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Azaphilone pigments and macrodiolides from the coprophilous fungus *Coniella fragariae*

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ABSTRACT

Two azaphilone pigments (**1** and **2**), two dihydrobenzofurans (**3** and **4**), two macrodiolides (**5** and **6**), and a dimeric alkyl aromatic constituent (**7**) were isolated from the goose dung-derived fungus *Coniella fragariae*. Compounds **1–3** proved to be new natural products. Coniellins H and I (**1** and **2**) feature a tetracyclic core and an aldehyde group at C-5, which is unusual for azaphilone derivatives. The X-ray structure of pyrenophorin (**5**) is reported for the first time. Pyrenophorin (**5**) showed strong cytotoxicity against several cancer cell lines with IC_{50} values ranging from 0.07 to 7.8 μM.

1. Introduction

Secondary metabolites of fungi are known for their high structural diversity and pronounced biological activities [1]. Azaphilones as a large group of fungal pigments assembled via the polyketide pathway are found in numerous species of fungi including the genera *Penicillium, Aspergillus, Talaromyces, Chaetomium, and Monascus* [2,3]. The structures of azaphilones are based on a highly oxygenated pyranoquinone bi- or tri-cyclic core, which is responsible for their red or yellow colors [2,3]. Several azaphilones have been reported to show pronounced bioactivities. Peyronellone B exhibited a strong hypoxia-protective effect [4]. Penicilone B displayed potent antibacterial activity against two strains of methicillin-resistant *Staphylococcus aureus* with MIC values of 3.13 μg/mL [5]. *epi*-Isochromophilone II showed strong cytotoxicity against three renal carcinoma cell lines ACHN, 786-O and OS-RC-2 with IC_{50} values of 4.4, 3.0 and 3.9 μM [6].

In our previous study, a series of new azaphilones was isolated from the goose dung-derived fungus *Coniella fragariae* [7]. Fungi of the genus *Coniella* (syn. *Pilidiella* and *Schizoparme*) are generally known as plant-pathogenic fungi [8]. However, chemical investigations of these fungi have rarely been conducted. In continuation of our previous study on *C. fragariae*, we report now two new azaphilone pigments, coniellins H (**1**) and I (**2**), and a new dihydrobenzofuran, conielldihydrobenzofuran (**3**) as well as four known compounds including a further dihydrobenzofuran (**4**), two macrodiolides (**5** and **6**) and a dimeric alkyl aromatic constituent (**7**) (Fig. 1). The structure elucidation of the new compounds and their cytotoxic activities are discussed in this paper.

2. Results and discussion

The molecular formula of **1** was determined as $C_{23}H_{28}O_7$ based on the HRMS data, indicating 10 degrees of unsaturation. The ^{13}C NMR data of **1** (Table 1) combined with HSQC data indicated the presence of three carbonyls at δ_C 204.7 (C-13), 196.6 (C-6) and 191.1 (C-10), an ester carbonyl at δ_C 169.0 (C-21), and four olefinic carbons at δ_C 170.3 (C-3), 99.0 (C-4), 154.9 (C-4a) and 115.1 (C-5), accounting for 6 degrees of unsaturation. Thus, compound **1** was suggested to be a tetracyclic natural product. The HMBC correlations from Me-11 (δ_H 2.16) to C-3 and C-4, from H-4 (δ_H 7.24) to C-5, C-4a and C-8a, from H-10 (δ_H 10.06) to C-4a and C-5, from Me-9 (δ_H 1.35) to C-6, C-7 and C-8, from

