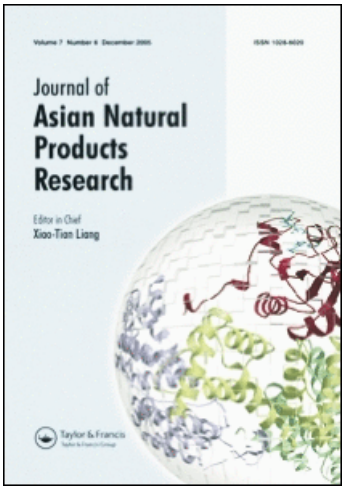

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Metabolites from endophytic fungus S20 of *Cephalotaxus hainanensis*

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Three new C-methylated acetogenins, (2*E*,6*Z*)-9,10-dihydroxy-4-hydroxymethyl-2,6-decadiene (**1**), (2*E*,6*Z*)-8,9,10-trihydroxy-4-hydroxymethyl-2,6-decadiene (**2**), and (2*E*,6*Z*)-9-hydroxy-4-hydroxymethyl-2,6-nonadiene (**3**), together with two known compounds, *p*-hydroxybenzyl alcohol (**4**) and indolyl-3-carboxylic acid (**5**), were isolated from endophytic fungus S20 of *Cephalotaxus hainanensis* Li. Their structures were determined based on HR-ESI-MS and spectroscopic techniques (IR, UV, 1D, and 2D NMR). Compound **5** showed inhibitory effects on *Staphylococcus aureus* and methicillin-resistant *S. aureus* by the filter paper disc agar diffusion method.

Keywords: *Cephalotaxus hainanensis* Li; endophytic fungus; acetogenins; antibacterial activity

1. Introduction

The cephalotaxine esters from the bark of *Cephalotaxus hainanensis* Li, such as harringtonine and homoharringtonine, have good effect in the treatment of leukemia. Since *C. hainanensis* is the national secondary protected plant on the brink of extinction, much attention has been paid to look for an alternative source of these compounds other than the bark of *C. hainanensis* [1]. Stierle *et al.* [2] claimed production of taxol and related compounds by means of an endophytic fungus, *Taxomyces andreanae*, isolated from the bark of *Taxus brevifolia*, and this observation led us to start our investigation of the metabolites produced by endophytic fungi of *C. hainanensis*. In our previous study, 72 strains of endophytic fungi were isolated from the healthy bark, branches, and leaves of *C. hainanensis* tree collected in Jianfengling tropical rainforest reserve [3]. Further investigation on the secondary metabolites from endophytic fungus S20 of *C. hainanensis* led to the isolation of three new C-methylated acetogenins (**1**–**3**) (Figure 1) and two known compounds; their structures were unambiguously elucidated as (2*E*,6*Z*)-9,10-dihydroxy-4-hydroxymethyl-2,6-decadiene (**1**), (2*E*,6*Z*)-8,9,10-trihydroxy-4-hydroxymethyl-2,6-decadiene (**2**), (2*E*,6*Z*)-9-hydroxy-4-hydroxymethyl-2,6-nonadiene (**3**), *p*-hydroxybenzyl alcohol (**4**), and indolyl-3-carboxylic acid (**5**) by extensive spectroscopic analysis (2D NMR experiments and HR-ESI-MS). In this paper, we describe the isolation and identification of compounds **1**–**5**, as well as their antibacterial activity.

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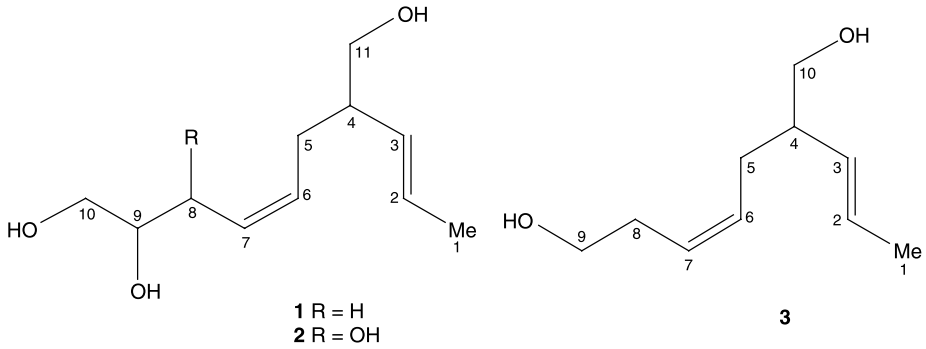

Figure 1. Structures of compounds **1**–**3**.

