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Antifungal polyketide derivatives from the endophytic fungus *Aplosporella javeedii*

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ABSTRACT

Six new polyketides aplojaveediins A–F (**1**–**6**) were isolated from the endophytic fungus *Aplosporella javeedii* associated with the host plant *Orychophragmus violaceus* (Brassicaceae). The structures of the new metabolites were elucidated by analysis of their NMR and MS data. Compound **1** exhibited antifungal activity against the hyphae form of *Candida albicans* strain ATCC 24433 in the agar plate diffusion assay and the microbroth dilution assay. The kinetic of killing of *C. albicans* cells for compound **1** was considerably faster than that of the positive control hygromycin B. Compounds **1** and **6** also exhibited moderate antibacterial activities against sensitive (ATCC 29213) and drug-resistant (ATCC 700699) strains of *Staphylococcus aureus*.

1. Introduction

Endophytic fungi are firmly established as sources of new bioactive metabolites and have been shown to accumulate diverse groups of compounds such as alkaloids, terpenoids, steroids, phenols, quinones, xanthones, and peptides.[1,2,3,4,5] Due to this pronounced chemical diversity, endophytic fungi represent an important potential source of new medicinal and biotechnological agents. During our ongoing research on new bioactive secondary metabolites from endophytic fungi,[6,7] *Aplosporella javeedii* was isolated from stem tissue of *Orychophragmus violaceus* (L.) O. E. Schul (*Brassicaceae*) collected around Beijing. *O. violaceus* is an edible wild herb as well as a medicinal plant that is used in Traditional Chinese Medicine (TCM).[8,9] It is recorded in the TCM literature for dissipating swelling and for treating unknown pyrogenic infections.[10] In recent years, researchers also reported hepatoprotective effects for this plant.[11] *A. javeedii* is a member of the fungal family *Aplosporellaceae* and is usually associated with canker and dieback disease of woody plants. It was first isolated and identified from wood sections of *Celtis africana* Burm.f. (*Cannabaceae*) and *Searsia lancea* (L.f.) F.A. Barkley (*Anacardiaceae*) in South Africa in 2013.[12] Other records are from China and came from woody trees of the *Fabaceae*, *Cupressaceae*,[13] *Rhamnaceae*,[14] and *Moraceae*.[15] To our best knowledge, this is the first record of *A. javeedii* from a host plant of the *Brassicaceae*. Until now, there are no reports on secondary metabolites of *A. javeedii* which prompted us to investigate this fungus. When grown on solid rice medium *A. javeedii* yielded six new polyketides (**1**–**6**) (Fig. 1). In this paper, we report the isolation and structure elucidation of these polyketides, as well as their antifungal and antibacterial activities.

2. Results and discussion

Compound **1** was obtained as colorless crystals, with UV absorptions at λ_{max} 208, 220 and 298 nm. Its molecular formula was established as $C_{13}H_{18}O_3$ on the basis of prominent pseudomolecular ion peaks at *m/z* 223.1332 $[M+H]^+$ and 221.1178 $[M-H]^+$ in the HRESIMS spectrum, indicating five degrees of unsaturation. The ^{1}H NMR data of **1** (Table 1) showed one aldehyde proton at δ_H 10.01 (s, H-7), one aromatic proton at δ_H 6.24 (s, H-5), one aromatic methyl group at δ_H 1.99 (s, Me-8). The ^{13}C NMR data of **1** (Table 1) displayed one aldehyde carbon at δ_C 194.1 (C-7) and six aromatic carbons at δ_C 165.2 (C-2), 165.0 (C-6), 147.9 (C-4), 112.7 (C-1), 110.2 (C-5), and 110.1 (C-3). The HMBC correlations (Fig. 2) from H-7 to C-1, C-2, and C-6, from Me-8 to C-2, C-3, and C-4, and from H-5 to C-1, C-3, and C-6 established the presence of a pentasubstituted benzene ring with an aldehyde group and a methyl group at C-1 and C-3, respectively. The remaining NMR data are characteristic signals of a *n*-pentyl chain, which was further confirmed by the COSY correlations between H_2-9 (δ_H 2.80)/H_2-10 (δ_H 1.61), H_2-10/H_2-11 (δ_H 1.35), H_2-12 (δ_H 1.36)/Me-13 (δ_H 0.91) as well as by the HMBC correlations from Me-13 to C-11 (δ_C 32.7) and C-12 (δ_C 23.5). In addition,