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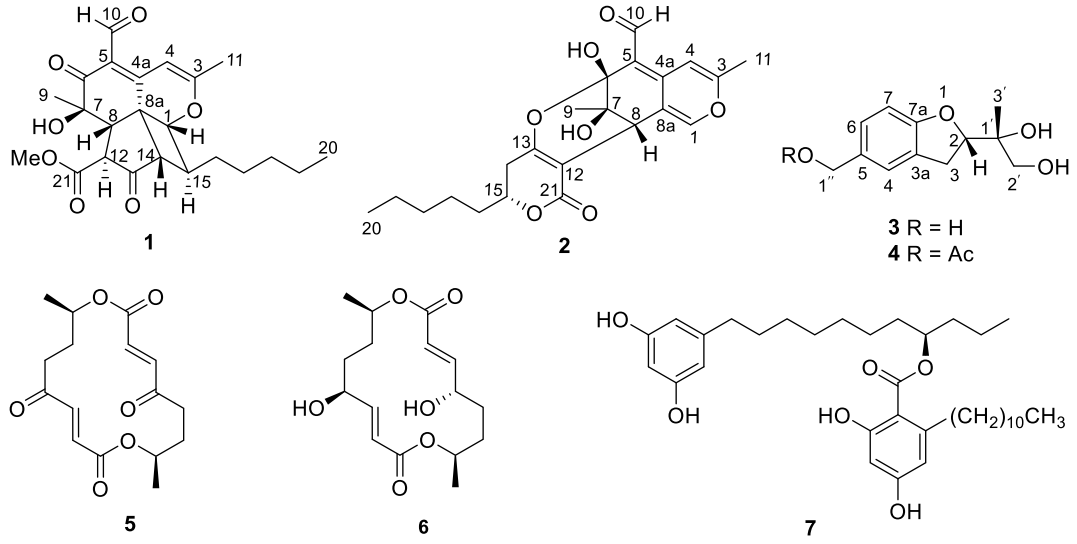

Fig. 1. Structures of isolated compounds.

Table 1
^{1}H and ^{13}C NMR Data of Compounds **1** and **2**.

Position	**1**[a]		**2**[b]	
	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)
1	80.6, CH	4.86, d (4.8)	146.8, CH	7.47, s
3	170.3, C		160.9, C	
4	99.0, CH	7.24, s	101.2, CH	6.77, s
4a	154.9, C		147.8, C	
5	115.1, C		111.4, C	
6	196.6, C		104.1, C	
7	74.1, C		67.4, C	
8	50.5, CH	3.51, d (11.2)	39.3, CH	3.69, s
8a	42.0, C		121.6, C	
9	21.6, CH_3	1.35, s	19.3, CH_3	1.36, s
10	191.1, CH	10.06, s	188.5, CH	9.94, s
11	22.1, CH_3	2.16, s	20.0, CH_3	2.24, s
12	58.7, CH	3.13, dd (11.2, 1.2)	102.7, C	
13	204.7, C		164.6, C	
14	63.5, CH	3.00, dd (8.9, 1.2)	31.6, CH_2	2.46, ddd (17.0, 11.1, 1.3) 2.32, dd (17.0, 4.2)
15	43.3, CH	3.09, m	75.5, CH	4.25, m
16	28.4, CH_2	1.63, m 1.55, m	34.7, CH_2	1.75, m 1.57, m
17	25.9, CH_2	1.28, m	24.5, CH_2	1.45, m 1.34, m
18	31.5, CH_2	1.27, m	31.4, CH_2	1.27, m
19	22.4, CH_2	1.29, m	22.4, CH_2	1.29, m
20	14.0, CH_3	0.89, t (7.0)	13.9, CH_3	0.87, t (7.0)
21	169.0, C		166.0, C	
6-OH				9.01, s
21-OMe	53.3, CH_3	3.83, s		

[a] Recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$.
[b] Recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$.

H-8 (δ_H 3.51) to C-6, C-4a and C-1, and from H-1 (δ_H 4.86) to C-3 and C-8 indicated the presence of a pyranoquinone bicyclic ring, in which two methyl groups are attached to C-3 and C-7 and an aldehyde group is connected to C-5. A third cyclopentanone ring was established from the COSY correlation between H-8 and H-12 (δ_H 3.13) together with the HMBC correlations from H-12 to C-13, and from H-14 (δ_H 3.00) to C-13, C-8 and C-4a. The HMBC correlations from H-8, H-12 and a methoxy group (δ_H 3.83) to C-21 confirmed the attachment of a methoxycarbonyl group to C-12. The remaining signals accounted for a *n*-hexanyl side chain from C-15 to C-20 based on the COSY correlations

