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Metabolites from the endophytic fungus *Cylindrocarpon* sp. isolated from tropical plant *Sapium ellipticum*

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ABSTRACT

Three new polyketides, cylindrocarpones A − C (**1–3**), two new pyridone alkaloids, cylindrocarpyridones A − B (**5–6**), a new pyrone cylindropyrone (**7**), together with seven know compounds were isolated from the endophytic fungus, *Cylindrocarpon* sp., obtained from the tropical plant *Sapium ellipticum*. The structures of the new compounds were elucidated by extensive analysis of their spectroscopic data (1D and 2D NMR, HRESIMS). The absolute configuration of 19-*O*-methyl-pyrrocidine B (**13**) was confirmed by X-ray analysis. All isolated compounds were screened for their cytotoxic and antibacterial activities. Pyrrocidine A (**12**) exhibited potent cytotoxicity against the human ovarian cancer cell line A2780 with an IC_{50} value of 1.7 μM. 19-*O*-Methyl-pyrrocidine B (**13**) showed moderate antibacterial activity against *S. aureus* ATCC25923 and ATCC700699 with MIC values of 50 and 25 μM, respectively.

1. Introduction

Endophytic fungi are known as an important source of polyketides [1,2]. This class of natural products exhibits a broad range of bioactivities, including antibiotic, anticancer, antifungal, antiparasitic and immunosuppressive properties, such as erythromycin, eribulin, bryostatin, and spongistatin [3–5]. Fungi of the genus *Cylindrocarpon* have been reported to produce structurally diverse secondary metabolites such as two inhibitors of pollen development in *Arabidopsis thaliana*, roridin A and verrucarin A [6], inhibitors of dihydroxynaphthalene-melanin biosynthesis, fusarins [7], a cytotoxic cyclopeptide, cylindrocyclin A [8], and an ascochlorin congener, cylindrol A_5 [9].

In our ongoing search for structurally novel and bioactive metabolites from endophytic fungi isolated from African tropical rain forest plants, cytotoxic penicolinate A, and two new *o*-aminobenzoic acid derivatives, bionectriamines A and B were isolated from the fungus *Bionectria* sp. [10], while a new cyclohexapeptide, penitropeptide and a new polyketide, penitropone were obtained from *Penicillium tropicum* [11]. We have now analyzed the endophytic fungus *Cylindrocarpon* sp. that was isolated from the tropical plant *Sapium ellipticum*.

We obtained three new polyketides, cylindrocarpones A − C (**1–3**), two new pyridone alkaloids, cylindrocarpyridone (**5–6**), a new pyrone cylindropyrone (**7**), as well as seven known compounds which included lamellicolic anhydride (**4**) [12], 5-chloro-6,8,10-trihydorxy-1-methoxy-3-methyl-9(10*H*)-anthracenone (**8**) [13], 1-*O*-methylemodin (**9**) [14], 5-chloro-1-*O*-methylemodin (**10**) [15], dihydroramulosin (**11**) [16], pyrrocidine A (**12**) [17,18], and 19-*O*-methyl-pyrrocidine B (**13**) [18] (Fig. 1). Here we report the structure elucidation of the new metabolites and the biological activities of all isolated compounds.

2. Results and discussion

Compound **1** was isolated as a yellow powder. The molecular formula $C_{14}H_{12}O_6$, indicating nine degrees of unsaturation, was deduced from the HREIMS data. The ^{13}C and ^{1}H NMR spectrum of **1** (Table 1) displayed signals of a methyl group at δ_C 25.8 (Me-11) and δ_H 2.76 (s, Me-11), of a methoxy group at δ_C 55.9 (OMe-1) and δ_H 3.91 (s, OMe-1), of two aromatic methines at δ_C 117.8 (C-7), 96.9 (C-2) and δ_H 6.66 (H-7) and 6.45 (H-2), of an oxygenated methine at δ_C 101.3 (C-10) and δ_H 6.47 (H-10), as well as the signal of a carbonyl carbon at δ_C 171.3 (C-9) in addition to signals of eight aromatic quaternary carbons. The UV and NMR data of **1** showed similarities to those of the co-isolated known

