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A-*seco*-oleane-type triterpenes from *Phomopsis* sp. (strain HKI0458) isolated from the mangrove plant *Hibiscus tiliaceus*

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Abstract

From the fermentation broth of an unidentified *Phomopsis* sp. (strain HKI0458) isolated from the mangrove plant *Hibiscus tiliaceus*, four A-*seco*-oleane-type triterpenes, namely 3,4-*seco*-olean-11,13-dien-4,15α, 22β,24-tetraol-3-oic acid (**1**), 3,4-*seco*-olean-11,13-dien-4,7β,22β,24-tetraol-3-oic acid (**2**), 3,4-*seco*- olean-13-en-4,7,15,22,24-pentaol-3-oic acid (**3**), and 3,4-*seco*-olean-13-en-4,15,22,24-tetraol-3-oic acid (**4**) were obtained. Their structures were elucidated by extensive spectroscopic (UV, IR, FABMS, and 2D NMR) data analyses.

Keywords: Fungus; *Phomopsis* sp.; Mangrove plant; *Hibiscus tiliaceus*; Malvaceae; A-*seco*-oleane-type triterpenes; Spectroscopic analyses

1. Introduction

Mangrove forests are distributed in most tropical and subtropical coast regions of the world, with several mangrove species being a valuable source of useful metabolites for medicinal usage (Miles et al., 1998). Some of the potency of mangrove plants may be due to mutualistic fungal endophytes associated with host plants. In fact fungi from mangroves are the second largest group among marine fungi (Manoharachary et al., 2005). In general, the production of secondary metabolites that are potentially useful for pharmaceutical and agricultural applications is widespread among endophytic fungi (Petrini et al., 1992). Our previous investigation of endophytic microorganisms from mangrove plants resulted in a number of bioactive and structurally unique metabolites (Guan et al., 2005). In the course of a continuous investigation of natural products for drug oriented lead compound discovery and the metabolic relationship between mangrove plants and their endophytes, a fungus *Phomopsis* sp. (HKI0458) was isolated from mangrove *Hibiscus tiliaceus* (L.), collected from a mangrove forest in Dongzaigang, Hainan province of China. *H. tiliaceus* L. (Malvaceae) is used in folk medicine for treating fevers, to soothe coughs, dysentery, and ear infections (Ali et al., 1980). A primary chemical examination of this plant indicated that the main components were oleane-type triterpenes, with these co-occurring with the friedelin-type (Li et al., 2006). An examination of the fermentation broth of the *Phomopsis* sp. (HKI0458) resulted in the isolation and characterization of four new A-*seco*-oleane-type triterpenes (**1**–**4**). Their structures were elucidated by spectroscopic data analyses.

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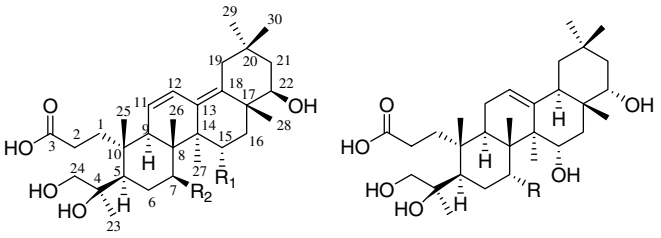


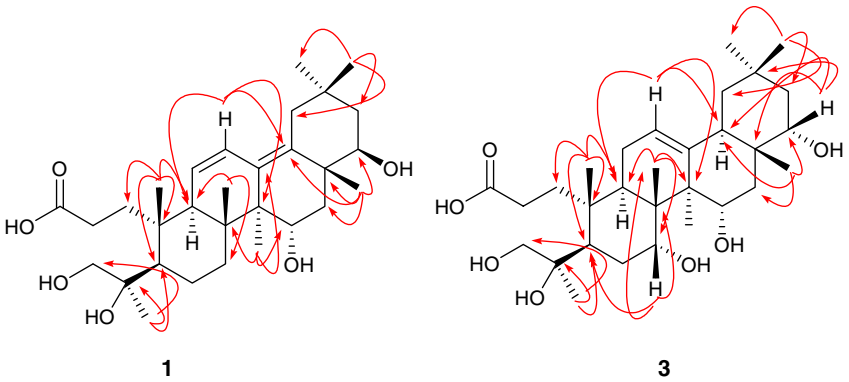
1. $R_1 = OH$, $R_2 = H$
2. $R_1 = H$, $R_2 = OH$