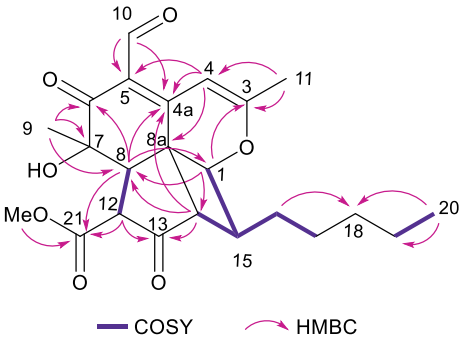

Fig. 2. COSY and key HMBC correlations of compound **1**.

between H-15/H_{ab}-16/H_{ab}-17 and between H_{ab}-19 and Me-20 as well as on the HMBC correlations from H_{ab}-16 to Me-18 and from Me-20 to C-18 and C-19. In addition, the COSY correlations between H-14/H-15/H-1 and the HMBC correlation from H-1 to C-14 indicated the presence of a fourth cyclobutane ring. Thus, the planar structure of **1** was elucidated as shown (Fig. 2), representing a new tetracyclic azaphilone derivative, for which the trivial name coniellin H is proposed.

The NOE correlations between H-14/H-1, H-1/H-8, H-8/H-14 suggested that these protons were proximate in space (Fig. 3). The large coupling constant (11.2 Hz) between H-8 and H-12 indicated that these two protons are oriented on the opposite sides of the cyclopentanone ring. In addition, the NOE correlations from Me-9 to both H-8 and H-12

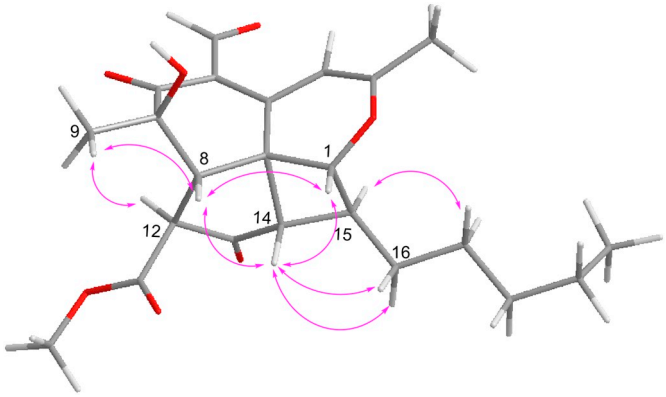

Fig. 3. Key ROESY correlations of compound **1**.

Table 2
^{1}H and ^{13}C NMR Data of Compound **3**.[a]

Position	δ_C, type	δ_H, (J in Hz)
2	85.7, CH	4.81, dd (9.6, 8.3)
3	30.2, CH_2	3.26, dd (16.0, 8.3) 3.20, dd (16.0, 9.6)
3a	127.4, C	
4	124.4, CH	7.21, d (1.8)
5	133.5, C	
6	127.5, CH	7.10, dd (8.1, 1.8)
7	109.0, CH	6.74, d (8.1)
7a	159.0, C	
1′	73.6, C	
2′	67.0, CH_2	3.78, d (11.0) 3.54, d (11.0)
3′	19.3, CH_3	1.20, s
1″	65.3, CH_2	4.60, s

[a] recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$.

assigned Me-9 on the α-face of the cyclohexenone ring. Furthermore, H-14 exhibited NOE correlations to H_{ab}-16 whereas H-15 showed NOE correlations to H_{ab}-17, indicating opposite orientation between H-14 and H-15. The absolute configuration at C-7, C-8, and C-12 in **1** is suggested to be identical to that of the previously reported coniellin A (Fig. 8) based on the close biogenetic relationship of both compounds [7]. Thus, the stereochemistry of **1** was determined as shown.

