Research Paper

Tricycloalternarene derivatives produced by an endophyte *Alternaria alternata* isolated from *Maytenus hookeri*

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A new tricycloalternarene derivative, named tricycloalternarenal (**1**), together with three known compounds was isolated from the cultures of *Alternaria alternata*, an endophytic fungus in the leaves of the plant *Maytenus hookeri* (Celastraceae). Structure of **1** was established as (2*E*)-6-(3,3a,5,6,7,8,9,9a-octahydro-5-hydroxy-3a-methyl-8-oxo-cyclo-penta[b]chromen-1-yl)-2-methylhept-2-enal by spectroscopic methods, including 2D-NMR experiments. The remaining compounds were determined as tricycloalternarene-3b (**2**), alterperylenol (**3**), and dihydroalterperylenol (**4**).

Introduction

Endophytes are bacteria or fungi that live in the intercellular spaces of the tissues of host plants without causing discernible manifestation of disease (Strobel 2002, Strobel and Long 1998). Recently, endophytes have been recognized as important sources of a variety of structurally novel and biologically active secondary metabolites, including terpenoids, steroids, alkaloids, isocoumarin derivatives, quinines, and lipids (Strobel *et al.* 2004, Tan and Zou 2001, Zhang *et al.* 2006). Our ongoing research on new biologically active metabolites (e.g. maytansine) from endophytic microorganisms residing in *Maytenus hookeri* (Celastraceae), has shown that the culture material of some *Streptomyces* sp. on *M. hookeri* contained compounds that exhibited potent activities against several pathogenic micro-organisms (Zhao *et al.* 2005) and also had antineoplastic activity (Lu and Shen 2003).

The objective of our present research is to explore new bioactive substances from the endophytic fungus *Alternaria alternata* of *M. hookeri*; in particular, four compounds, including a new tricycloalternarene derivative,

tricycloalternarenal (**1**), along with tricycloalternarene-3b (**2**), alterperylenol (**3**), and dihydroalterperylenol (**4**), were isolated from the fermentation products of this fungus and characterized. In this paper, we describe the isolation and structure elucidation of this new isolate with a distinct isoprenoid side chain (Figure 1).

Materials and methods

General procedures

Mass spectra were measured using a VG Auto Spec-3000 spectrometer and Thermo Finnigan LCQ Advantage. NMR spectra were measured on Bruker DRX-500 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. IR spectra were obtained with a Bruker Tensor 27 FT-IR with KBr pellets. The reversed-phase (RP) C18 silica gel for column chromatography (CC) was obtained from Merck and the Sephadex LH-20 from Amersham Biosciences. Silica gel (200–300 mesh) for CC and silica gel GF$_{254}$ for TLC were purchased from Qingdao Marine Chemical Ltd. China.

Fungal material

The leaves of *M. hookeri* were collected at Xishuangbanna, Yunnan, P.R. China, in April 2003. The strain was isolated from the sterilized leaf of *M. hookeri*, iden-

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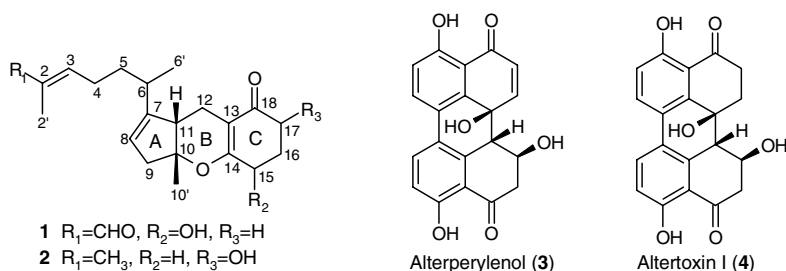
Figure 1. The structures of compounds **1–4**.

tified as *Alternaria alternata,* and deposited at the Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, China. The fungus was cultured using liquid medium: KNO_3 9.0 g, $CaCl_2$ 0.8 g, KH_2PO_4 0.2 g, KCl 0.05 g, $MgSO_4 \cdot 7\,H_2O$ 0.15 g, $FeSO_4 \cdot 7\,H_2O$ 0.05 g, $ZnSO_4 \cdot 7\,H_2O$ 0.02 g, glucose 40.0 g. The pH was adjusted to 5.5 before autoclaving. Fermentation was carried out in 1000 ml flasks containing 100 ml medium. After static cultivation for two weeks at 24 °C, the cultures were used for the extraction and isolation experiments below.