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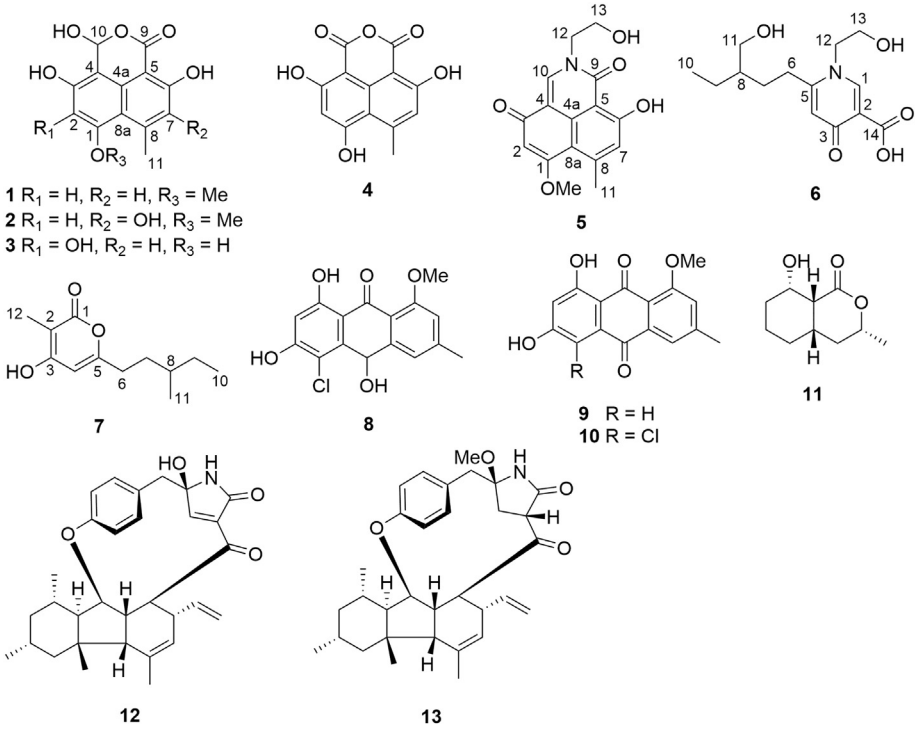

Fig. 1. Structures of isolated compounds.

Table 1
NMR data of compounds **1**–**3**.[a]

No.	1		2		3	
	δ_C, type	δ_H	δ_C, type	δ_H	δ_C, type	δ_H
1	162.3, C		161.4, C		160.3, C	
2	96.9, CH	6.45, s	97.7, CH	6.48, s	n.d.[b]	
3	157.6, C		154.9, C		157.3, C	
4	101.9, C		101.5, C		100.5, C	
4a	133.5, C		127.2, C		133.4, C	
5	97.7, C		97.6, C		97.0, C	
6	164.8, C		155.2, C		165.0, C	
7	117.8, CH	6.66, s	141.8, C		116.7, CH	6.63, s
8	149.4, C		130.4, C		150.0, C	
8a	113.4, C		113.8, C		112.5, C	
9	171.3, C		171.6, C		171.2, C	
10	101.3, CH	6.47, s	101.6, CH	6.50, s	101.3, CH	6.47, s
11	25.8, CH_3	2.76, s	15.4, CH_3	2.73, s	25.2, CH_3	2.82, s
OMe-1	55.9, CH_3	3.91, s	55.9, CH_3	3.90, s		

[a] Recorded at 600 MHz for ^1H and 150 MHz for ^{13}C in CD_3OD.
[b] Not detected.