2. Results and discussion

Compound **1**, isolated as colorless oil, has a molecular formula $C_{11}H_{19}O_3$ based on its HR-ESI-MS at *m/z* 199.1337 $[M-H]^-$. The ^{13}C NMR and DEPT spectra of **1** presented 11 carbon signals for six methines (δ 45.5, 71.2, 126.9, 128.2, 131.9, 132.4), four methylenes (δ 34.8, 36.7, 66.2, 65.7), including two oxygenated carbons and one methyl (δ 18.1). The corresponding proton signals were assigned from HMQC spectrum. In the $^1H-^1H$ COSY spectrum, the methyl protons at δ 1.70 (H-1) showed correlation to the olefinic proton at δ 5.58 (H-2), while another olefinic proton at δ 5.23 (H-3) showed correlations to both the olefinic proton at δ 5.58 (H-2) and the methine proton at δ 2.20 (H-4), thus the chains of C1–C4 were determined. On the other hand, the methylene protons at δ 2.09 and 2.02 (H-5) showed correlations to the olefinic proton at δ 5.52 (H-6), while another olefinic proton at δ 5.46 (H-7) showed correlation to the methylene proton at δ 2.15 (H-8), and both the methylene proton at δ 2.15 (H-8) and at δ 3.66, 3.48 (H-10) showed correlations to the methine proton at δ 3.71 (H-9), thus the chains of C5–C10 were determined. The methylene protons at δ 3.55, 3.43 (H-11) showed correlations to the methine proton at δ 2.20 (H-4) in the $^1H-^1H$ COSY spectrum, and the same methylene protons (H-11) showed correlations to the C5 at δ 34.8 in the HMBC spectrum, so the methylene was connected with C4, and the above two chains of C1–C4 and C5–C10 were connected through C4 and C5. Thus, the primary structure of **1** was obtained by the analysis of the HMQC, $^1H-^1H$ COSY, and HMBC spectra (Figure 2), while the

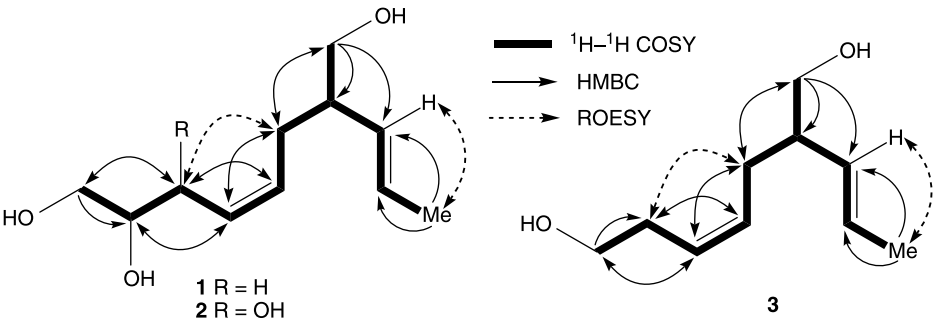

Figure 2. Key $^1H-^1H$ COSY, HMBC, and ROESY correlations of compounds **1**–**3**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compounds **1**–**3** (δ, ppm and J, Hz).

	1 ($CDCl_3$)		**2** (CD_3OD)		**3** ($CDCl_3$)	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	18.1 q	1.70 (d, 6.4)	18.3 q	1.66 (d, 6.4)	17.8 q	1.64 (d, 6.0)
2	128.2 d	5.58 (m)	127.6 d	5.49 (m)	127.0 d	5.50 (m)
3	131.9 d	5.23 (dd, 15.2, 8.4)	133.5 d	5.29 (m)	131.8 d	5.21 (dd, 15.6, 8.0)
4	45.5 d	2.20 (m)	46.6 d	2.20 (m)	45.0 d	2.18 (m)
5	34.8 t	2.09 (m), 2.02 (m)	35.6 t	2.25 (m), 2.05 (m)	34.6 t	2.07 (m), 2.01 (m)
6	132.4 d	5.52 (m)	132.3 d	5.68 (m)	131.3 d	5.46 (m)
7	126.9 d	5.46 (m)	132.0 d	5.56 (m)	127.0 d	5.42 (m)
8	36.7 t	2.15 (m)	74.8 d	3.99 (dd, 6.5, 4.8)	34.7 t	2.12 (m)
9	71.2 d	3.71 (m)	76.2 d	3.53 (m, overlap)	65.2 t	3.47 (m, overlap), 3.39 (m, overlap)
10	66.2 t	3.66 (dd, 7.2, 3.2), 3.48 (dd, 7.2, 3.2)	64.5 t	3.62 (m), 3.53 (m, overlap)	65.2 t	3.47 (m, overlap), 3.39 (m, overlap)
11	65.7 t	3.55 (dd, 10.5, 5.3), 3.43 (dd, 10.8, 5.3)	66.4 t	3.52 (m, overlap), 3.44 (dd, 10.6, 5.7)	–	–

stereochemistry of the double bond was given by the values of the coupling constants as well as the ROESY spectra: H-5 correlated with H-8 and H-1 correlated with H-3. This clearly suggested an *E* and *Z* stereochemistry for Δ2 and Δ6, respectively. Therefore, the structure of **1** was established as (2*E*,6*Z*)-9,10-dihydroxy-4-hydroxymethyl-2,6-decadiene.

