Leptosphaeric Acid, a Metabolite with a Novel Carbon Skeleton from *Leptosphaeria* sp. IV403, an Endophytic Fungus in *Artemisia annua*

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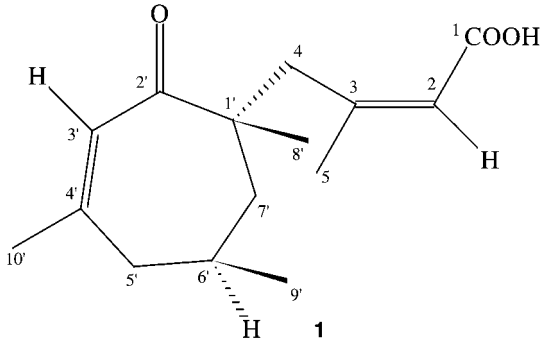
Leptosphaeric acid (**1**), a metabolite with a novel carbon skeleton, was isolated from the AcOEt extract of the culture of *Leptosphaeria* sp., an endophytic fungus (strain number: IV403) of *Artemisia annua*. The structure of acid **1** was elucidated on the basis of spectral analyses including homo- and heteronuclear correlation NMR experiments (HMQC and HMBC). The absolute configuration of the metabolite was determined by its CD and NOED studies.

1. Introduction. – Endophytes, microorganisms living inside the healthy plant tissue, have been confirmed to be a rich source of functional biomolecules [1]. In the continuation of our characterization of chemically new and/or biologically active metabolites from the cultures of endophytic fungi of *Artemisia* species [2–4], another metabolite with a new carbon framework named leptosphaeric acid (**1**) was obtained from the AcOEt extract of the re-culture broth of *Leptosphaeria* sp., an endophytic fungus (strain number: IV403) of *Artemisia annua*. We herewith present the establishment of the structure and absolute configuration of compound **1**.



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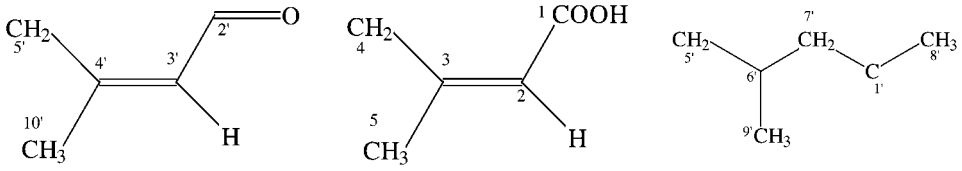
2. Results and Discussion. – Compound **1** was obtained as white gum. Its high-resolution ESI mass spectrum (positive-ion mode) showed intense quasimolecular ions at *m/z* 251.1641 ($[M+H]^+$) and 273.1640 ($[M+Na]^+$), respectively, corresponding to the molecular formula $C_{15}H_{22}O_3$ (the index of unsaturation: 5), which was well consistent with a total of 15 discrete signals in its ^{13}C-NMR spectrum edited by the DEPT pulse sequences. The presence of a conjugated COOH group and an α,β-unsaturated ketone was implied by the UV peak at 242 nm, and the IR absorption

bands at 3500–2500, 1713.3, 1692.9, 1668.4, and 1642.9 cm^{-1}, indicative of two C=O groups (C(1) and C(2′)) and a pair of C=C bonds (between C(2) and C(3) as well as between C(3′) and C(4′)) disclosed also by the ^{13}C-NMR spectrum. Subtraction of the four indexes of unsaturation involved in the above-mentioned moieties from that of the molecule demonstrated that compound **1** had to be monocyclic.

The conclusion were drawn on the basis of detailed NMR analyses (DEPT, HMQC, HMBC, and NOED) leading to the unambiguous assignments of ^{1}H- and ^{13}C-NMR data (*Table*). The discerned HMBC correlations (*Table*), along with coupling constants and ^{13}C-NMR data, established three substructures **A**, **B**, and **C** (the C-atoms were numbered in the same manner as for **1**). Specifically, the HMBC correlations of H–C(3′) to C(5′) and C(10′), of H–C(10′) with C(3′) and C(5′), and of H–C(5′) to C(3′) demonstrated the partial structure **A**, while substructure **B** was deduced from the HMBC correlations of H–C(2) to C(4) and C(5), and of H–C(5) to C(2) and C(4). Furthermore, the fragment **C** afforded by the sequential couplings from H–C(5′) to H–C(7′) (*Table*) was also reinforced by the HMBC correlations of H–C(7′) with C(5′), of H–C(6′) to C(1′), of H–C(9′) to C(7′), and of H–C(8′) with C(7′). The substructure **A** sharing a pivotal CH_2 (C(5′)) with fragment **C** established the connection between the two subunits. In view of the imperative presence of a monocycle in the molecule, both C(2′) in substructure **A** and C(4) in fragment **B** had to