3. R = OH
4. R = H

2. Result and discussion

Isolate HKI0458 has been assigned to *Phomopsis* sp. based on the formation of two types of one-celled and hyaline conidia namely filiform, hamate macroconidia and ellipsoidal microconidia, the simple or sparingly branched conidiophores bearing long, tapering phialides, and dark brown eustromatic conidiomata. Comparison of the internal transcribed spacer sequence of nuclear ribosomal DNA (ITS) confirmed the identification based on morphological features. The ITS sequence of strain HKI 0458 (GenBank accession no. EU046615) matched with an ITS sequence of *Phomopsis loropetali* (GenBank Accession No. AY601917, 99% identity), and an ITS sequence of *Phomopsis phyllanthicola* (GenBank Accession No. AY620819, 99% identity).

The molecular formula of **1** was determined as $C_{30}H_{48}O_6$ from HRFABMS and NMR spectroscopic data, indicating seven degrees of unsaturation. The IR absorptions at 3418 and 1709 cm^{-1} suggested the presence of carbonyl and hydroxy groups which was supported by the ^{13}C NMR signals at δ_C 177.1 (s), 77.3 (s), 76.5 (d), 68.7 (t), and 65.5 (d) for one carbonyl and four hydroxylated carbons. The ^1H NMR spectrum exhibited seven methyl singlets at δ_H 0.68 (s, H_3-26), 0.71 (s, H_3-29), 0.87 (s, H_3-27), 0.91 (s, H_3-25), 0.93 (s, H_3-30), 0.94 (s, H_3-28), and 1.16 (s, H_3-23), which are characteristic of an oleane-type skeleton. The ^{13}C NMR and DEPT spectra displayed altogether 30 carbons, including four olefinic signals at δ_C 126.6 (d, C-12), 127.8 (d, C-11), 135.4 (s, C-13), and 137.6 (s, C-18), and the HMQC spectrum permitted assignment of all attached protons. Since three of the seven elements of unsaturation were attributed to two vinyl and one carbonyl groups, the molecule was thus assumed to contain a tetracyclic nucleus. The HMBC correlations traced from the methyl and olefinic protons (Fig. 1) enabled establishment of hexa-numbered cyclic rings B to E of an oleane backbone. Thus, ring A was considered to be cleaved. The HMBC correlations observed between carbons at δ_C 38.0 (t, C-19), 32.7 (s, C-20), 44.2 (t, C-21) and H_3-29 and H_3-30, respectively, along with the HMBC correlations between a proton at δ_H 3.21 (dd, $J = 5.0$, 12.0 Hz, H-22) and C-20 and C-21, indicated a hydroxy group to be located at C-22. This was further supported by HMBC correlations from H_3-28 to C-16 (δ_C 43.7, t), C-18, C-17 (δ_C 42.6, s), and C-22 (δ_C 76.5, d) (Fig. 1). Moreover, the HMBC correlations between H_3-27 and C-8 (δ_C 48.5, s), C-14 (δ_C 41.8, s), and C-15 (δ_C 65.5, d), between H_3-26 and C-7 (δ_C 35.3, t), C-9 (δ_C 46.3, d), C-8, and C-14, and between H-11 (δ_H 6.30, d, $J = 10.5$ Hz) and C-9, C-8, and C-13 allowed determination of two double bonds at C-11/C-12 and C-13/C-18 and a second hydroxy group at C-15. The methyl group H_3-25 was established to be located at C-10, as in other oleane-type compounds, from the HMBC correlations. The HMBC correlations of the remaining methyl singlet (H_3-23) with carbons at δ_C 68.7 (t, C-24), 77.3 (s, C-4), and 49.0 (d, C-5) and, in turn, the geminal protons at δ_H 3.36 and 3.29 (2H, d, $J = 11.0$ Hz, H-24) correlating to C-4, C-5, and C-23 (δ_H 27.2, q), indicated the presence of a 1,2-dihydroxyisopropyl unit to be linked to C-5. The remaining moiety was attributed to a propionic acid constituent, as evidenced by the DQFCOSY correlation between H_2-1 (δ_H 2.24, ddd; 1.78, m) and H_2-2 (δ_H 2.38, ddd; 1.97, ddd), and the protons of both methylenes showing a HMBC correlation with a carbonyl carbon at δ_C 177.1 (s, C-3). Accordingly, this moiety was assumed to be positioned at C-4, which was further supported by the HMBC relationship between H_3-25 and C-1 (δ_C 34.7, t). The relative stereochemistry of **1** was mainly assigned through NOESY data. The NOE effects between H_3-29/