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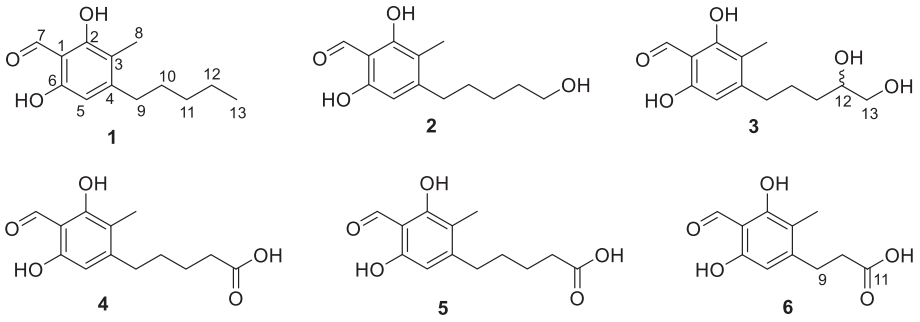

Fig. 1. Structures of new polyketides isolated from *A. javeedii*.

Table 1
^{1}H and ^{13}C NMR data for compounds **1**–**3** in methanol-d_4.

NO.	**1**[a]		**2**[a]		**3**[b]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type[c]	δ_H (J in Hz)
1	112.7, C		112.7, C		112.7, C	
2	165.2, C		165.3, C		165.3, C	
3	110.1, C		110.1, C		110.1, C	
4	147.9, C		147.8, C		147.6, C	
5	110.2, CH	6.24, s	110.3, CH	6.25, s	110.3, CH	6.26, s
6	165.0, C		165.3, C		165.2, C	
7	194.1, C	10.01, s	194.1, C	10.03, s	194.2, C	10.05, s
8	7.1, CH_3	1.99, s	7.1, CH_3	1.99, s	7.1, CH_3	1.99, s
9	32.5, CH_2	2.80, m	32.5, CH_2	2.82, m	32.5, CH_2	2.88, ddd (13.9, 9.5, 6.1) 2.81, ddd (13.9, 9.3, 6.2)
10	33.7, CH_2	1.61, m	33.8, CH_2	1.64, m	30.0, CH_2	1.80, m 1.67, m
11	32.7, CH_2	1.35, m	26.7, CH_2	1.44, m	34.0, CH_2	1.59, m 1.43, m
12	23.5, CH_2	1.36, m	33.4, CH_2	1.56, m	72.9, CH	3.59, m
13	14.3, CH_3	0.91, t (6.9)	62.8, CH_2	3.55, t (6.4)	67.4, CH_2	3.45, dd (11.1, 4.8) 3.42, dd (11.1, 6.3)

[a] Recorded at 300 (^{1}H) and 75 MHz (^{13}C).
[b] Recorded at 600 (^{1}H) and 150 MHz (^{13}C).
[c] Data extracted from HSQC and HMBC spectra.

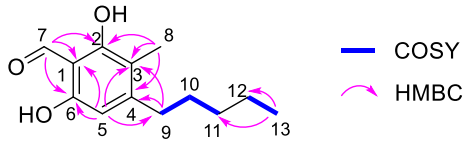

Fig. 2. COSY and key HMBC correlations for compound **1**.

the HMBC correlations from H-5 to C-9 (δ_C 32.5) and from H_2-9 to C-3, C-4, and C-5 indicated the location of the *n*-pentyl chain at C-4. The substitution of two hydroxy group at C-2 and C-6 of the benzene ring was suggested by the chemical shifts of C-2 and C-6 as well as the molecular formula of **1**. Thus, compound **1** was determined as 2,6-dihydroxy-3-methyl-4-pentylbenzaldehyde, for which the trivial name aplojaveediin A is proposed.