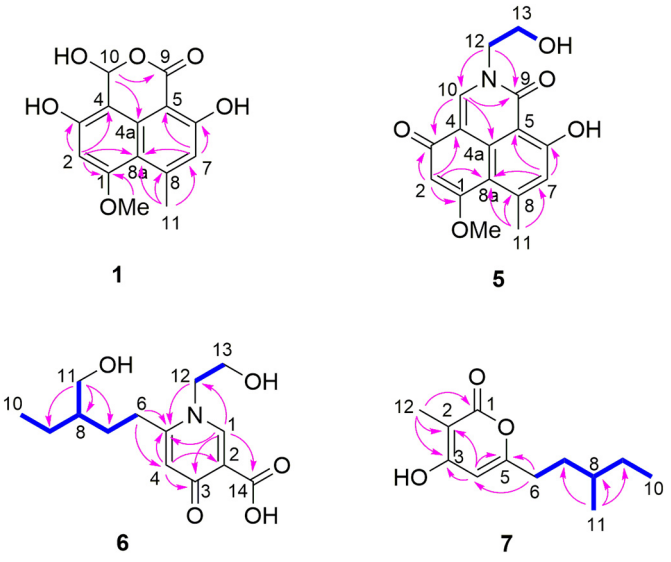

Fig. 2. COSY and key HMBC correlations of compounds **1**, **5**, **6** and **7**.

compound lamellicolic anhydride (**4**) [12], suggesting a similar skeletal structure. The HMBC correlations from H-2 to C-1 (δ_C 162.3), C-3 (δ_C 157.6), C-4 (δ_C 101.9) and C-8a (δ_C 113.4), from H-7 to C-5 (δ_C 97.7), C-6 (δ_C 164.8), C-8a and C-11 (δ_C 25.8), and from Me-11 to C-7 (δ_C 117.8), C-8 (δ_C 149.4) and C-8a established a naphthalene core structure with a methyl group at C-8 and two hydroxy groups at C-3 and C-6 (Fig. 2). In contrast to the known compound **4**, the HMBC correlations of **1** from OMe-1 to C-1 and from H-10 to C-9 (δ_C 171.3) and C-4a (δ_C 133.5) indicated the presence of a methoxy substituent at C-1 and of a hydroxy group at C-10 in the latter compound. Thus, the planar structure of **1** was elucidated as shown, for which the trivial name cylindrocarpone A is proposed.

The molecular formula of cylindrocarpone B (**2**) was determined as $C_{14}H_{12}O_7$ by HRESIMS thus containing an additional oxygen atom compared to **1**. The NMR data of **2** were similar to those of **1** except for the replacement of a methine by an aromatic quaternary carbon at δ_C 141.8 in **2**. The HMBC correlations from Me-11 (δ_H 2.73) to C-7 (δ_C 141.8), C-8 (δ_C 130.4) and C-8a (δ_C 113.8) indicated that an additional hydroxy group was attached at C-7. The remaining substructure of **2** was elucidated to be identical to that of **1** by detailed analysis of the 2D NMR of **2**.

Cylindrocarpone C (**3**) has the molecular formula $C_{13}H_{10}O_7$ as deduced from HRESIMS data, thus differing by the loss of a CH_2 moiety compared to **2**. The signals of the two methines at δ_H 6.63 (s) and 6.47 (s) were assigned to H-7 and H-10, respectively, based on the HMBC correlations from H-7 (δ_H 6.63, s) to C-5 (δ_C 97.0), C-6 (δ_C 165.0), C-8a (δ_C 112.5) and C-11 (δ_C 25.2), and from H-10 (δ_H 6.47, s) to C-3 (δ_C 157.3), C-4 (δ_C 100.5), C-9 (δ_C 171.2) and C-4a (δ_C 133.4). These data along with the disappearance of signals of the methoxy group suggested the presence of two hydroxy groups at C-1 and C-2 in compound **3**.

Table 2
NMR data of compounds **5–7**.