1 **3**

Fig. 1. Key HMBC correlations of **1** and **3** (H→C).

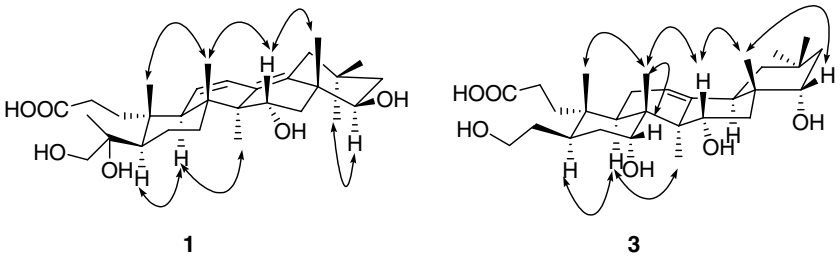


Fig. 2. Key NOE correlations of **1** and **3**.

H-22, H-15 (δ_H 3.85, dd, $J = 4.0$, 12.0 Hz)/H_3-26, H_3-28/H-15, H_3-26/H_3-25, H-9 (δ_H 2.04, brs)/H_3-27, and H-9/H-5 (δ_H 1.43, m) (Fig. 2), in association with the axial-axial and axial-equatorial coupling of H-15 (dd, $J = 4.0$, 12.0 Hz) to H_2-16, and H-22 (dd, $J = 5.0$, 12.0 Hz) to H_2-21, were in agreement with β-orientation of H-15, H_3-25, H_3-26, and H_3-28, while H-5, H-9 and H-22 to be α-oriented. Thus, structure **1** was determined to be 3,4-*seco*-olean-11,13-dien-4,15α, 22β,24- tetraol-3-oic acid.

The molecular formula of **2** was determined to be the same as that of **1** through analysis of HRFABMS and NMR spectroscopic data. The ^{1}H and ^{13}C NMR data of **2** (Table 1) closely resembled those of **1**, indicating that **2** shared the same basic skeleton of a 3,4-*seco*-olean-11,

Table 1
The ^{1}H and ^{13}C NMR spectroscopic data of **1** to **4** in DMSO-d_6