The molecular formula of **2** was determined as $C_{13}H_{18}O_4$ by the HRESIMS data, containing an additional oxygen atom when compared to **1**. The NMR data of **2** (Table 1) were similar to those of compound **1** except for the replacement of signals of the terminal methyl group in the side chain by signals of an oxygenated methylene resonating at δ_C 62.8 (C-13) and δ_H 3.55 (2H, t, J = 6.4 Hz, H_2-13). The COSY correlations between H_2-13/H_2-12 (δ_H 1.56), H_2-12/H_2-11 (δ_H 1.44), H_2-11/H_2-10 (δ_H 1.64), H_2-10/H_2-9 (δ_H 2.82) together with the HMBC correlations from H_2-13 to C-11 (δ_C 26.7) and C-12 (δ_C 33.4) indicated the location of a hydroxy group at C-13 in the side chain of **2**. Detailed analysis of the 2D NMR spectra of **2** revealed that the remaining substructure of **2** was identical to that of **1**. Thus, the structure of **2** was elucidated as shown.

Compound **3** has the molecular formula $C_{13}H_{18}O_5$ as deduced from the HRESIMS data, containing an additional oxygen atom when compared to **2**. Comparison of the NMR data of **2** and **3** (Table 1) suggested that they are structurally similar. The major difference is the observation of an additional oxygenated methine at δ_C 72.9 (C-12) and δ_H 3.59 (H-12) in **3**. Besides, the protons of the oxygenated methylene at C-13 appeared as two dd peaks in **3** instead of two triplet peaks in **2**. The above finding suggested the attachment of an additional hydroxy group at C-12, which was further confirmed by the COSY correlations between H_2-13/H-12/H_2-11/H_2-10/H_2-9. Due to the limited amount, the absolute configuration at C-12 of **3** was not determined.

Aplojaveediin D (**4**) was found to have the molecular formula $C_{13}H_{16}O_5$ on the basis of the HRESIMS data, accounting for six degrees of unsaturation. Its ^{1}H NMR data (Table 2) were similar to those of **1** but lacked signals of the terminal methyl group in the side chain. Meanwhile, the ^{13}C NMR spectrum of **4** exhibited the signal of one additional carbonyl carbon at δ_C 178.0 (C-13). The HMBC correlations from H_2-12 (δ_H 2.32, t, J = 6.5 Hz) to C-13, C-11 (δ_C 25.9), and C-10 (δ_C 33.3), together with the COSY correlations between H_2-12/H_2-11 (δ_H 1.67) and between H_2-10 (δ_H 1.66)/H_2-9 (δ_H 2.84) indicated a terminal carboxylic acid group in the side chain that replaced the methyl substituent of compound **1**. The remaining substructure of **4** was

Table 2
^{1}H and ^{13}C NMR data for compounds **4**–**6** in methanol-d_4.

NO.	**4**[a]		**5**[a]		**6**[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	112.8, C		112.7, C		112.7, C	
2	165.3, C		165.3, C		165.4, C	
3	110.1, C		110.2, C		110.2, C	
4	147.5, C		147.3, C		147.2, C	
5	110.2, CH	6.26, s	110.3, CH	6.25, s	110.1, CH	6.28, s
6	164.8, C		165.0, C		165.2, C	
7	194.2, C	10.04, s	194.1, C	10.02, s	194.3, C	10.07, s
8	7.1, CH_3	1.99, s	7.1, CH_3	1.99, s	7.1, CH_3	1.97, s
9	32.2, CH_2	2.84, t (7.0)	32.1, CH_2	2.83, t (7.3)	28.9, CH_2	3.10, t (7.8)
10	33.3, CH_2	1.66, m	33.2, CH_2	1.65, m	41.2, CH_2	2.46, t (7.8)
11	25.9, CH_2	1.67, m	25.7, CH_2	1.67, m	181.8, C	
12	35.0, CH_2	2.32, t (6.5)	34.4, CH_2	2.36, t (7.0)		
13	178.0, C		175.7, C			
13-OMe			52.0, CH_3	3.65, s		