A **B** **C**

Table. *^{1}H- and ^{13}C-NMR Data* ($CDCl_3$) *of Compound* **1**

Position	$\delta_{(C)}$ (DEPT)	$\delta_{(H)}$ (*J* in Hz)	HMBC
1	170.3 (C)		
2	128.9 (CH)	5.93 (br. *s*)	H–C(5)
3	167.4 (C)		H–C(5), H–C(8′)
4	42.2 (CH_2)	2.33 (br. *s*)	H–C(2), H–C(5), H–C(8′)
5	20.5 (Me)	2.00 (br. *s*)	H–C(2)
1′	42.4 (C)		H–C(6′)
2′	198.6 (C)		H–C(4)
3′	115.0 (CH)	5.74 (br. *s*)	H_α–C(5′), H_β–C(5′), H–C(10′)
4′	162.3 (C)		H_α–C(5′), H–C(10′)
5′	36.1 (CH_2)	2.29 (*dd*, *J* = 13.2, 5.7, H_α), 2.37 (*dd*, *J* = 13.2, 9.9, H_β)	H–C(3′), H_β–C(7′), H–C(10′)
6′	34.0 (CH)	1.88 (*m*)	
7′	34.5 (CH_2)	1.73 (br. *d*, *J* = 10.8, H_α), 1.79 (*t*, *J* = 10.8, H_β)	H–C(4), H–C(8′), H–C(9′)
8′	19.6 (Me)	1.09 (br. *s*)	
9′	15.7 (Me)	1.01 (*d*, *J* = 6.3)	
10′	19.9 (Me)	2.22 (br. *s*)	H–C(3′)

be anchored at the quaternary C-atom (C(1′)) in subunit **C**. As anticipated, this was also in accordance with the HMBC correlation of H–C(4) to C(2′), and the observed NOE effect between H–C(4) and H–C(8′). Regarding the geometrical configuration of the C=C bonds, both 2,3-dihydro and 3′,4′-dihydro olefins were shown to be (*Z*)-configured by the NOE enhancements discerned between H–C(2) and H–C(5), as well as between H–C(3′) with H–C(10′) (*Fig. 1*). In conclusion, the planar structure of metabolite **1** is 3-methyl-4-(1,4,6-trimethyl-2-oxocyclohept-3-enyl)but-2-enoic acid, which possesses a hitherto undescribed C skeleton.

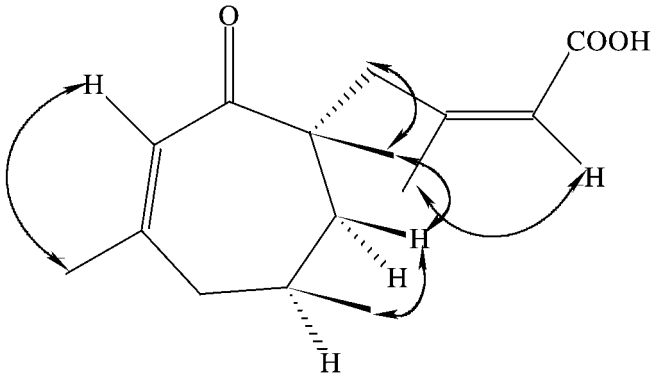


Fig. 1. *Key NOED correlations of compound* **1**

The absolute configuration of compound **1** was established by its circular dichroism (CD) spectrum and NOE difference spectroscopy. A negative *Cotton* effect at 320 nm ($\Delta\varepsilon = -0.121$) ascribable to the $n \rightarrow \pi^*$ transition of conjugated ketone (C(2′)) allowed the assignment of (1′*S*) for metabolite **1** [4–6]. In the NOE difference spectrum of compound **1**, pronounced NOE enhancements were observed with H_β–C(7′) *triplet* at δ 1.79 ($J(6',7'\beta) = 10.8$ Hz) upon irradiating both H–C(8′) *singlet* at δ 1.09 and H–C(9′) *doublet* ($J(6',9') = 6.3$ Hz) at δ 1.01, allowing the assignment of the (6′*R*)-configuration for acid **1**.

Based on the above information, a computer-generated plot for the 3D structure of **1** (*Fig. 2*) was obtained according the process reported in [4]. The calculated distances between H–C(3′)/H_α–C(10′) (3.387 Å), H–C(3′)/H_β–C(10′) (2.345 Å), H–C(3′)/H_p–C(10′) (3.501 Å), H–C(2)/H_α–C(4) (3.502 Å), H–C(2)/H_β–C(4) (2.291 Å), H–C(2)/H_p–C(4) (3.281 Å), H_β–C(7′)/H_α–C(8′) (3.663 Å), H_β–C(7′)/H_β–C(8′) (2.704 Å), H_β–C(7′)/H_p–C(8′) (2.493 Å), H_β–C(7′)/H_α–C(9′) (2.990 Å), H_β–C(7′)/H_β–C(9′) (2.513 Å), H_β–C(7′)/H_p–C(9′) (3.741 Å), H_β–C(4)/H_α–C(8′) (3.234 Å), H_β–C(4)/H_β–C(8′) (3.867 Å), H_β–C(4)/H_p–C(8′) (2.671 Å), H_α–C(4)/H_α–C(8′) (3.757 Å), H_α–C(4)/H_β–C(8′) (4.386 Å), and H_α–C(4)/H_p–C(10′) (3.829 Å), are less than 4.00 Å, consistent with the well-defined NOEs observed for each of these H-atom pairs. Thus, the structure of **1** is (2*Z*)-3-methyl-4-[(1*S*,6*R*,3*Z*)-1,4,6-trimethyl-2-oxocyclohept-3-enyl]but-2-enoic acid.