No.	**5**[a]		**6**[b]		**7**[b]	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	169.3, C		149.0, CH	8.68, s	168.3, C	
2	101.3, CH	5.91, s	115.5, C		98.5, C	
3	180.6, C		179.3, C		167.5, C	
4	111.1, C		118.7, CH	6.75, s	100.8, CH	5.99, s
4a	133.8, C					
5	107.9, C		159.2, C		164.7, C	
6	162.5, C		30.3, CH_2	2.89, m	32.0, CH_2	2.52, m 2.45, m
7	116.9, CH	6.91, s	30.5, CH_2	1.77, m 1.67, m	34.7, CH_2	1.68, m 1.45, m
8	147.8, C		42.5, CH	1.53, m	35.1, CH	1.40, m
8a	111.4, C					
9	164.9, C		24.3, CH_2	1.44, m	30.3, CH_2	1.40, m 1.21, m
10	141.7, CH	8.52, s	11.3, CH_3	0.96, t (7.4)	11.6, CH_3	0.90, t (7.3)
11	24.5, CH_3	2.70, s	64.2, CH_2	3.63, dd (11.0, 4.8) 3.51, dd (11.0, 6.2)	19.2, CH_3	0.93, d (6.6)
12	51.3, CH_2	4.24, t (5.2)	56.8, CH_2	4.33, m	8.2, CH_3	1.85, s
13	58.0, CH_2	3.74, m	61.6, CH_2	3.88, m		
14			169.0, C			
OMe-1	55.8, CH_3	3.93, s				
OH-6		12.98, s				
OH-13		4.98, t (5.4)				

[a] Recorded at 600 MHz for ^1H and 150 MHz for ^{13}C in DMSO-d_6.
[b] Recorded at 600 MHz for ^1H and 150 MHz for ^{13}C in CD_3OD.

Thus, the structure of cylindrocarpone C (**3**) was elucidated as shown. The ECD spectra of compounds **1–3** were baseline curves, which proved them to be racemic mixtures.

Compound **5** was obtained as a yellow powder. The molecular formula was established as $C_{16}H_{15}NO_5$ by HRESIMS, indicating ten degrees of unsaturation. The NMR data of **5** (Table 2) were similar to those of **1**, suggesting their structural similarity. Compared to **1**, the HMBC correlations from H-2 (δ_H 5.91, s) to C-1 (δ_C 169.3), C-3 (δ_C 180.6), C-4 (δ_C 111.1) and C-8a (δ_C 111.4), and from H-10 (δ_H 8.52, s) to C-3, C-4a (δ_C 133.8) and C-9 (δ_C 164.9) in **5** indicated the presence of an additional ketone group at C-3 and an additional double bond at C-4/C-10. Moreover, the presence of a hydroxyethyl group was established through signals of two methylenes at δ_C 51.3 (C-12), 58.0 (C-13) and δ_H 4.24 (H_2-12), 3.74 (H_2-13), and a hydroxy group at δ_H 4.98 (OH-13) as well as from the COSY correlations between H_2-12/H_2-13/OH-13. The HMBC correlation from H-10 to C-12 and from H_2-12 to C-9 and C-10 (δ_C 141.7), together with the chemical shift of C-12 and the molecular formula of **5**, confirmed the hydroxyethyl group as a substituent of a nitrogen atom which replaced the oxygen atom of compounds **1–4**. The remaining structure of **5** was identical to that of **1** as shown by detailed analysis of the 2D NMR spectra (Fig. 2).

Cylindrocarpyridone B (**6**) possessed the molecular formula $C_{14}H_{21}NO_5$ as determined by HRESIMS data, being indicative of five degrees of unsaturation. A ketodihydronicotinic acid substructure was established by the HMBC correlations from H-1 (δ_H 8.68, s) to C-3 (δ_C 179.3), C-5 (δ_C 159.2) and C-14 (δ_C 169.0), and from H-4 (δ_H 6.75, s) to C-2 (δ_C 115.5), C-3 and C-5. The substitution of the nitrogen atom of the ketodihydronicotinic acid substructure by a hydroxyethyl group was confirmed by the COSY correlations between H_2-12 (δ_H 4.33) and H_2-13 (δ_H 3.88), the HMBC correlations from H-1 to C-12 (δ_C 56.8) and from H_2-12 to C-1 (δ_C 149.0) and C-5 (Fig. 2) as well as based on the chemical shifts of C-12 and C-13 (δ_C 61.6). In addition, the COSY correlations between H_2-6/H_2-7/H-8/H_2-9/H_2-10 and between H-8/H_2-11, together with the HMBC correlations from Me-10 (δ_H 0.96) to C-8 (δ_C 42.5) and C-9 (δ_C 24.3), from H_2-11 (δ_H 3.63 and 3.51) to C-7 (δ_C 30.5), C-8 and C-9, and from H_2-6 (δ_H 2.89) to C-4 (δ_C 118.7) and C-5 indicated the presence of a 3-(hydroxymethyl)pent-1-yl side chain at C-5. Thus, the structure of compound **6** was elucidated as shown. The absolute configuration of C-8 remained unsolved due to the limited amount of compound isolated.