Coniellin I (**2**) had the molecular formula $C_{22}H_{26}O_7$ as determined by the HRMS data, corresponding to ten degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **2** (Table 2) were similar to those of **1**, suggesting structural similarity between both compounds. For instance, the correlations in the HMBC spectrum of **2** from Me-11 (δ_H 2.24) to C-3 and C-4, from H-4 (δ_H 6.77) to C-5 and C-8a, from H-10 (δ_H 9.94) to C-5 and C-6, from 6-OH (δ_H 9.01) to C-5, C-6 and C-7, from Me-9 (δ_H 1.36) to C-6, C-7 and C-8, from H-8 (δ_H 3.69) to C-4a, C-8a and C-1, and from H-1 (δ_H 7.47) to C-3, C-4a, C-8a and C-8 established a pyranoquinone bicyclic ring similar to that in **1** except for the presence of a double bond at C-1/C-8a and a hydroxy group at C-6 in **2**. A third α,β-unsaturated δ-lactone ring was deduced from the COSY correlation between H-15/H_{ab}-14 as well as from the HMBC correlations from H-8 to C-12, C-13 and C-21, from H_{ab}-14 to C-12 and C-13, and from H-15 to C-21. The COSY correlations between H-15/H_{ab}-16/H_{ab}-17/H_{ab}-18 and between H_{ab}-19 and Me-20 along with the HMBC correlations from Me-20 to C-18 and C-19 indicated the attachment of a *n*-pentanyl side chain at C-15. The above NMR data accounted for nine degrees of unsaturation, suggesting the presence of a fourth ring in **2**. Based on the chemical shifts of C-6 (δ_C 104.1) and C-13 (δ_C 164.6) and the molecular formula of **2**, an ether bridge between C-6 and C-13 was suggested. Thus, the planar structure of **2** was elucidated as shown (Fig. 4).

The relative configuration of **2** was determined by NOE correlations and coupling constants. In the ROESY spectrum, Me-19 exhibited

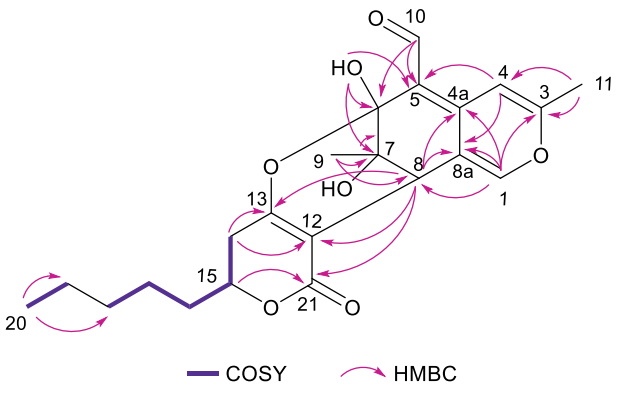

Fig. 4. COSY and key HMBC correlations of compound **2**.

correlations to 6-OH, H-8 and H_b-14 (δ_H 2.32), indicating that 6-OH and H-8 were on the same side and Me-9 was on the α-face of the cyclohexene ring whereas 7-OH was on the opposite side (Fig. 5). In addition, the NOE correlation from H-15 to H_b-14 and H_b-16 (δ_H 1.57) together with the coupling constants between H-15 to H_{ab}-14 ($^{2}J_{H\text{-}15/Ha\text{-}14}$ = 11.1 Hz, $^{2}J_{H\text{-}15/Hb\text{-}14}$ = 4.2 Hz) suggested that H-15 and H_b-14 were on the same side of the lactone ring whereas H_a-14 was on the opposite side. Based on the biogenetic relationship between **2** and coniellin A (Fig. 8), whose absolute configuration has been determined by ECD calculations [7], compound **2** is suggested to share the same absolute configuration at C-7 and C-8. Accordingly, the absolute configuration of **2** was assigned as 6*R*,7*R*,8*R*,15*R*.

The molecular formula of **3** was established as $C_{12}H_{16}O_4$, suggesting five degrees of unsaturation. The ^{13}C NMR spectrum of **3** (Table 2) showed six olefinic carbons at δ_C 127.4 (C-3a), 124.4 (C-4), 133.5 (C-5), 127.5 (C-6), 109.0 (C-7) and 159.0 (C-7a), three methylene groups at δ_C 30.2 (C-3), 67.0 (C-2′) and 65.3 (C-1″), an oxygenated methine at δ_C 85.7 (C-2), a non-protonated carbon at δ_C 73.6 (C-1′), at and a methyl group at δ_C 19.3 (C-3′). An ABX benzene ring system was detected at δ_H 7.21 (d, J = 1.8 Hz, 1H, H-4), 7.10 (dd, J = 8.1, 1.8 Hz, 1H, H-6), 6.74 (d, J = 8.1 Hz, 1H, H-7). A dihydrobenzofuran bicyclic core was established by the COSY correlations between H-2/H_{ab}-3, and H-6/H-7 in addition to the HMBC correlations from H-2 to H-7a, from H_{ab}-3 to C-3a and C-7a, from H-4 to C-3, C-6 and C-7a, from H-6 to C-4 and C-7a, and from H-7 to C-3a and C-5. Furthermore, the HMBC correlations from Me-3′ to C-2, C-1′ and C-2′, from H_{ab}-2′ to C-2, C-1′ and C-3′, and from H_{ab}-1″ to C-4, C-5 and C-6 indicated the attachment of a 1,2-dihydroxyisopropyl group and a hydroxymethyl group at C-2 and C-5, respectively. Thus, the planar structure of **3** was elucidated as shown (Fig. 6), for which the trivial name conielldihydrobenzofuran is proposed.