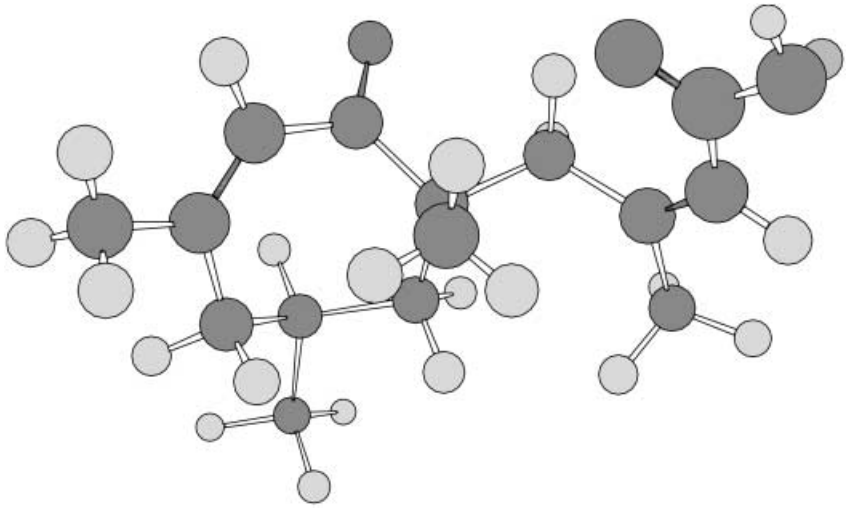


Fig. 2. *3D Structure of compound* **1**

Experimental Part

General. All chemicals used in the study were of anal. grade. Optical rotations: *JASCO DIP-181* spectrometer. UV Spectra: *Hitachi U-3000* spectrophotometer. CD Spectrum: *J-20C* automatic spectropolarimeter. IR Spectra: *Nexus 870 FT-IR*, ν in cm^{-1}. ^1H- and ^{13}C-NMR, DEPT, HMQC, HMBC, NOED Spectra: *Bruker DPX-300* spectrometer; at 300 and 75 MHz, resp.; δ in ppm rel. to Me_4Si as an internal standard, coupling constants *J* in Hz. HR-ESI-MS (positive-ion mode): *VG-ZAB-HS* mass spectrometer.

Material. Leptosphaeria sp., strain number IV403, is an endophytic fungus isolated from fresh stems of apparently healthy *Artemisia annua* collected in May, 1997, in the suburb of Nanjing, China [7]. The re-culture broth of *Leptosphaeria* sp. was obtained according to the process reported in [4].

Extraction and Isolation. The culture filtrate (total volume 60 l) and mycelium were extracted with AcOEt. Evaporation of solvent *in vacuo* gave a residue (11.0 g), which was then subjected to chromatography on silica-gel column (250 g, 200–300 mesh) eluting with petroleum ether/acetone gradient (1:0–0:1) to give five fractions (*Fr. 1:* 5.1 g; *Fr. 2:* 1.3 g; *Fr. 3:* 1.7 g; *Fr. 4:* 2.1 g; *Fr. 5:* 0.7 g). *Fr. 4* was subjected to silica-gel chromatography (60 g, 200–300 mesh) with $CHCl_3$/MeOH (15:1 → 5:1). Three fractions were obtained (*Fr. 4.1:* 0.8 g; *Fr. 4.2:* 0.6 g; *Fr. 4.3:* 0.6 g). Further CC separation of *Fr. 4.2* over silica gel with $CHCl_3$/MeOH (8:1; 0.3 l), followed by repeated gel filtration over *Sephadex LH-20* with $CHCl_3$/MeOH 1:1 gave **1** (4 mg).

Leptosphaeric Acid (=*(2Z)-3-Methyl-4-[(1S,6R,3Z)-1,4,6-trimethyl-2-oxocyclohept-3-enyl]but-2-enoic Acid*, **1**: white gum. $[\alpha]_D^{25} = +63.0$ ($CHCl_3$, $c = 0.073$); UV: ($CHCl_3$) λ_{max} 242; IR: 3500–2500 (br.), 2957.4, 2922.7, 1713.3, 1692.9, 1668.4, 1642.9, 1456.5, 1379.1, 1252.7. CD ($c = 0.10$ mg/ml in $CHCl_3$): λ 320 ($\Delta\varepsilon - 0.121$). ^1H- and ^{13}C-NMR (300 and 75 MHz, resp.; $CHCl_3$): see *Table*. HR-ESI-MS: 251.1641 ($[M+H]^+$, $C_{15}H_{23}O_3^+$; calc. 251.1642).

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