Compound **7** was obtained as a white powder, and the molecular formula was determined as $C_{12}H_{18}O_3$ by HRESIMS. Its UV maximum absorption at 291 nm together with the HMBC correlations from Me-12 (δ_H 1.85, s) to C-1 (δ_C 168.3), C-2 (δ_C 98.5) and C-3 (δ_C 167.5), and from H-4 (δ_H 5.99, s) to C-2, C-3 and C-5 (δ_C 164.7) indicated the presence of a *α*-pyrone ring with a methyl and hydroxy substituent at C-2 and C-3, respectively. A 3-methylpent-1-yl side chain linked to C-5 was established by the COSY correlations between H_2-6/H_2-7/H-8/H_2-9/H_2-10 and between H-8/Me-11 in addition to the HMBC correlations from Me-10 (δ_H 0.90, t) to C-8 (δ_C 35.1) and C-9 (δ_C 30.3), from Me-11 (δ_H 0.93, d) to C-7 (δ_C 34.7), C-8 and C-9, and from H_2-6 (δ_H 2.52 and 2.45) to C-4 (δ_C 100.8) and C-5 (Fig. 2). Thus, the planar structure of compound **7** was elucidated, for which the trivial name cylindropyrone is proposed. Attempts for crystallization of **7** failed, which left the absolute configuration of **7** unknown.

The known compounds included pyrrocidine A (**12**) and 19-*O*-methylpyrrocidine B (**13**) formerly reported from the endophytic fungus *Neonectria ramulariae* [18]. Here the absolute configuration of **13** as 3*R*, 6*R*, 7*S*, 9*R*, 11*S*, 12*R*, 13*S*, 14*S*, 15*S*, 17*S*, and 19S is reported for the first time using X-ray analysis (Fig. 3). Crystallographic data of compound **13** has been deposited to the Cambridge Crystallographic Data Center (CCDC 1589981).

The cytotoxicity of compounds **1–13** was evaluated against the human ovarian cancer cell line A2780 using the MTT assay. Only pyrrocidine A (**12**) exhibited potent cytotoxic activity with an IC_{50} value of 1.7 μM whereas the remaining compounds proved to be inactive at the range of doses analyzed. Compared to compound **12**, the absence of the double bond in the lactam ring in compound **13** led to loss of cytotoxicity. In addition, 19-*O*-methyl-pyrrocidine B (**13**) exhibited moderate antibacterial activity against *S. aureus* ATCC25923 and ATCC700699 with MIC values of 50 and 25 μM, respectively whereas compound **12** showed strong to moderate inhibitory effects against all tested strains including persistent *S. aureus* with MIC values ranging from 0.78 to 25 μM (Table 3). The other isolated metabolites were inactive up to a dose of 100 μM.

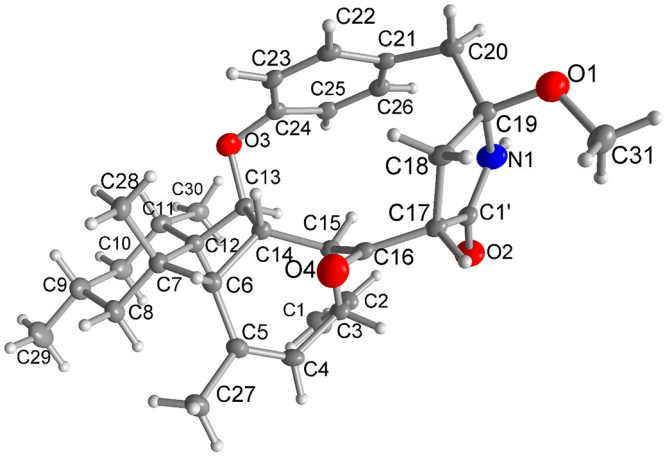

Fig. 3. Molecular structure of compound **13**.

