1:20), which was then incubated at 30 °C with shaking at 200 rpm to mid exponential growth phase. To the rice medium was added a 10 mL volume of the bacterial culture, followed by incubation for 6 days at 30 °C. Then the same procedures as previously described for *B. subtilis* 168 trpC2 were taken.

3.4. Extraction and isolation

The axenic fungal culture was extracted three times (500 mL EtOAc each) to give a crude extract (4.3 g), which was then subjected to vacuum liquid chromatography (VLC) on silica gel (20 cm × 6 cm) using a gradient of *n*-hexane-EtOAc (100:0, 75:25, 50:50, 25:75, 0:100, 1000 mL each), then CH_2Cl_2–MeOH (10:0, 9:1, 7:3, 0:10, 500 mL each) to give nine fractions (FrA-FrI). FrB (45.7 mg) was chromatographed over a Sephadex LH-20 column (50 cm × 3 cm) using 100% MeOH, followed by purification using semi-preparative RP-HPLC with MeOH and 0.1% HCOOH in H_2O (gradient sequence: 0–2 min 50% MeOH, 2–20 min from 80% to 100% MeOH, 20–24 min 100% MeOH) to yield **1** (2.0 mg), **2** (2.4 mg), **3** (6.5 mg) and **5** (3.0 mg). Following similar procedures as above, compounds **4** (10.4 mg), and **6** (3.5 mg) were isolated from FrD (60.7 mg).

Table 1
^{1}H and ^{13}C NMR data of compound **1**.

Position	**1**[a]	
	δ_C, type	δ_H (*J* in Hz)
1	53.2, CH	3.92, dd (7.0, 6.3)
2	75.5, CH	4.95, d (7.0)
3	175.8, C	
5	42.5, CH_2	3.44, m
		3.13, m
6	26.5, CH_2	1.93, m
		1.77, m
7	26.7, CH_2	1.63, m
		0.98, m
7a	62.3, CH	4.13, dt (8.8, 6.3)
1′	137.0, C	
2′, 6′	130.8, CH	7.03, d (7.2)
3′, 5′	129.3, CH	7.28, t (7.2)
4′	128.2, CH	7.25, t (7.2)

[a] Recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C in CD_3OD.

The crude extracts obtained from co-cultures of *Bionectria* sp. with *B. subtilis* or with *S. lividans* were combined due to their similar HPLC profiles. The obtained crude extract (3.4 g) was dissolved in 90% MeOH and partitioned against *n*-hexane to yield 2.2 g of MeOH extract, which was fractionated over a VLC column (21 × 3.5 cm) using a gradient of *n*-hexane-EtOAc (100:0; 75:25; 50:50; 25:75; 100:0) as mobile phase to give five subfractions (V1–V5). Subfraction V2 (50 mg) and V4 (65 mg) were purified by semi-preparative RP-HPLC using 50% MeOH − H_2O to give **7** (0.8 mg) and **8** (0.9 mg), respectively. Subfraction V6 (35 mg) was separated by semi-preparative RP-HPLC with 60% MeOH − H_2O to afford compounds **9** (2.1 mg) and **10** (2.0 mg).

3.4.1. 1,2-Dihydrophenopyrrozin (1)

White powder; UV (MeOH) λ_{max} 225 and 318 nm; $[\alpha]_D^{25}$ + 9 (*c* 0.125, MeOH); ^{1}H and ^{13}C NMR data see Table 1; HRESIMS $[M + H]^+$ *m/z* 218.1176 (calcd for $C_{13}H_{16}NO_2$, 218.1176).

3.4.2. Bionectriamine A (7)

Colorless oil; UV (MeOH) λ_{max} 221, 255 and 352 nm; $[\alpha]_D^{25}$ -234 (*c* 0.4, MeOH); ^{1}H and ^{13}C NMR data see Table 2; HRESIMS $[M + H]^+$ *m/z* 272.1129 (calcd for $C_{12}H_{18}NO_6$, 272.1129).

Table 2
^{1}H and ^{13}C NMR data of compound **7**.

Position	**7**[a]	
	δ_C, type[b]	δ_H (*J* in Hz)
1	111.7, C	
2	152.0, C	
3	111.9, CH	6.84, dd (8.4, 0.8)
4	135.2, CH	7.36, ddd (8.4, 8.0, 1.6)
5	115.2, CH	6.60, ddd (8.0, 8.0, 0.8)
6	133.0, CH	7.89, dd (8.0, 1.6)
7	171.5, C	
1′	46.4, C H_2	3.43, dd (13.1, 5.6)
		3.36, dd (13.1, 7.7)
2′	69.5, C	4.10, ddd (7.7, 5.6, 1.7)
3′	72.8, C	3.52, dd (8.2, 1.7)
4′	72.6, C	3.71, ddd (8.2, 5.9, 3.5)
5′	64.7, CH_2	3.81, dd (11.2, 3.5)
		3.63, dd (11.2, 5.9)

[a] Recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C in CD_3OD.
[b] Data extracted from HSQC and HMBC spectra.

Table 3
^{1}H and ^{13}C NMR data of compound **8**.

Position	**8**[a]	
	$\delta_{C,\ type}$[b]	δ_{H} (J in Hz)
1, 1″	118.3, C	
2, 2″	139.4, C	
3, 3″	120.8, CH	8.43, dd (8.3, 1.1)
4, 4″	133.5, CH	7.63, ddd (8.3, 7.9, 1.6)
5, 5″	123.5, CH	7.24, ddd (7.9, 7.9, 1.1)
6, 6″	130.8, CH	8.00, dd (7.9, 1.6)
7, 7″	168.9, C	
1′, 1‴	161.6, C	
2′, 2‴	134.3, CH	7.09, s

[a] Recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C in DMSO-d_6.
[b] Data extracted from HSQC and HMBC spectra.

3.4.3. *Bionectriamine B* (**8**)

White powder; UV (MeOH) λ_{max} 255 and 298 nm; ^{1}H and ^{13}C NMR data see Table 3; HRESIMS $[M + H]^{+}$ m/z 355.0920 (calcd for $C_{18}H_{15}N_2O_6$, 335.0925).

3.5. Cytotoxicity assay

Cytotoxicity against human ovarian cancer cell line A2780 was tested by MTT method as previously described [33]. All experiments were carried out in triplicate using cis-diamminedichloroplatinum (CDDP) as positive control.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2017.10.021.

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