[a] Recorded at 300 (^{1}H) and 75 MHz (^{13}C).
[b] Recorded at 600 (^{1}H) and 150 MHz (^{13}C).

identical to that of **1** as confirmed by detailed analysis of the 2D NMR spectra of **4**.

Compound **5** exhibited the molecular formula $C_{14}H_{18}O_5$ as determined by the HRESIMS data. The ^1H and ^{13}C NMR data of **5** were similar to those of **4** (Table 2). Analysis of the 2D NMR spectra revealed that both compounds shared the same benzene ring core structure. Compound **5** was identified as the C-13*O*-methyl derivative of **4**, as evident from the presence of an additional methoxy group at δ_H 3.65 (3H, s) and δ_C 52.0, together with the HMBC correlations from the protons of this additional methoxy group and H_2-12 (δ_H 2.36, t, J = 7.0 Hz) to the carbonyl carbon at δ_C 175.7 (C-13), and from H_2-12 to C-10 (δ_C 33.2) and C-11 (δ_C 25.7). Compound **5** could already be detected in the HPLC chromatogram of the crude fungal extract which argues for **5** being a natural product and not an artefact arising from **4** in the presence of MeOH. Moreover, incubation of **4** in MeOH for several days at room temperature failed to yield **5**.

The molecular formula of aplojaveediin F (**6**) was established as $C_{11}H_{12}O_5$ from the HRESIMS data, requiring six degrees of unsaturation. Comparison of the NMR data (Table 2) indicated compound **6** to be closely related to compound **4** except for that the side chain of **6** lacked two methylene groups when compared to **4**. The HMBC correlations from H_2-10 (δ_H 2.46, t, J = 7.8 Hz) to C-11 (δ_C 181.8) and C-4 (δ_C 147.2), and from H_2-9 (δ_H 3.10, t, J = 7.8 Hz) to C-11, C-3, C-4 and C-5 as well as the COSY correlations between H_2-10 and H_2-9 indicated the presence of a *n*-propanoic acid side chain at C-4 in **6**. Thus, the structure of **6** was elucidated as shown.

Compounds **1–6** were tested for their antibacterial activity against a panel of bacterial strains. Compound **1** exhibited moderate antibacterial activity against the sensitive *Staphylococcus aureus* strain ATCC 29213, the methicillin-resistant and vancomycin intermediate sensitive (MRSA/VISA) *S. aureus* strain ATCC 700699 and *Bacillus subtilis* (ATCC 169) with minimal inhibitory concentrations (MICs) of 50, 50 and 25 μM, respectively. Compound **6** also exhibited moderate antibacterial activity against *S. aureus* ATCC 29213 and ATCC 700699 with MICs of 25 and 50 μM, respectively. No or only a very weak antibacterial effect was observed for compounds **1** and **6** against the other tested bacterial strains (Table S1). Compounds **2–5** showed no antibacterial activity.