	1		**2**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	34.7	1.78 m 2.24 ddd 4.0,11.0,14.0	35.0	1.66 m 2.14, m	35.3	1.48 m 2.06 m	35.5	1.48 m 2.10 m
2	29.9	1.97 ddd 4.0, 11.0,14.5 2.38 ddd 4.5,11.0,14.5	30.0	1.98 m 2.35 m	30.0	2.04 m 2.48 m	30.1	1.96 m 2.40 m
3	177.1		175.4		176.6		176.7	
4	77.3		76.1		77.1		77.1	
5	49.0	1.43	46.3	1.61 m	41.6	1.96 brd 12.5	49.9	1.35 m
6	21.6	1.45 m 1.56 m	32.1	1.58 m 1.65 m	29.9	1.50 brd 13.5 1.80 dd 12.5,13.5	21.6	1.34 m 1.44 m
7	35.3	1.40 dd 12.0, 13.5 1.58 m	70.9	3.50 dd 4.5, 12.0	74.3	3.71 br	36.2	1.43 m 1.74 m
8	48.5		45.8		44.1		41.4	
9	46.3	2.04 brs	45.4	1.95 brs	35.8	2.18 dd 8.5, 9.0	39.7	1.62 dd 6.0, 11.0
10	40.1		39.8		41.0		41.3	
11	127.8	6.30 d 10.5	127.0	6.35 d 10.5	24.4	1.86 m	24.8	1.80 m
12	126.6	5.52 d 10.5	125.5	5.50 d 10.5	123.3	5.15, br	124.3	5.28 s
13	135.4		135.4		149.5		146.3	
14	41.8		43.9		49.5		49.1	
15	65.5	3.85 dd 4.0, 12.0	28.0	1.40 m 1.78 m	66.9	4.05, dd, 5.5, 11.5	67.0	3.92, dd 4.5, 11.0
16	43.7	1.42 dd 12.0,12.5 1.75 dd 4.0, 12.5	33.8	1.36 m 1.66 m	38.3	1.28 dd, 5.5, 13.0 1.69 dd 11.5, 13.0	40.1	1.24 m 1.63 m
17	42.6		40.2		38.4		38.5	
18	137.6		138.0		44.3	2.00 brd 13.0	46.5	1.96 brd 13.5
19	38.0	1.63 d 14.0 2.24 d 14.0	37.7	1.62 d 13.5 2.35 d 13.5	47.8	0.92 brd 14.5 1.63 dd 13.0, 14.5	46.6	0.94 brd 14.5 1.68 dd 13.5, 14.5
20	32.7		32.5		31.3		31.3	
21	44.2	1.30 dd 5.0, 12.0 1.37 dd 12.0,12.0	44.4	1.30 dd 4.5,12.0 1.36 dd 12.0,12.0	42.1	1.32 dd 3.0, 12.0 1.33 dd 4.0, 12.0	42.2	1.26 dd 2.5 14.0 1.31 dd 3.0 14.0
22	76.5	3.21 dd 5.0, 12.0	76.1	3.20 dd 4.5, 12.0	74.8	3.21 dd 3.0, 4.0	74.7	3.23 dd 2.5, 3.0
23	27.2	1.16 s	28.0	1.12 s	27.9	1.15 s	27.6	1.16 s
24	68.7	3.29 d 11.0 3.36 d 11.0	68.5	3.30 d 10.5 3.38 d 10.5	68.2	3.24 d, 11.0 3.32 d 11.0	68.5	3.30 d 10.5 3.33 d 10.5
25	22.9	0.91 s	22.3	0.87 s	20.7	1.00 s	21.3	1.01 s
26	17.6	0.68 s	11.8	0.64 s	20.0	0.85 s	18.2	0.97 s
27	15.2	0.87 s	20.9	0.94 s	25.1	1.34 s	19.7	1.03 s
28	19.7	0.94 s	18.7	0.91 s	22.4	0.81 s	22.3	0.79 s
29	26.0	0.71 s	25.9	0.72 s	29.2	0.98 s	29.4	0.96 s
30	33.1	0.93 s	32.9	0.94 s	34.2	0.83 s	33.8	0.85 s

13-dien-3-oic acid. However, the hydroxylated methine at C-15 of **1** was replaced by a methylene group of **2**, which was evidenced by the HMBC correlations between H_3-27 (δ_H 0.94, s) and δ_C 28.0 (t, C-15). In turn, an additional hydroxylated methine signal observed at δ_H 3.50 (dd, $J = 4.5$, 12.0 Hz) and δ_C 70.9 (d) was assignable to C-7 through the HMBC correlations between H_3-26 (δ_H 0.64, s) and C-7, C-8 (δ_C 45.8, s), C-9 (δ_C 45.4, d), and C-14 (δ_C 43.9, s). The relative configuration of H-7 was determined to be α-oriented on the basis of NOESY correlations between H-9 (δ_H 1.95, brs) and H-7, and between H-7 and H_3-27 (δ_H 0.94, s), as well as axial–axial and axial-equatorial couplings of H-7 (dd, $J = 4.5$, 12.0 Hz). The remaining relative stereochemistry was identical to that of **1** due to the similar NOE correlations. Accordingly, structure **2** was identified as 3, 4-*seco*-olean-11,13-dien-4,7β,22β,24-tetraol-3-oic acid.