The absolute configuration of **3** was suggested to be (2*S*,1′*R*) by comparison of its ^{13}C NMR data and optical rotation (Table 3) with those of (2*R*,1′*S*)-, (2*S*,1′*R*)-, (2*R*,1′*R*)-, and (2*S*,1′*S*)-2,3-dihydro-2-(1′,2′-dihydroxy-1′-methylethyl)-6- methoxybenzofuran [9,10].

The four known compounds were identified as 5-(1″-acetyloxymethylene)-2-(1,2-dihydroxyisopropyl)-2,3-dihydrobenzofuran (**4**) [11], pyrenophorin (**5**) [12], pyrenophorol (**6**) [13], and 15-dehydroxyintegracin B (**7**) [14] based on their NMR and MS data as well as by comparison with the literature. Here, the absolute configuration of the C_2-symmetric ring in pyrenophorin (**5**) with *R*-configuration at C-6 and the symmetry-related C-6′ is reported for the first time using X-ray analysis (Fig. 7).

All isolated compounds (**1**–**7**) were tested for their cytotoxicity against the MDA-MB-231 human breast cancer cell line (Table 4). Only pyrenophorin (**5**) showed cytotoxicity with an IC_{50} value of 7.8 μM. Pyrenophorin (**5**) as well as the analogue pyrenophorol (**6**) were further tested against additional cell lines (Table 3). Pyrenophorin (**5**) showed significant cytotoxicity against A2780 *cis*-platin sensitive and *cis*-platin resistant cells, Ramos cells, and against Jurkat J16 cells with IC_{50} values of 0.5, 0.9, 0.6, and 0.07 μM, respectively. Pyrenophorol (**6**) in contrast was inactive. Comparison of the structures of pyrenophorin (**5**) and pyrenophorol (**6**) indicated the importance of the α,β-unsaturated ketone unit of **5** for cytotoxicity.

Coniellins H and I (**1** and **2**) feature a tetracyclic core and an aldehyde group at C-5, which is unusual for azaphilone derivatives. A proposed biosynthetic pathway is shown in Fig. 8. The azaphilone intermediate A is formed through esterification of a β-ketoacid (from the FAS pathway) to the polyketide chromophore (from the PKS pathway) followed by a C-8/C-12 Knoevenagel cyclization and reduction [15]. Hydrolysis of intermediate A gives the lactone ring-opened product B. From the intermediate B, coniellin H (**1**) is obtained by elimination of 15-OH, [2 + 2]cycloaddition and methylation. The second branch of the pathway from intermediate B to dihydropyrone C is suggested to be catalysed by type III PKS [16]. Then attack of OH-13 to the carbonyl at C-6 affords the hemiketal group of coniellin H (**2**).

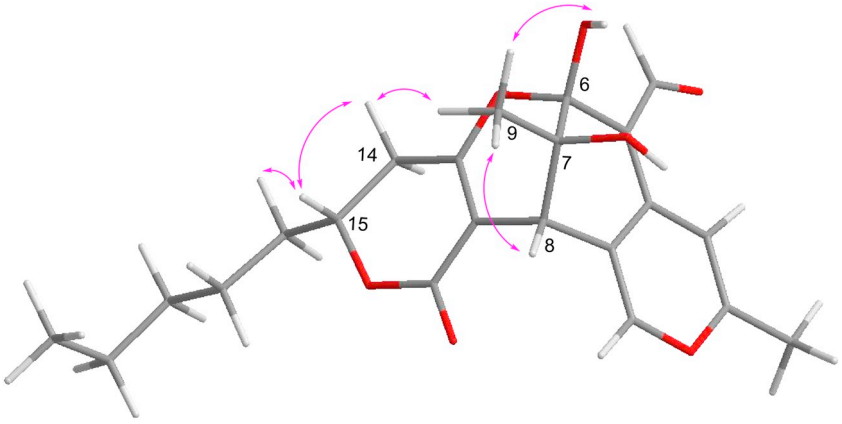

Fig. 5. Key ROESY correlations of compound **2**.