In addition, compounds **1–6** were tested for their antifungal activity against *Candida albicans* grown in the yeast or the hyphae form. While compounds **2–6** were inactive, compound **1** exhibited antifungal activity against the hyphae form of *C. albicans* strain ATCC 24433 with an inhibition diameter of 8 mm in the agar plate diffusion assay at a concentration of 1 mM. The compound was also active against the yeast *Saccharomyces cerevisiae* resulting in an inhibition diameter of 18 mm (Fig. S44). The MIC of compound **1** against the hyphae form of *C. albicans* strain ATCC 24433 in liquid medium was 100 μM as determined by the microbroth dilution assay. Moreover, compound **1** showed no substantial cytotoxicity against the three tested human cell lines (HUH7, THP-1, CLS-54) up to a concentration of 100 μM (Fig. 3). As an extension of the antifungal assay, a time-kill assay was performed (Fig. 4). Incubation of cells of the hyphae form of *Candida albicans* strain ATCC 24,433 with compound **1** at 400 μM (=4-fold MIC) resulted in a rapid decrease of viability by 3.5-log over a period of 6 h, after which a plateau was reached. In contrast, the positive control hygromycin B (474 μM = 4-fold MIC), which has antifungal activity against *C. candida*,[16,17] exhibited only a largely static growth inhibitory effect (Fig. 4). This finding highlights the fungicidal property of compound **1**. When comparing the antifungal activity of compounds **1–6**, it is obvious that addition of polar groups to the side chain (**2–5**) as well as shortening of the side chain (**6**) weakens the antifungal activity, which might be due to hindered uptake by the fungus. Based on its fungicidal activity and lack of cytotoxicity against human cells, compound **1** could be a promising candidate for the development of new antifungal agents.