Table 3
Antibacterial activity of compounds **12** and **13**.

Compound	MIC (μM)						
	S. aureus, ATCC 25923	*S. aueus*, ATCC 700699	Persistent *S. aueus*, ATCC 700699	*E. faecalis*, ATCC 29212	*E. faecalis*, ATCC 51299	*E. faecium*, ATCC 35667	*E. faecium*, ATCC 700221
12	1.6	0.78	25	3.1	1.6	1.6	3.1
13	50	25	> 100	100	100	100	100
Moxifloxacin	< 0.9	3.8	–	0.9	0.9	1.9	7.8

3. Experimental section

3.1. General experimental procedures

A JASCO p-1020 polarimeter was used to measure the optical rotations. 1D and 2D NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer. HRESIMS data were obtained on a Bruker UHR-QTOF maxis 4G mass spectrometer. HPLC analysis was performed on a Dionex P580 system coupled with an UVD340s photodiode array detector. The analytical column (125 × 4 mm) was prefilled with Eurospher-10 C_{18} (Knauer, Germany) using MeOH and 0.1% HCOOH in H_2O as mobile phase at a flow rate of 1 mL/min. Semi-preparative RP-HPLC was carried out with a Merck Hitachi system (Pump L7100 and UV detector L7400) and a Eurospher 100-10 C_{18} column (300 × 8 mm) at a flow rate of 5 mL/min. Silica gel $60F_{254}$ plates (Merck, Germany) were used for analytical TLC.

3.2. Fungal material and fermentation

The fungus was isolated from fresh roots of *S. ellipticum* collected in January 2015 in Haut Plateaux region, Cameroon according to previous protocol [19]. It was identified as *Cylindrocarpon* sp. by DNA amplification, sequencing of ITS region and by comparison with GenBank data (GeneBank accession No. KY211868) as previously described [20]. A voucher strain (No. SAR-2) is kept in the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Germany. The fungal fermentation was carried out in 15 Erlenmeyer flasks (1 L each) 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.

3.3. Extraction and isolation

The crude extract (9.2 g) was subjected to a liquid-liquid partition between *n*-hexane and EtOAc. The EtOAc fraction (4.2 g) was subjected to vacuum liquid chromatography (VLC) on silica gel with a gradient solvent system (*n*-hexane-EtOAc 100:0 to 0:100) to afford ten fractions (Fr.A to Fr.J). Fr·B (1.5 g) was chromatographed over a Sephadex LH-20 column eluting with MeOH to give four subfractions (Fr·B-1 to Fr·B-4). Fr·B-1 was fractionated by semi-preparative HPLC using MeOH and 0.1% TFA in H_2O (gradient sequence: 0–2 min 50% MeOH, 2–20 min from 80% to 100% MeOH, 20–24 min 100% MeOH, flow rate 5 mL/min) to afford compounds **1** (2.1 mg), **2** (3.3 mg), **3** (2.0 mg), **4** (3.2 mg), **5** (2.5 mg), Fr·B-4 was separated by semi-preparative HPLC with MeOH and 0.1% HCOOH in H_2O (MeOH:H_2O = 1:1, flow rate 5 mL/min) to afford compounds **10** (6.7 mg), **11** (3.0 mg), **12** (4.4 mg) and **13** (3.8 mg). Fr·C was fractionated by a Sephadex column eluting with CH_2Cl_2 − MeOH (1:1) and further purified by semi-preparative HPLC with 30% MeOH-H_2O (0.1% TFA) to give compound **7** (4.5 mg). Fr·H was purified by semi-preparation HPLC with MeOH and 0.1% TFA in H_2O as mobile phase (gradient sequence: 0–2 min 60% MeOH, 2–8 min from 70% to 100% MeOH, 8–10 min 100% MeOH, flow rate 5 mL/min) to afford compounds **6** (1.9 mg), **8** (1.8 mg) and **9** (1.2 mg).