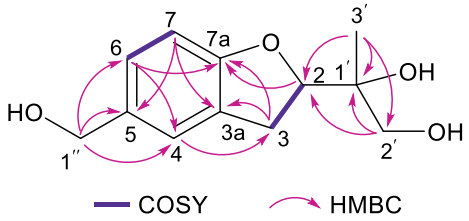

Fig. 6. COSY and key HMBC correlations of compound **3**.

Table 3
Comparison of ^{13}C NMR data in $CDCl_3$ and optical rotation in $CHCl_3$.

	3	2*R*,1′*S*	2*S*,1′*R*	2*R*,1′*R*	2*S*,1′*S*
C-2	85.7	86.13		88.60	
C-1′	73.6	73.76		73.17	
C-2′	67.0	66.96		68.73	
[α] in $CHCl_3$	+17	−27.6	+27.9	−21.2	+20.3

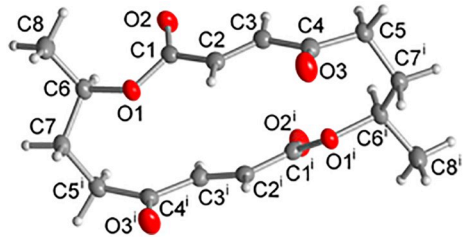

Fig. 7. Molecular structure of compound **5**; the ring lies on the special position around a twofold proper rotation axis (C_2) (symmetry code: $-x + 1, -y + 1, z$).

Table 4
Cytotoxity (IC_{50}, μM) of pyrenophorin (**5**) and pyrenophorol (**6**).

	MDA-MB-231	*cis*-platin sensitive A2780	*cis*-platin resistant A2780	Ramos	Jurkat J16
Pyrenophorin (**5**)	7.8	0.5	0.9	0.6	0.07
Pyrenophorol (**6**)	> 100	> 100	> 100	> 100	> 100
STS [a]	0.039	–	–	0.037	0.059
cDDP [b]	–	2.1	11.1	–	–

[a] Staurosporin as positive control.
[b] *cis*-Diammindichloridoplatin as positive control.

3. Experimental section

3.1. General experimental procedures

1D and 2D NMR spectra were recorded with Bruker Avance III 300 or 600 spectrometers. Mass spectra were obtained by a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest and HRESIMS data were recorded on a Bruker Daltonics UHR-QTOF Maxis 4G mass spectrometer. Optical rotations were measured with a JASCO P-2000 polarimeter. HPLC analysis was performed using a Dionex UltiMate-3400SD system coupled with a LPG-3400SD Pump, a photodiode array detector (DAD3000RS) and a Knauer Eurospher C_{18} analytical column (125 × 4 mm, 5 μm). Semi-preparative HPLC was performed with a Lachrom-Merck Hitachi system (UV detector L-7400, pump L-7100, 300 × 8 mm Knauer Eurospher-100 C_{18} column). Column chromatography was carried out using Sephadex LH-20, Merck MN Silica gel 60 M or LiChroprep RP-18 as stationary phases. Plates precoated with Merck silica gel F254 were used for TLC with detection under 254 and 365 nm followed by spraying with anisaldehyde reagent.