The molecular formula of **3** was determined as $C_{30}H_{50}O_7$ from HRFABMS, indicating six degrees of unsaturation. The IR absorptions at 3386 and 1709 cm^{-1} suggested the presence of carbonyl and hydroxy groups. The NMR spectroscopic data of **3** were similar to those of **1**, characteristic of a 3, 4-*seco*-olean-3-oic acid nucleus. However, the ^{13}C NMR spectrum indicated only two olefinic carbons at δ_C 123.3 (d) and 149.5 (s) for a trisubstituted double bond, but five hydroxylated carbons at δ_C 77.1 (s), 74.3 (d), 66.9 (d), 74.8 (d), and 68.2 (t). The HMBC correlations between H_3-27 (δ_H 1.34, s) and C-8 (δ_C 44.1, s), C-13 (δ_C 149.5, s), C-14 (δ_C 49.5, s), and C-15 (δ_C 66.9, d) indicated the presence of a double bond at C-12/C-13, and a hydroxy group at C-15. With exception of two hydroxylated carbons at δ_C 77.1 (s) and 68.2 (t) assignable to C-4 and C-24 as indicated in **1** and **2**, there were still two oxygenated carbons left at δ_C 74.3 (d) and 74.8 (d). The similar HMBC correlations traced from methyl protons H_3-26 (δ_H 0.85, s) and H_3-28 (δ_H 0.81, s) as those of **1** and **2** permitted the locations of the remaining two hydroxy groups at C-7 and C-22. The relative stereochemistry of **3** was determined mainly through analysis of NOESY data and coupling constants. The NOESY correlations between H_3-28/H-22 (δ_H 3.21, dd, $J = 3.0$, 4.0 Hz), H-7 (δ_H 3.71, brs)/H_3-26, H-15 (δ_H 4.05, dd, $J = 5.5$, 11.5 Hz)/H_3-26, H-7/H-15, H_3-27 (δ_H 1.34, s)/H-9 (δ_H 2.18, dd), and H-9/H-5 (δ_H 1.96, d) (Fig. 2) suggested H-7, H-15, and H-22 to be in β-configuration, while H-5, H-9, and H_3-27 were in α-face. The small coupling constants of H-7 (br) and H-22 (dd, $J = 3.0$, 4.0 Hz) were indicative of equatorial-equatorial and equatorial-axial coupling, whereas H-15 was in agreement with axial–axial and axial-equatorial coupling due to the J value ($J_{H\text{-}15/H\text{-}16} = 5.5$, 11.5 Hz). The structure of **3** was thus determined to be 3,4-*seco*-olean-12-en-4,7α,15α, 22α,24-pentaol-3-oic acid.

The molecular formula of **4** was determined as $C_{30}H_{50}O_6$ from HRFABMS, being 16 amu less than that of **3**. The ^1H and ^{13}C NMR spectroscopic data of **4** (Table 1) closely resembled those of **3**, with exception of a hydroxylated methine group at C-7 in **3** being replaced by a methylene carbon δ_C 36.2 (t, C-7) of **4**. This was evidenced by HMBC correlations between H_3-26 (δ_H 0.97, s) and C-7, C-8 (δ_C 41.4, s), C-9 (δ_C 39.7, d), and C-14 (δ_C 49.1, s). The similar HMBC relationship of both **4** and **3** indicated that the four hydroxylated carbons at δ_C 77.1 (s), 67.0 (d), 74.7 (d), and 68.5 (t) of **4** were located at C-4, C-15, C-22, and C-24, respectively. The relative stereochemistry of H-15 and H-22 was identical to that of **3** due to similar NOE correlations and coupling constants. Therefore, structure **4** was identified as 3,4-*seco*-olean-12-en-4,15α, 22α,24-tetraol-3-oic acid.