Compound **2**, obtained as colorless oil, has a molecular formula $C_{11}H_{19}O_4$ based on its HR-ESI-MS at *m*/*z* 215.1287 $[M-H]^-$. A careful comparison of the ^{13}C and ^1H NMR spectral data of compound **2** with those of compound **1** indicated that the two structures were very similar except for the appearance of an oxygenated methine and the absence of a methylene in compound **2** (Table 1). In the $^1H-^1H$ COSY spectrum, the oxygenated methine at δ 3.99 (H-8) showed correlations to both the olefinic proton at δ 5.56 (H-7) and the methine proton at δ 3.53 (H-9), thus the oxygenated methine was located between C7 and C9, which has been confirmed by the correlations between the oxygenated methine at δ 3.99 (H-8) to C10 at δ 64.5, C9 at δ 76.2, and C6 at δ 132.3 in the HMBC spectrum. The primary structure of **2** was obtained by the analysis of the HMQC, $^1H-^1H$ COSY, and HMBC spectra (Figure 2), while the stereochemistry of the double bond was given as same as for compound **1** by the ROESY spectra. Therefore, the structure of **2** was established as (2*E*,6*Z*)-8,9,10-trihydroxy-4-hydroxymethyl-2,6-decadiene. Unfortunately, the yields of compounds **1** and **2** were too low to determine the configuration of the stereocenter at C-4 and C-9 for **1** and C-4, C-8, and C-9 for **2**.

Compound **3**, obtained as colorless oil, has a molecular formula $C_{10}H_{18}O_2$ based on its HR-ESI-MS at *m*/*z* 169.1235 $[M-H]^-$. Comparison of the ^{13}C and ^1H NMR spectral data of compound **3** with those of compound **1** revealed that the structure of compound **3** was similar to that of compound **1**, except for the lack of the oxygenated methane at δ 71.2 (Table 1). And the consistent difference between the $^1H-^1H$ COSY spectra of the two compounds is that the methylene proton of H-8 showed correlations to both the olefinic proton of H-7 and the oxygenated methylene proton at δ 3.47, 3.39 (H-9) of compound **3**, instead of the oxygenated methine at δ 3.71 (H-9) of compound **1**. Thus, the primary structure of **3** was determined and confirmed by the HMQC, $^1H-^1H$ COSY, and HMBC spectra (Figure 2), while the stereochemistry of the double bond was given by the values of the coupling constants as well as the ROESY spectra similar to that of compound **1**. Therefore, the structure of **3** was established as (2*E*,6*Z*)-9-hydroxy-4-hydroxymethyl-2,6-nonadiene.

It is reported that some analogous C-methylated acetogenins have been isolated from *Pestalotiopsis* spp., endophytic fungi of *T. brevifolia*, and the possible biosynthesis pathways have been discussed [4].

Antibacterial tests demonstrated that compounds **1**–**4** showed no antibacterial activity to the test strains, while compound **5** showed inhibitory effects on *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), diameters of inhibition zones of which were 12 and 8 mm, respectively. The diameter of inhibition zone of the positive control, kanamycin sulfate, was 31 mm.

3. Experimental

3.1 General experimental procedures

Melting points were obtained on a Beijing Taike X-5 stage apparatus and are uncorrected. Optical rotation was recorded using a Rudolph Autopol III polarimeter (Rodolph Research Analytical, Hackettstown, NJ, USA). UV spectra were measured on a Shimadzu UV-2550 spectrometer. IR spectra were obtained on a Nicolet 380 FT-IR instrument, as KBr pellets. NMR spectra were recorded on a Bruker AV-400 spectrometer, using TMS

as the internal standard. HR-ESI-MS spectra were measured with an API QSTAR Pulsar mass spectrometer. Column chromatography was performed with silica gel (Marine Chemical Industry Factory, Qingdao, China) and Sephadex LH-20 (Merck, Darmstadt, Germany). TLC was performed with silica gel GF254 (Marine Chemical Industry Factory).

3.2 Fungal material

Endophytic fungus S20 was isolated from the bark of *C. hainanensis* tree collected in Jianfengling tropical rainforest reserve, Hainan Province, China (2005). This fungus was deposited at the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, and maintained on potato dextrose agar (PDA) slant at 4°C.