Extraction and isolation

The fermentation broth of *A. alternata* was filtered and the filtrate was exhaustively extracted with ethyl acetate three times. The crude organic fraction (0.885 g) was chromatographed over RP-C18 silica gel column with aqueous MeOH (30%, 50%, 65%, 85%, 100%) as elutent to provide five fractions designated A−E. Fraction D (0.258 g) was subjected to silica gel column, eluted with petroleum ether-acetone (100, 50:1, 30:1),

and the resulting portion (60 mg) obtained by eluting with petroleum ether-acetone (30:1) was subjected to Sephadex LH-20 (acetone) to give a residue (48 mg), followed by Sephadex LH-20 column (MeOH), and then subjected to RP-18 column by eluting with aqueous MeOH (55%, 60%, 65%, 70%) to give subfraction F−I. Subfraction H (32 mg) was further purified by preparative TLC (petroleum ether-ethyl aceate, 4:1 to give tricycloalternarene-3b (**2**, 10 mg). Fraction B (178 mg) was subjected to silica gel (12 g) CC with petroleum ether-ethyl aceate (5:1, 4:1, 2:1, 1:1) and pure MeOH to provide 10 subfractions (subfrs 1−10). The combined subfrs 4−6 (38 mg) were repeatedly chromatographed over Sephadex LH-20 with acetone to obtain alterperylenol (compound **3**, 10 mg), altertoxin I (compound **4**, 8 mg). Purification of subfrs 7 (18 mg) by preparative TLC with chloroform-acetone (5:1) yielded tricycloalternarenal (compound **1**, 2 mg).

Tricycloalternarenal (**1**) colorless oil; IR (KBr) $\nu_{\max}$ 3420, 1686, 1635 cm^{-1}; ^1H- and ^{13}C-NMR data, see Table 1; positive ESI-MS *m/z*: 344.20 $[M+H]^+$, 345.20, 346.21; posi-

Table 1. ^1H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Data of Compound **1** ($CDCl_3$, TMS, δ ppm).

| No.	δ_C	δ_H (*J* in Hz)	Selected HMBC	Selected ROESY
1	195.3 d	9.37 s	C-2′, 3	H-3
2	138.0 s		C-4, 2′	
2′	9.2 q	1.70 br.s	C-2, 3, 1	
3	154.3 d	6.44 t (*J* = 7.2)	C-2′, 4, 5, 1	H-5
4	27.0 t	2.27 m	C-2, 3	
5	33.5 t	1.48 m, 1.64 m	C-4, 6, 6′	H-6′, 8
6	32.4 d	2.02 m	C-6′, 5, 8, 7	H-4
6′	20.1 q	1.00 d (*J* = 6.8)	C-5, 7	H-9, 11
7	149.5 s		C-9, 12, 6, 6′	
8	120.3 d	5.35 m	C-9, 10, 6	H-5
9	44.9 t	2.50 m, 2.65 m	C-10, 8	H-6, 10′
10	88.6 s		C-8, 12, 10′	
10′	23.6 q	1.50 s	C-9, 11, 10, 7	H-11, 9
11	46.6 d	2.79 m	C-13	H-6′, 10′, 9
12	15.2 t	2.27 m, 2.60 m	C-18	
13	107.7 s		C-11, 12	
14	169.8 s		C-15, 12, 16	
15	66.4 d	4.35 m	C-14	H-17
16	28.9 t	1.95 m	C-15	
17	33.3 t	2.27 m, 2.60 m	C-15, 13, 18	
18	196.7 s		C-17, 16	

tive HR-ESI-MS *m/z*: 367.1885 (calcd. for $C_{21}H_{28}O_4Na$ 367.1936).