3. Concluding remarks

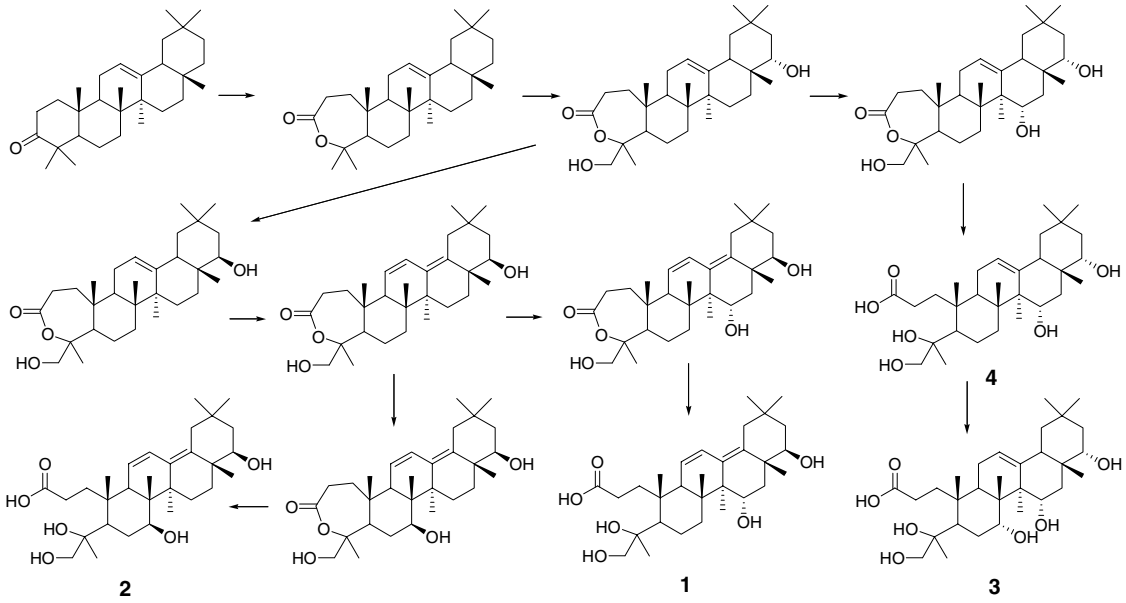
A-*seco*-oleane-type triterpenoids are a group of unique structures mainly discovered from some medicinal plants such as *Ligularia intermedia* (Ma et al., 1997), *Sandoricum koetjape* (Kosela et al., 1995; Soleh et al., 1995), *Maytenus undata* (Muhammad et al., 2000), genus *Junellia* (Caldwell et al., 2000), and also co-occur in other plants which could produce oleane derivatives (Kaneda et al., 1992; Ismail et al., 2003; Baas, 1983; Klinto et al., 1973). Some of the A-*seco*-oleane-type derivatives with 3,28-dioic acid showed cytotoxic (Yolanda et al., 2001), antibacterial, and anti-inflammatory (Caldwell et al., 2000) activities. Compounds **1** and **4** were evaluated in our general bioactivity profiling program, e.g. antimicrobial, cytotoxic and antiviral testing, as well as various enzyme assays. They only showed very weak cytotoxic activity ($IC_{50} > 50$ μg/mL).

Oleane-type triterpenes are rarely found from fungi, but were frequently isolated from terrestrial plants. This is the first report to find A-*seco*-triterpenes from a microbial source. Shirane et al. (1996) and Laskin et al. (1964) reported that some fungi could convert oleane-type triterpenes into A-*seco*-oleanes, but there is no oleane reported from microbial sources. The endophytic *Phomopsis* sp. might have obtained the biosynthetic machinery from the oleane skeleton from the host plant during evolution. We suggest ring A cleavage and hydroxylation biosynthetic steps as shown in Scheme 1. The structurally related A-*seco*-oleanes in a mangrove endophyte may play an important role to protect the host plant against environmental infections.

4. Experimental

4.1. General

Optional rotations were measured on a JASCO DIP-370 polarimeter. IR spectra were recorded on a Perkin-Elmer Nicol FT-50X spectrometer. NMR spectra were measured on a Varian 300 (1D) and Varian 500 (2D) without TMS as internal standard. HRFABMS spectra were obtained on a VG Autospec spectrometer. Column chromatography was



Scheme 1. Possible biotransformation from oleane precursor in *Phomopsis* sp.

carried out on Merck silica gel (200–400 mesh), and the HF_{254} silica gel for TLC was provided by Sigma Co. Ltd. Sephadex LH-20 (18–110 cm) were obtained from Pharmacia Co. High pressure liquid chromatography (HPLC) was performed on an Alltech-426 apparatus using Kromasil prepack column (ODS, 10 × 250 mm, for reverse-phase) and monitored by UV detection.