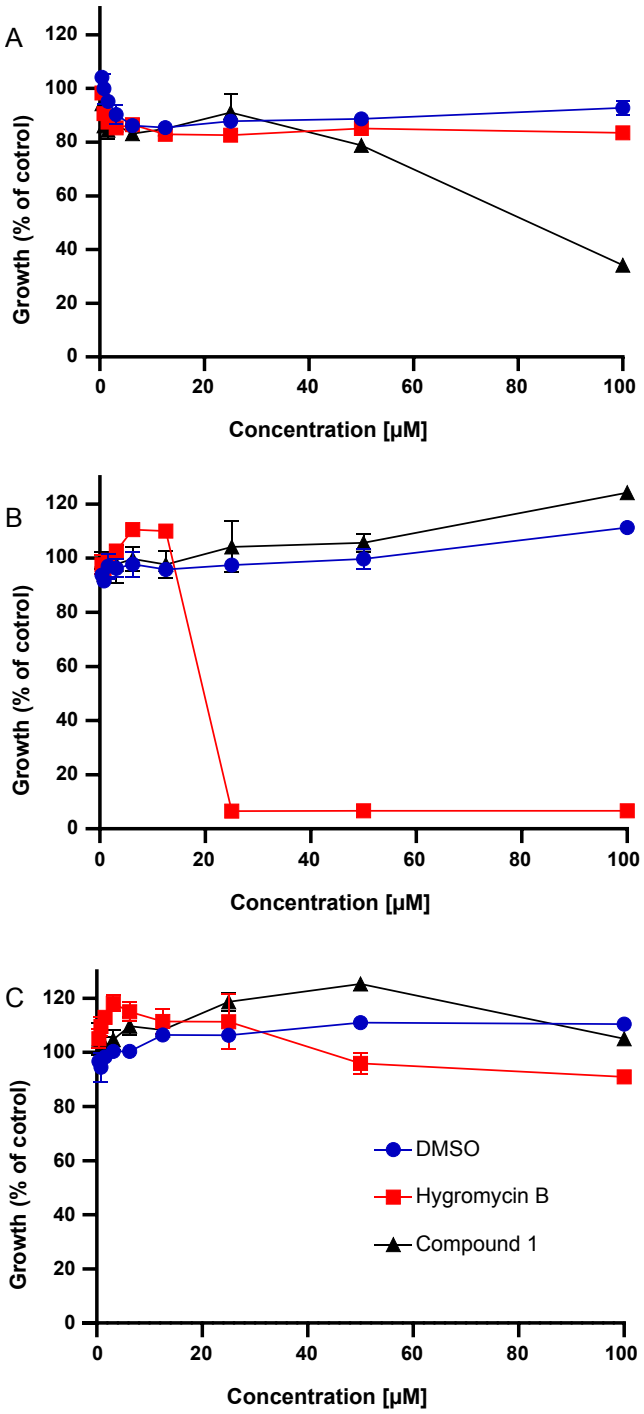

Fig. 3. Evaluation of cytotoxicity of compound **1** against different human cell lines. (A) Effect against the human liver cell line HUH7, (B) the human lung epithelial cell line CLS-54, and (C) the human monocytic leukemia cell line THP-1. DMSO was used as solvent control, the antifungal compound hygromycin B as reference. Data represent means from two replicates ± standard error.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Perkin-Elmer-241 MC polarimeter. NMR spectra were recorded at 25 °C on Bruker ARX 300 or 600 NMR spectrometers. Chemical shifts were referenced to the solvent residual peaks. Mass spectra (ESI) were recorded with a Finnigan LCQ

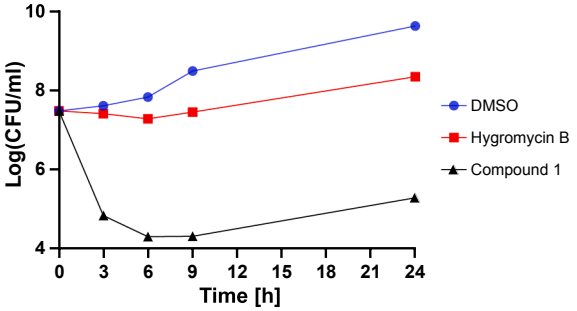

Fig. 4. Time-kill curve of compound **1** (400 μM, black) against the hyphae form of *Candida albicans* strain ATCC 24433. The antifungal compound hygromycin B (474 μM, red) was used as positive control; DMSO (blue) was used as the solvent control. Colony forming units (CFU) were quantified after the indicated time points of incubation. The medium was replaced after 6 h incubation with fresh medium containing compounds at the initial concentration to avoid effects by potential compound degradation.

Deca mass spectrometer while HRESIMS were recorded with a UHR-QTOF maXis 4G (Bruker Daltonics) mass spectrometer. HPLC analysis was performed with a Dionex UltiMate-3400SD system with a LPG-3400SD pump and a photodiode array detector (DAD 3000RS). The analytical column (125 × 4 mm) was prefilled with Eurosphere-10 C_{18} (Knauer, Germany). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L7400; pump L7100; Eurosphere-100 C_{18}, 300 × 8 mm, Knauer, Germany). Normal phase column chromatography included Merck MN silica gel 60 M (0.04–0.063 mm) or Sephadex LH-20. TLC plates precoated with silica gel F_{254} (Merck, Germany) were used to monitor fractions following column chromatography with UV detection at 254 and 366 nm or by spraying the plates with anisaldehyde reagent followed by heating. Distilled and spectral grade solvents were used for column chromatography and spectroscopic measurements, respectively.

3.2. Fungal material and identification

The endophytic fungus was isolated from fresh, healthy stems of *O. violaceus* (L.) O. E. Schul (*Brassicaceae*), which were collected in April 2018 around Beijing, China. After 70% ethanol surface sterilization, the disinfected stems were dissected into small pieces of 0.5 cm length and placed on the fungal isolation medium (malt agar medium). The isolation of the fungal strain was achieved according to a standard procedure as described before.[18] It was identified as *Aplosporella javeedii* according to the DNA amplification and sequencing of the ITS region as described previously.[19] The sequence data were submitted to GenBank with the accession number MN720704. The fungal strain is kept in the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Duesseldorf, Germany, with the ID code ZGB-B.