Results and discussion

Compound **1** was isolated as a colorless oil. Its molecular composition was determined to be $C_{21}H_{28}O_4$ by the quasimolecular ion peak at *m/z* 367.1885 [M+Na]$^+$ in the HR-ESI-MS and ^{13}C NMR spectra. The IR spectrum indicated the presence of both α,β-unsaturated aldehyde and α,β-unsaturated ketone at 1686 and 1635 cm^{-1}. Analysis of the ^{13}C NMR and DEPT spectra (Table 1) established 21 carbons in the molecule, consisting of three methyls, six methylenes, six methines, and six quaternary carbons. The 8 degree of unsaturation of the molecule indicated that compound **1** has three rings in addition to five double bonds mentioned above.

In the ^1H NMR spectrum, one methyl doublet of H-6′ (δ 1.00, J = 6.8 Hz) due to the coupling with the H-6 (δ 2.02) and two methyl singlets of H-10′ (δ 1.50) and H-2' (δ 1.70) were observed. In addition, an oxygenated methine proton was observed at δ 4.35 (m), and two olefinic protons were seen at δ 5.35 (m) and 6.44 (t, J = 7.2 Hz). These ^1H NMR data implied that compound **1** possessed the features of tricycloalternarene derivatives (Kono *et al.* 1986, Liebermann *et al.* 1997, Nussbaum *et al.* 1999)]. The ^1H and ^{13}C NMR data for compound **1** were very similar to those reported for a known analogue ACTG-toxin E, which was previously isolated from cultures of *Alternaria citri* and *A. alternata* (Kono *et al.* 1986, Liebermann *et al.* 1997, Nussbaum *et al.* 1999)]; however, the NMR spectra of compound **1**, compared with those of ACTG-toxin E, lacked a hydroxymethyl resonance, contained an aldehyde proton singlet (δ 9.37) in ^1H NMR spectrum and a carbon resonance (δ 195.3) in ^{13}C NMR spectrum. The ^1H and ^{13}C NMR data of compound **1** were unambiguously assigned by analysis of HMQC, HMBC, and ROESY experiments (Fig. 2, Table 1). The assignment of the aldehyde function was made from the HMBC correlations between H-3 and C-1, and H-1 and C-3. Moreover, HMBC correlation of H-15 (δ 4.35) with C-14 (δ 169.8) revealed that the secondary hydroxyl group in the ring C was placed at C-15.

The relative stereochemistries were determined with the aid of ROESY experiment (Fig. 2). The ROESY spectrum displayed NOE couplings between the H-1 and H-3, suggesting that the geometry of the double bond at C-2 in the side chain was *trans*. In addition, NOE correlation between H-10′ and H-11 (δ 2.79), and H-9β (δ 2.50) exhibited that the junction of A/B ring system in **1** was *cis*-fused. Additionally, the absolute configuration at
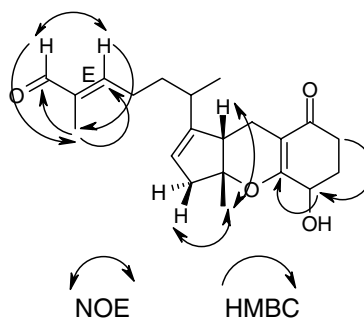
Figure 2. Key HMBC and selected ROESY correlations for compound **1**.

C-15 of compound **1** was determined as *R* because the CD spectrum of its analogue 15*R*-ACTG-toxin E exhibited a positive Cotton effect (Kono *et al.* 1986); this is corroborated by comparison of the ^1H and ^{13}C NMR data with C-15 of ACTG-toxin E reported in the literature (Kono *et al.* 1986), as well as from the biogenetic point of view (Kono *et al.* 1986, Liebermann *et al.* 1997, 2000). Therefore, the structure of compound **1** was elucidated as (2E)-6-(3,3a,5,6,7,8,9,9a-octahydro-5-hydroxy-3a-methyl-8-oxo-cyclopenta[b]chromen-1-yl)-2-methylhept-2-enal.