3.3.1. Cylindrocarpone A (1)

Yellow powder; $[\alpha]_D^{25}$ + 6 (*c* 0.18, MeOH); UV (MeOH) λ_{max} 234, 273 and 343 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M+Na]^+ m/z$ 299.0525 (calcd for $C_{14}H_{12}O_6Na$, 299.0526).

3.3.2. Cylindrocarpone B (2)

Yellow powder; $[\alpha]_D^{25}$ + 3 (*c* 0.30, MeOH); UV (MeOH) λ_{max} 234, 274 and 344 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M-H]^- m/z$ 291.0499 (calcd for $C_{14}H_{11}O_7$, 291.0499).

3.3.3. Cylindrocarpone C (3)

Yellow powder; $[\alpha]_D^{25}$ + 9 (*c* 0.35, MeOH); UV (MeOH) λ_{max} 227, 276 and 344 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M+H]^+ m/z$ 279.0499 (calcd for $C_{13}H_{11}O_7$, 279.0499).

3.3.4. Cylindrocarpyridone A (5)

Yellow powder; UV (MeOH) λ_{max} 215, 321 and 410 nm; for ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M+H]^+ m/z$ 302.1024 (calcd for $C_{16}H_{16}NO_5$, 302.1023).

3.3.5. Cylindrocarpyridone B (6)

White powder; $[\alpha]_D^{25}$ + 10 (*c* 0.20, MeOH); UV (MeOH) λ_{max} 240 and 290 nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M+H]^+ m/z$ 284.1493 (calcd for $C_{14}H_{22}NO_5$, 284.1492).

3.3.6. Cylindropyrone (7)

White powder; $[\alpha]_D^{25}$ + 8 (*c* 0.51, MeOH); UV (MeOH) λ_{max} 201 and 291 nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M+H]^+ m/z$ 211.1327 (calcd for $C_{12}H_{19}O_3$, 211.1329).

3.4. Cytotoxicity assay

Cytotoxicity against the human ovarian cancer cell line A2780 was tested by MTT method as previously described [21]. All experiments were carried out in triplicate using *cis*-diamminedichloroplatinum (CDDP, IC_{50} 1.2 µM) and DMSO as positive and negative control, respectively.

3.5. Antimicrobial assay

The antibacterial activity of isolated compounds were evaluated by calculating their minimal inhibitory concentration (MIC) against *S. aureus* ATCC25923, *S. aureus* ATCC700699, *E. faecalis* ATCC29212, *E. faecalis* ATCC 51299, *E. faecium* ATCC35667, *E. faecium* ATCC700221. MIC for each strain was determined by the broth micro dilution method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) [22]. Moxifloxacin and DMSO were used as positive and negative control, respectively.

3.6. Anti-persister activity assay

Sterile U-bottom 96-well polystyrene plates were used for the anti-persister assay. The wells were prepared with a final volume of 50 µL phosphate-buffered saline (PBS, NaCl = 137 mM, KCl = 2.7 mM, $NaHPO_4$ = 10 mM, KH_2PO_4 = 1.8 mM, pH = 7.4) containing a two-

fold serial dilution of compounds ranging from 100 µM to 0.78 µM. *Staphylococcus aureus* (ATCC strain 700,699, MRSA-VISA) was cultivated in Mueller-Hinton broth overnight shaking at 37 °C. The cells were then washed three times with PBS and optical density was measured with a photometer to adjust OD_{600} = 0.08 to yield a cell density of approx. 2×10^7 cell per mL. To the prepared U-bottom 96-well polystyrene plates, 50 µL of the diluted cell suspension was added to each well. The plate was statically incubated for 24 h at 37 °C.

Viability of bacterial cells was estimated employing the resazurin dye reduction assay. Briefly, 10 µL of a 100 µg/mL resazurin solution were added to each well and were resuspended carefully. The plates were then incubated for several hours at 37 °C. Finally, a 10% formalin solution was added to each well and fluorescence was quantified in a plate reader with 535 nm excision and 590 nm emission wavelengths. Persiter eradication was used as positive control with an MIC value of 4 µg/mL.

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