4.1.1. Strain isolation and taxonomy

The strain HKI0458 was isolated from a leaf of the mangrove plant *Hibiscus tiliaceus*, which was collected at Dongzaigang, Province Hainan of China, in January 2003, and authenticated by Prof. P. Lin from Xiamen University, China. A voucher sample of the plant (MP-057) is deposited at the State Key Laboratory of Natural and Biomimetic Drugs, Peking University. The fungal isolate is deposited as HKI0458 in the strain collection of the Leibniz-Institute for Natural Products Research and Infection Biology, Kans-Knöll-Institute (HKI; Jena, Germany).

For microbial isolation, freshly harvested leaves were rinsed with sterile H_2O, sterilized by soaking in 70% EtOH-H_2O (7:3, v/v, 1 min) and 3.5% NaOCl-solution (3 min) and rinsed again with sterile H_2O. Tiny pieces obtained by cutting with a sterile knife were placed on agar plates with ISP2 medium, which was prepared with artificial sea salt solution (Sigma S-9883, 34 g/L) and supplemented with nalidixic acid (0.5 g/L), streptomycin (0.1 g/L), and chloramphenicol (0.2 g/L), and incubated at 22 °C until growth appeared. Fungal colonies were transferred to fresh agar plates for further growth. General laboratory cultivation was performed on malt agar (malt extract 20.0 g/L, yeast extract 2.0 g/L, glucose 10.0 g/L, $(NH_4)_2HPO_4$ 0.5 g/L, pH 6.0) or the respective liquid medium at 22 °C. For long term preservation, cultures grown on agar plates supplemented with 5% glycerol were maintained in the vapour phase of liquid N_2. The fungal isolate (HKI0458) is deposited in the strain collection of the Leibniz-Institute for Natural Products Research and Infection Biology.

The fungus was taxonomically assigned as *Phomopsis* sp. (Teleomorph: *Diaporthe*, Diaporthales, Ascomycetes) on the basis of macro- and micromorphological criteria. On malt extract agar, the strain produces a luxuriant white to cream-coloured and felty aerial mycelium consisting of septate, branched, hyaline, and guttulate hyphae that are 0.8–10 μm in diameter. After 3–4 weeks, the mycelium develops dark brown, spherical, separate or aggregated eustromatic conidiomata. They arise superficial or semi-immersed either from the normal mycelium or from dark brown areas and reach up to 1 mm in diameter. The conidiophores are simple or sparingly branched at the base and bear long, tapering phialides that form two types of hyaline and one-celled conidia. The macroconidia are filiform, curved or hamate, 15–36 × 1–1.5 μm in size, and eguttulate, while the microconidia are ellipsoidal, mostly attenuated towards the base, 5–10 × 1.5–2.5 μm in size, and guttulate (often biguttulate). The conidial slime leaking from the conidiomata is creamy white.

Extraction of genomic DNA from the pure culture of strain HKI 0458 was done according to the extraction method 1 described in Burik et al. (1998) with minor modifications. PCR amplification of nuclear rDNA comprising internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA gene, and ITS 2 involved the primers V9G (5′-TTACGTCCCTGCCCTTTGTA-3′; Hoog and Gerrits, 1998), and LR5 (5′-TCCTGAGGGAAACTTCG-3′; Vilgalys and Hester, 1990). Amplicons were purified with GFX columns (GE Healthcare UK Ltd., Buckinghamshire, England) and cycle-sequenced using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Nieuwerkerk,

The Netherlands). Reaction products were purified with Sephadex G-50 Fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and sequenced on ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, USA) using the primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′; Gardes and Bruns, 1993), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990).

The ITS sequence of strain HKI 0458 (GenBank Accession No. EU046615) was deposited at the sequence database of National Center for Biotechnology Information (GenBank; http://www.ncbi.nlm.nih.gov) and compared to GenBank sequences by means of the Basic Local Alignment Search Tool (BLAST).

4.1.2. Strain cultivation

General laboratory cultivation was performed on malt agar (malt extract 20.0 g/L, yeast extract 2.0 g/L, glucose 10.0 g/L, $(NH_4)_2HPO_4$ 0.5 g/L, pH 6.0) or the respective liquid medium at 22 °C. For long term preservation cultures grown on agar plates supplemented with 5% glycerol were maintained in the vapour phase of liquid N_2.