Comparison of the physicochemical properties and spectral data with reported values allowed for identification of additional compounds as tricycloalternarene-3b (**2**) [KONO et al, 1986], alterperylenol (**3**) (Okuno *et al.* 1983, Stack *et al.* 1986), and altertoxin I (dihydroalterperylenol, **4**) (Okuno *et al.* 1983, Stack *et al.* 1986, Hradil *et al.* 1989). Both **1** and **2** are tricycloalternarene-type metabolites, which appear to be characteristic of the genus *Alternaria*.

Species of the genus *Alternaria* (Fungi Imperfecti) such as *A. alternata* and its pathotypes have been known to produce a variety of secondary metabolites (Liebermann *et al.* 1997, Nussbaum *et al.* 1999), many of which are phytotoxic. The fungus *A. alternata* from *Brassica sinensis* yielded a series of tricycloalternarenes (Liebermann *et al.* 1997, Nussbaum *et al.* 1999). These three-isoprene unit-containing tricycloalternarenes are associated closely with ACTG-toxins (Kono *et al.* 1986, Liebermann *et al.* 2000). So far, the exact biological function of tricycloalternarenes has not been clarified. It is likely that together with other compounds, they serve as nonspecific phytotoxins.

Acknowledgements

References

Hradil, C.M., Hallock, Y.F., Clardy, J., Kenfield, D.S. and Strobel, G.A., 1989. Phytotoxins form *Alternaria cassiae*. Phytochemistry, **28**, 73−75.

Kono, Y., Gardner, J.M., Suzuki, Y. and Takeuchi, S., 1986. Studies on host-selective toxins produced by a pathotype of *Alternaria citri* causing brown spot disease of mandarins. Agric. Biol. Chem., **50**, 1597−1606.

Liebermann, B., Ellinger, R., Günther, W., Ihn, W. and Gallander, H., 1997. Tricycloalternarenes produced by *Alternaria alternata* related to ACTG-toxins. Phytochemistry, **46**, 297−303.

Liebermann, B., Nussbaum, R.P. and Günther, W., 2000. Bicycloalternarenes produced by the phytopathogenic fungus *Alternaria alternata*. Phytochemistry, **55**, 987−992.

Lu, C. and Shen, Y., 2003. A new macrolide antibiotic with antitumor activity produced by *Streptomyces* sp. CS, a commensal microbe of *Maytenus hookeri*. J. Antibiot., **56**, 415−418.

Nussbaum, R.P., Günther, W., Heinze, S. and Liebermann, B., 1999. New tricycloalternarenes produced by the phytopathogenic fungus *Alternaria alternata*. Phytochemistry, **52**, 593−599.

Okuno, T., Natsume, I., Sawai, K., Sawamura, K., Furusaki, A. and Matsumoto, T., 1983. Structure of agriculture and phytotoxin pigments produced by *Alternaria* sps. Tetrahedron Letters, **24**, 5653−5656.

Stack, M.E., Mazzola, E.P., Page, S.W., Pohland, A.E., Highet, R.J., Tempesta, M.S. and Corley, D.G., 1986. Mutagenic perylenequinone metabolites of *Alternaria alternata*: altertoxins I, II, and III. J. Nat. Prod., **49**, 866−871.

Strobel, G.A., 2002. Rainforest endophytes and bioactive products. Crit. Rev. Biotechnol., **22**, 315−333.

Strobel, G.A. and Long, D.M., 1998. Endophytic microbes embody pharmaceutical potential. ASM News, **64**, 263−268.

Strobel, G.A., Daisy, B., Castillo, U. and Harper, J., 2004. Natural products from endophytic microorganisms. J. Nat. Prod., **67**, 257−268.

Tan, R.X. and Zou, W.X., 2001. Endophytes: a rich source of functional metabolites. Nat. Prod. Rep., **18**, 448−459.

Zhang, H.W., Song, Y.C. and Tan, R.X., 2006. Biology and chemistry of endophytes. Nat. Prod. Rep., **23**, 753−771.

Zhao, P.J., Fan, L.M., Li, G.H., Zhu, N. and Shen, Y.M., 2005. Antibacterial and antitumor macrolides from *Streptomyces* sp. Is9131. Arch. Pharm. Res., **28**, 1228−1232.