3.2. Identification, cultivation and isolation

In September 2016, goose (*Anser anser*) dung was collected at the North Sea coast close to Garding, Germany. The fungus was identified as *C. fragariae* (GenBank No. KJ710465.1) by DNA amplification and sequencing of its ITS region [17]. The fungus was fermented on rice medium in 50 Erlenmeyer flasks (each containing 100 g of rice and 110 mL of demineralized water, autoclaved at 121 °C for 20 min) for two weeks at 20 °C under static conditions. 500 mL EtOAc were added to each flask to terminate fermentation followed by shaking for 8 h at 150 rpm. 52 g EtOAc crude extract was divided into a *n*-hexane fraction and a MeOH fraction (27 g) by liquid-liquid separation. The MeOH fraction was separated by vacuum liquid chromatography on a RP-18 column (60 × 200 mm) using a solvent gradient (from 100% H_2O to 100% MeOH) to give seven fractions (Fr.1 to Fr.7). Compound **5** (100 mg) was obtained from Fr.1 (4.1 g) as needle crystals. The rest of Fr.1 was fractionated on a silica gel column (30 × 600 mm) eluted with a gradient of CH_2Cl_2 and MeOH (1:0 to 8:2) to give 6 subfractions (Fr.1.1 to Fr.1.6). Fr.1.2 was purified by semipreparative HPLC using 30% MeOH-H_2O to afford **6** (4.8 mg). Fr.1.5 was separated by semipreparative HPLC using 25% MeOH-H_2O to give **3** (5.1 mg) and **4** (4.7 mg). Fr.2 (0.6 g) was subjected to a Sephadex LH-20 column (20 × 1000 mm) with MeOH as mobile phase, followed by separation using semipreparative HPLC to give **2** (5.8 mg). Fr.4 (0.9 g) was fractionated on a silica gel column (30 × 300 mm) with *n*-hexane and EtOAc as mobile phase to give three subtractions (Fr.4.1 to Fr.4.3). Fr.4.3 was separated by semipreparative HPLC using 50% MeOH-H_2O to afford **1** (5.0 mg). Fr.6 (2.6 g) was subjected to a silica column