For screening purposes, the strain was grown in 250 mL Erlenmeyer flasks with 100 mL of the production culture medium consisting of saccharose 20 g/L, soybean flour 10 g/L, cornsteep 10 g/L and KCl 8 g/L, adjusted to pH 6.5 prior to sterilization. It was inoculated with pieces (1×1 cm^2) from an agar plate of a well grown culture and cultivated as resting culture for 17 days at 22 °C. The production culture was carried out in a 300 L fermentor filled with 200 L of the above production medium, with this being grown for 10 days at 22 °C (aeration 50 L/Min, pH 6.5) and stirred at 125 rpm. The inocculum (2 L) was obtained after several steps of resting cultures with increasing cultivation volume in a modified malt extract medium (malt extract 10.0 g/L, yeast extract 4.0 g/L, glucose 4.0 g/L, $(NH_4)_2HPO_4$ 0.5 g/L, pH 5.5).

4.2. Extraction and isolation

The fermentation broth (200 L) was obtained by filtration and loaded onto an Amberchrom 161c resin LC column (200×20 cm, 6 L). Elution was performed with a linear gradient of MeOH–H_2O (from 30% to 100% v/v, flow rate 0.5 L/min, in 58 min) to afford seven fractions (F1–F7), each of which was collected with 4.5 L, and then was lyophilized. By monitoring using TLC, fractions F4–F6 were combined and concentrated to afford a fraction F4–F6 (10 g), which was subjected to silica gel CC using a gradient of $CHCl_3$–MeOH (from 15:1 to 1:1) as eluant to obtain **4** (15 mg), and a mixture of **1**, **2**, and **3** (35 mg) as detected by ^{1}H NMR spectroscopic analyses, but showing one spot by TLC. The mixture of **1**–**3** was applied to a semi-preparative HPLC column (ODS) eluted with MeOH–H_2O–HOAc (67:33:0.1, v/v), as mobile phase to obtain **1** (12 mg), **2** (3 mg), and **3** (10 mg).

4.2.1. 3,4-seco-olean-11,13-dien-4,15α,22β,24-tetraol-3-oic acid (*1*)

Amorphous powder. $[\alpha]_D^{25}$ – 61.2 (*c* 1.45, MeOH). UV (MeOH) λ_{max} nm: 245, 250, 260. IR (KBr) ν_{max} cm^{-1}: 3418, 2950, 1709, 1464, 1384, 1390, 1023. ESIMS (positive ion) *m/z* 527.27 $[M + Na]^+$. HRFABMS *m/z* 527.3353 (calcd. for $C_{30}H_{48}O_6Na$, 527.3354). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

4.2.2. 4-seco-olean-11,13-dien-4,7β,22β,24-tetraol-3-oic acid (*2*)

Amorphous powder. $[\alpha]_D^{25}$ – 45.8 (*c* 0.5, MeOH). UV (MeOH) λ_{max} nm: 252, 263. IR (KBr) ν_{max} cm^{-1}: 3424, 2957, 1709, 1571, 1423, 1152. HRFABMS *m/z* 527.3345 (calcd. for $C_{30}H_{48}O_6Na$, 527.3354). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

4.2.3. 3,4-seco-olean-13-en-4,7α,15α,22α,24-pentaol-3-oic acid (*3*)

Amorphous powder. $[\alpha]_D^{25}$ +35.2 (*c* 0.37, MeOH). UV (MeOH) λ_{max} nm: 280. IR (KBr) ν_{max} cm^{-1}: 3386, 2951, 1709, 1507, 1463, 1387, 1026. HRFABMS (negative) *m/z* 521.3483 (calcd. for $C_{30}H_{49}O_7$, 521.3479). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

4.2.4. 3,4-seco-olean-13-en-4, 15α, 22α-triol-3-oic acid (*4*)

Amorphous powder. $[\alpha]_D^{25}$ + 76.2 (*c* 0.22, MeOH). UV (MeOH) λ_{max} nm: 280. IR (KBr) ν_{max} cm^{-1}: 3426, 2952, 1710, 1465, 1384, 1024. ESIMS *m/z* 529 $[M + Na]^+$; HRFABMS *m/z* 529.3510 $[M + Na]^+$. For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

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