3.3 Fermentation, extraction, and isolation

The endophytic fungus S20 was grown on PDA at room temperature for 5 days. Three pieces of mycelial agar plugs ($0.5 \times 0.5\,cm^2$) were inoculated into 11 Erlenmeyer flasks containing 400 ml potato dextrose broth. The cultivation was shaken at 160 rpm at room temperature for 7 days, and then kept in still at room temperature for 21 days. The culture broth (86 l) was filtered to give the filtrate and mycelia. The filtrate was evaporated *in vacuo* to small volume and then partitioned in succession between H_2O and petroleum ether, EtOAc. The EtOAc solution was evaporated under reduced pressure to give a crude extract (6.3 g), which was separated into nine fractions on a silica-gel column using a step gradient elution of $CHCl_3$/MeOH (1:0 → 0:1). Fraction 2 (1.2 g) was submitted to chromatography on a silica-gel column with $CHCl_3$/MeOH (20:1) as eluent and further separated by column chromatography over Sephadex LH-20 with 95% EtOH as eluent, yielding compound **5** (2.7 mg). Fraction 4 (1.1 g) was submitted to chromatography on a silica-gel column with $CHCl_3$/MeOH (16:1) as eluent, yielding eight subfractions. Subfraction 2 (67.3 mg) was submitted to repeated column chromatography over silica gel with petroleum ether/acetone (2:1) and further separated by column chromatography over Sephadex LH-20 with 95% EtOH as eluent, yielding compounds **1** (5.8 mg) and **3** (4.3 mg). Subfraction 4 (139.4 mg) was submitted to chromatography on a silica-gel column with petroleum ether/acetone (2:1) as eluent, yielding compound **4** (2.5 mg). Subfraction 6 (32.6 mg) was submitted to repeated column chromatography over silica gel with petroleum ether/acetone (1:1) as eluent, yielding compound **2** (2.5 mg).

3.3.1 (2E,6Z)-9,10-Dihydroxy-4-hydroxymethyl-2,6-decadiene (*1*)

Colorless oil; $[\alpha]_D^{31} + 39.2$ ($c = 0.36$, $CHCl_3$); UV ($CHCl_3$) λ_{max} (nm) (log ε): 231 (2.35), 206 (3.34); IR (KBr) ν_{max} (cm^{-1}): 3382, 2923, 2855, 1635; For ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 199.1337 $[M-H]^-$ (calcd for $C_{11}H_{19}O_3$, 199.1340).

3.3.2 (2E,6Z)-8,9,10-Trihydroxy-4-hydroxymethyl-2,6-decadiene (*2*)

Colorless oil; $[\alpha]_D^{31} + 23.8$ ($c = 0.67$, MeOH); UV (MeOH) λ_{max} (nm) (log ε): 231 (2.36), 206 (3.33); IR (KBr) ν_{max} (cm^{-1}): 3383, 2922, 2854, 1634; For ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 215.1287 $[M-H]^-$ (calcd for $C_{11}H_{19}O_4$, 215.1289).

3.3.3 (2E,6Z)-9-Hydroxy-4-hydroxymethyl-2,6-nonadiene (*3*)

Colorless oil; $[\alpha]_D^{31} + 52.5$ ($c = 0.57$, $CHCl_3$); UV ($CHCl_3$) λ_{max} (nm) (log ε):

233 (2.38), 206 (3.37); IR (KBr) ν_{max} (cm^{-1}): 3401, 2922, 2854, 1635; For ^{1}H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 169.1235 $[M-H]^-$ (calcd for $C_{10}H_{17}O_2$, 169.1234).

3.3.4 p-*Hydroxybenzyl alcohol (**4**)*

Colorless needles; mp 118–120°C; ^{1}H and ^{13}C NMR spectral data were consistent with the literature [5].

3.3.5 *Indolyl-3-carboxylic acid (**5**)*

Yellow powder; mp 215–217°C; ^{1}H and ^{13}C NMR spectral data were consistent with the literature [6].

3.4 Antibacterial activity

All compounds were tested for antibacterial activity against *S. aureus* and MRSA strains (obtained from Professor Kui Hong of the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences) by the filter paper disc agar diffusion method [7]. The strains were cultured using nutrient agar. Fifty microliters (10 mg/ml) of the compounds were impregnated on sterile filter paper discs (6-mm diameter), and then, aseptically applied to the surface of the agar plates. Ten microliters (0.08 mg/ml) of kanamycin sulfate were used as positive control. Then the diameters of inhibition zones were measured after 24-h incubation at room temperature. Experiments were done in triplicate, and the results were presented as mean values of the three measurements.

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