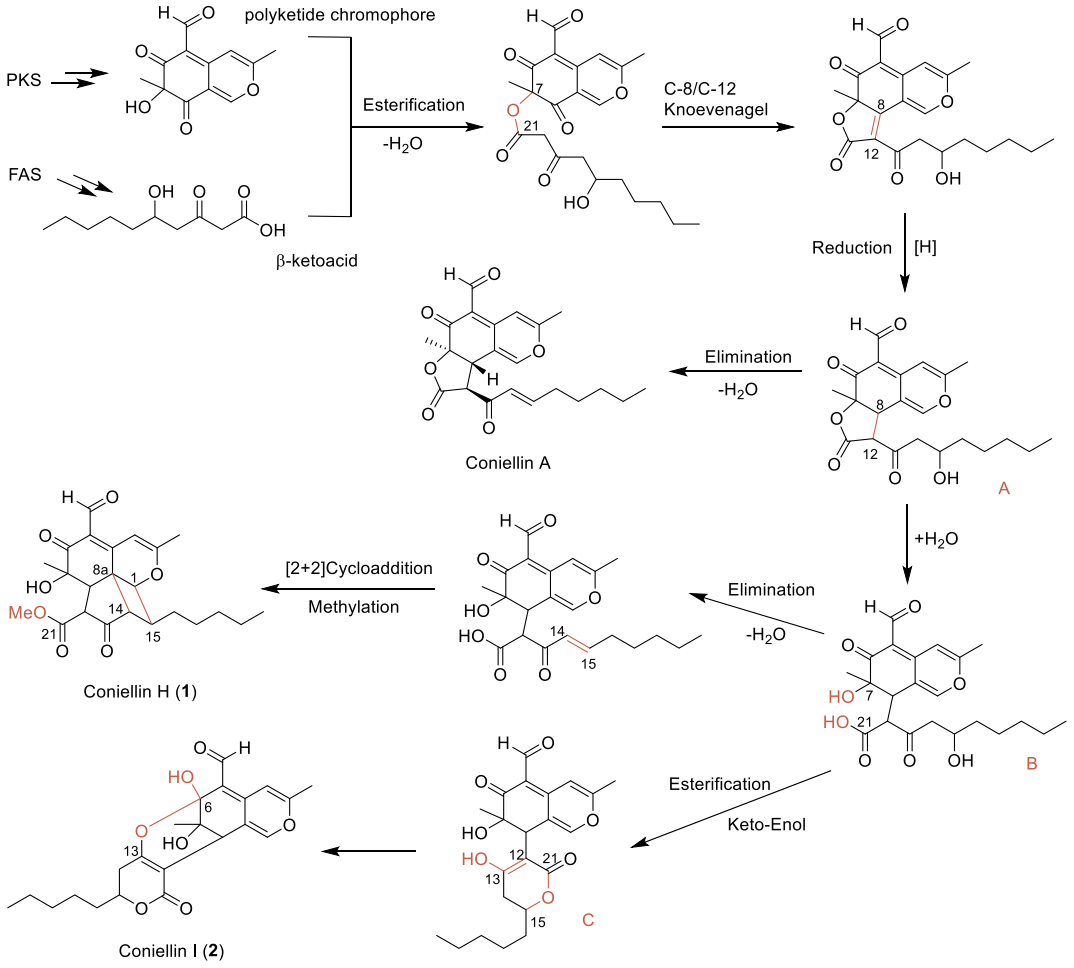

Fig. 8. Proposed biosynthetic pathway for compounds **1** and **2**.

(30 × 600 mm) using a gradient of *n*-hexane and EtOAc, followed by purification with semipreparative HPLC using 80% MeOH-H_2O to give **7** (3.2 mg).

Coniellin H (1): brown amorphous solid; [α]20*D* +413 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 258, 384 nm; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 417.1905 $[M + H]^+$ (calcd for $C_{23}H_{29}O_7$, 417.1908).

Coniellin I (2): brown amorphous solid; [α]20*D* -482 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 259, 384 nm; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 403.1794 $[M + H]^+$ (calcd for $C_{22}H_{27}O_7$, 403.1757).

Coniellidihydrobenzofuran (3): colorless powder; [α]20*D* +17 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 230, 283 nm; ^1H and ^{13}C NMR data, Tables 2; HRESIMS *m/z* 247.0941 $[M + Na]^+$ (calcd for $C_{12}H_{16}NaO_4$, 247.0941).

3.3. X-ray crystallographic analysis of compound 5

Data Collection: compound **5** was measured with a Bruker Kappa APEX2 CCD diffractometer with a microfocus tube using Cu Kα radiation (λ = 0.71073 Å). For data collection APEX2, for cell refinement and data reduction SAINT [18], and for experimental absorption correction SADABS were used [19]. The structure was solved by intrinsic phasing using SHELXT [20]. refinement was done by full-matrix least-squares on F^2 using SHELXL-2016/6 [21]. The hydrogen atoms were positioned geometrically (with C-H = 0.95 Å for aromatic and aliphatic CH, 1.00 Å for tertiary CH, 0.99 Å for CH_2 and 0.98 Å for CH_3) and refined using riding models (AFIX 43, 13, 23, 137, respectively), with $U_{iso}(H) = 1.2U_{eq}$ (CH, CH_2) and 1.5 U_{eq} (CH_3).

The absolute structure of **5** was solved using anomalous dispersion from Cu Kα, resulting in a Flack parameter of x = 0.12 [4] using Parsons quotient method [22,23].

All graphics were drawn using DIAMOND [24]. The analyses of hydrogen bonds (inter- and intramolecular) as well as π-π and CH-π interactions were done using PLATON for Windows [25–28]. The structural data has been deposited in the Cambridge Crystallographic Data Center (CCDC No. 1914503).

Crystal Data of ***5****:* $C_{16}H_{20}O_6$, M = 308.32, orthorhombic system, space group $P2_12_12$, *a* = 9.8268(14) Å, *b* = 15.824(2) Å, *c* = 5.1127(7) Å, *V* = 795.0(2) $Å^3$, *Z* = 2, D_{calc} = 1.288 g/cm^3, crystal size 0.26 × 0.10 × 0.10 mm, μ(Cu Kα) = 0.82 mm^{-1}, 5.3° < θ < 66.5°, N_t = 5254, *N* = 1383 (R_{int} = 0.025), R_1 = 0.026, wR_2 = 0.067, *S* = 1.08, Flack parameter = 0.12(4).

3.4. Cytotoxicity assay

Cytotoxicity tests using the human breast cancer cell line MDAMB-231, the cisplatin sensitive and resistant human ovarian cancer cell line A2780, the adult lymphoblastic leukemia T cells Jurkat J16, or Burkitt's lymphoma B cells (Ramos) were carried out as described before [7,29].

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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.2019.104249.

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