3.3. Cultivation, extraction and isolation

The fungus was cultivated on solid rice medium (100 g rice and 110 mL demineralized water) in ten Erlenmeyer flasks (1 L each). After autoclaving at 121 °C for 20 min and cooling down to room temperature, the fungal strain was added and cultivated for 20 days. After the fungus had completely overgrown the medium, the culture was extracted with 800 mL EtOAc followed by evaporation of the extract to dryness. The obtained brown extract (10.5 g) was subjected to a silica gel vacuum liquid chromatography column (VLC), using solvents in a gradient of increasing polarity (*n*-hexane, EtOAc, CH_2Cl_2, MeOH) to yield 12 fractions (V1 to V12). Fraction V3 (1.5 g) was subjected to a silica gel column with a gradient of *n*-hexane and EtOAc (20:1 to 0:100), affording eight subfractions (V3-S1 to V3-S8). Subfraction V3-S2 (80.2 mg) was purified by semi-preparative HPLC using MeOH-H_2O (70:30 to 100:0) to give **1** (8.3 mg). Fraction V4 (1.1 g) was also separated on a silica gel column with a *n*-hexane-EtOAc gradient (20:1 to 0:100), affording ten subfractions (V4-S1 to V4-S10). Subfraction V4-S2 (51.1 mg) was purified by semi-preparative HPLC using MeOH-H_2O (30:70 to 70:30) to give **5** (4.3 mg). Fraction V5 (235.9 mg) was subjected to a Sephadex LH-20 column using CH_2Cl_2-MeOH (1:1) as eluent to obtain seven subfractions (V5-S1 to V5-S7). Subfraction V5-S4 (27.8 mg) was purified by semi-preparative HPLC using MeOH-H_2O (30:70 to 70:30) to give **2** (3.6 mg) and **4** (10.5 mg). Fractions V7 (206.4 mg) and V8 (100.2 mg) were combined and further fractionated using a Sephadex LH-20 column with CH_2Cl_2-MeOH (1:1) as eluent to give four subfractions (V7-S1 to V7-S4). Subfraction V8-S3 (119.6 mg) were subjected to a silica gel column with a CH_2Cl_2-MeOH gradient (20:1 to 0:100), followed by purification with semi-preparative HPLC using MeOH-H_2O (10:90 to70:30) as mobile phase to give **3** (2.0 mg). Fraction V9 (1.0 g) was separated on a Sephadex LH-20 column using CH_2Cl_2-MeOH (1:1) to yielded six subfractions (V9-S1 to V9-S6). Subfraction V9-S4 (18.2 mg) was further purified by semi-preparative HPLC using MeCN-H_2O (10:90 to 20:80) to give **6** (1.5 mg).

Aplojaveediin A (**1**): Colorless crystal; UV (MeOH) λ_{max} 208, 220 and 298 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M+H]^+$ *m/z* 223.1332 (calcd for $C_{13}H_{19}O_3$ 223.1334), $[M-H]^-$ *m/z* 221.1178 (calcd for $C_{13}H_{17}O_3$ 221.1178).

Aplojaveediin B (**2**): Brown homogeneous oil; UV (MeOH) λ_{max} 218 and 301 nm; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS $[M+H]^+$ *m/z* 239.1280 (calcd for $C_{13}H_{19}O_4$ 239.1283), $[M-H]^-$ *m/z* 237.1130 (calcd for $C_{13}H_{17}O_4$ 237.1127).

Aplojaveediin C (**3**): Brown homogeneous oil; $[\alpha]_D^{20}$ + 6 (*c* 0.2, MeOH); UV (MeOH) λ_{max} 206, 220 and 297 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M-H]^-$ *m/z* 253.1084 (calcd for $C_{13}H_{17}O_5$ 253.1076).

Aplojaveediin D (**4**): Colorless crystal; UV (MeOH) λ_{max} 220 and 300 nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M-H]^-$ *m/z* 251.0923 (calcd for $C_{13}H_{15}O_5$ 251.0919).

Aplojaveediin E (**5**): Colorless crystals; UV (MeOH) λ_{max} 208, 220 and 298 nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M-H]^-$ *m/z* 265.1082 (calcd for $C_{14}H_{17}O_5$ 265.1076).

Aplojaveediin F (**6**): White amorphous solid; UV (MeOH) λ_{max} 220 and 297 nm; ^1H and ^{13}C NMR data, see Table 2; HRESIMS $[M-H]^-$ *m/z* 223.0607 (calcd for $C_{11}H_{11}O_5$ 223.0606).

3.4. Antibacterial assay

The antibacterial activities were tested by calculating the MICs against *Mycobacterium tuberculosis* H37Rv, *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 700699, *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *E. faecium* ATCC 35667, *E. faecium* ATCC 700221, *Bacillus subtilis* ATCC 169 and *Escherichia coli* ATCC 25922. The MIC values were determined by the broth microdilution method following the recommendation of the Clinical and Laboratory Standards Institute (CLSI).[20]

3.5. Antifungal assay

Compounds were tested against the nosocomial pathogen *Candida albicans* both grown in the yeast form and the hyphae form. The microbroth dilution method was done as recommended by CLSI guidelines.[20] *Candida albicans* was inoculated in YPD medium (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L) and incubated at 37 °C with shaking at 180 rpm overnight to obtain the yeast form. For the hyphae form, YP + Proline medium (yeast extract 10 g/L, peptone 20 g/L, proline 20 g/L) was used, and cells were incubated at 30 °C with shaking at 60 rpm overnight. Afterwards, cells were seeded at a density of 1 × 10^6 CFU/mL in 96 well microplate containing two fold serial dilutions of compounds at a concentration ranging from 100 to 0.78 μM in a total volume 100 μL. DMSO at a maximal concentration of 1% was

used as solvent control, while hygromycin B served as antifungal positive control.[16,17] The plates were incubated at 37 °C overnight aerobically as static cultures before being evaluated macroscopically. All tests were repeated twice.

The disc diffusion method was used as an additional sensitivity test. Briefly, a preculture of *Candida albicans* (hyphae form) was adjusted to 2×10^8 CFU/mL. Subsequently, 100 µL culture aliquots were plated out on the surface of YP + Proline agar plates. Then, 5 µL of compound **1** (1 mmol/L, 10MIC) was spotted onto a sterile filter disc. Hygromycin B (3.12 µg in 5 µL, 10MIC) was used as positive and DMSO (5 µL) as negative control. The plates were incubated at 30 °C overnight aerobically. Subsequently, the inhibition zones were measured by a caliper. All tests were repeated once.

3.6. Cytotoxicity assay

Cytotoxicity studies were conducted with three human cell lines THP-1 (human monocytic leukemia cell line), CLS-54 (human lung epithelial cell line), and HUH7 (liver cell line). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 for 5 days. Afterwards, the cells were suspended and adjusted to a density of 1×10^6 cells/ml. For the adherent cell lines HUH7 and CLS-54, prior trypsinization was done for cell detachment. Cells were then seeded into a 96-well plate in a total volume of 100 µL containing 2-fold serial dilutions of the tested compounds in a concentration ranging from 100 to 0.78 µM. DMSO and hygromycin B were used as negative and positive controls, respectively. After 48 h incubation at 37 °C in a humidified atmosphere of 5% CO_2, 10 µL resazurin solution (100 µg/mL) was added to each well and incubated for a further 4 h. A microplate reader (excitation 545 nm, emission 590 nm) was used to measure the fluorescence. Residual growth was calculated relative to uninoculated (0% growth) and untreated (100% growth) controls, respectively.

3.7. Determination of time-kill kinetic

Time-kill kinetic was tested for compound **1** against the hyphae form of *Candida albicans*. A preculture grown in YP + Proline medium was adjusted to a density of 3×10^7 CFU/ml and split into three aliquots, which were treated either with 4-fold MIC (400 µM) of compound **1**, hygromycin B (474 µM) as an antifungal positive control or DMSO as the solvent control. After 0, 3, 6, 9 and 24 h incubation, 100 µL culture aliquots were taken and plated on YPD agar plates, and CFU were quantified after overnight incubation at 30°C aerobically. In order to avoid the potential degradation of compounds, the medium was removed after 6 h incubation by centrifuging at 4000 rpm for 10 min and replaced with an equal volume of fresh medium containing the respective compound at the initial concentration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115456.

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