

A New Ergosterol-Type Steroid Isolated from the *Nicotiana tabacum*-Derived Endophytic Fungus *Aspergillus* sp. TE-65L

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Abstract: Chemical investigation on the fungal strain *Aspergillus* sp. TE-65L, which was previously isolated from cultivated tobacco (*Nicotiana tabacum* L.), led to the production of four steroids including a new ergosterol-type steroid, namely aspergosterol A (**1**). Their chemical structures were established by detailed spectroscopic analysis of 1D/2D NMR and HRESIMS data. Compound **1** was found to possess anti-phytopathogenic activities against *Alternaria alternate*, *Botrytis cinerea*, and *Fusarium oxysporum*, with MIC values of 4, 8, and 16 µg/mL.

Keywords: *Aspergillus* sp.; endophytic fungus; secondary metabolite; steroid; antifungal activity. © 2023 ACG Publications. All rights reserved.

1. Fungal Source

The endophytic fungus *Aspergillus* sp. TE-65L was isolated from inner leaves of cultivated tobacco, *N. tabacum* L., which was harvested in Hubei province (coordinated at 108°23'12"–110°38'08" E, 29°07'10"–31°24'13" N) in August 2016. The fungus was initially identified via morphological inspection and sequence amplification of the ITS-rDNA region, which exhibited a 99% similarity to that of *Aspergillus* sp. with a GenBank accession number of OQ629860 (This fungus could not be identified at the species level by ITS sequencing alone). The axenic strain was preserved in 40 % glycerol (v/v) at –80 °C and deposited at Tobacco Research Institute of Chinese Academy of Agricultural Sciences.

2. Previous Studies

Endophytes harmoniously living in healthy tissues of host plants have been considered as an important source of unexplored microorganisms capable of generating diversified specialised metabolites [1,2]. These novel metabolites, which were classified into alkaloids, quinones, steroids,

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terpenoids, peptides, and polyketides, possess not only intriguing chemical structures but also remarkable bioactivities [3,4]. Therefore, endophytes are being comprehensively prospected to explore new potential chemical entities for various therapeutic purposes [5]. Endophytic *Aspergillus* genus consisting of 378 species possess the capacity to produce a large number of specialised metabolites, such as anthraquinones, butenolides, cytochalasins, diphenyl ethers, diketopiperazines, steroids, terpenes, and xanthenes [6]. Among them, steroids representing a class of evolutionarily conserved lipid natural products have gained great attention due to their potential anti-tumor, anti-inflammatory, antiviral, and anti-obesity activities [7].

3. Present Study

In our continuous investigations on novel specialised metabolites from endophytic fungi [8–10], a fungal strain *Aspergillus* sp. TE-65L was isolated from cultivated tobacco (*Nicotiana tabacum* L.). Previous screening experiments indicated that this fungal strain TE-65L showed promising antifungal activity against *Botrytis cinerea* with an inhibitory zone diameter of 12 mm. Therefore, to search for novel antifungal specialised metabolites, this fungus was further selected for detailed chemical study. This fungus was statically cultivated in malt extract medium utilizing 100 × 1 L Erlenmeyer flasks at 28°C. A total of 30 L cultures were obtained after 30 days. Then, the whole fermented cultures (containing both broth and mycelia) were adequately extracted with EtOAc to afford a crude extract of 15.8 g, which, in turn, subjected to silica gel vacuum liquid chromatography using a stepwise gradient of petroleum ether (PE)-EtOAc (eluting from 30:1 to 1:1, v/v) and CH₂Cl₂-MeOH (eluting from 20:1 to 1:1, v/v). Seven fractions, namely Fr.1–Fr.7, were obtained subsequently. Fr.4 (1.5 g), eluted with PE-EtOAc 1:1, was further separated by open silica gel column chromatography (CC) using CH₂Cl₂-MeOH mixtures (from 40:1 to 10:1, v/v) to yield four subfractions Fr.4.1–Fr.4.4. Compound **2** (9.0 mg) and **4** (2.9 mg) were isolated from Fr.4.1 and Fr.4.3 by Sephadex LH-20 (MeOH), respectively. Fr.5 (3.0 g), eluted with CH₂Cl₂-MeOH 20:1, was initially separated by CC using PE-EtOAc mixtures from 30% to 100% (v/v) to yield three subfractions Fr.5.1–Fr.5.3. Fr.5.2 was separated by preparative thin-layer chromatography (plate: 20 × 20 cm; CH₂Cl₂-MeOH 20:1, v/v), followed by Sephadex LH-20 (MeOH) to finally afford the new ergosterol-type steroid **1** (9.8 mg). Fr.5.3 was separated by preparative thin-layer chromatography (plate: 20 × 20 cm; CH₂Cl₂-acetone 20:1, v/v) to yield compound **3** (3.8 mg).

Aspergosterol A (1): Colorless oil; $[\alpha]_D^{20} +16.8$ ($c = 0.10$, MeOH); UV (MeOH): λ_{\max} (log ϵ): 196 (3.31), 220 (3.77), 278 (3.98) nm; ¹H (500 MHz) and ¹³C (125 MHz) NMR data, see Table 1; (-)-HRESIMS: m/z 443.3145 [$M - H$]⁻ (calcd for C₂₈H₄₃O₄, 443.3161).

Antifungal assay: The antifungal activities against six phytopathogenic fungi—*Fusarium oxysporum* (Schl.) F.sp *cucumerinum* Owen, *Alternaria alternata* (Fries) Keisslar, *Fusarium graminearum* schw., *Alternaria mali* rob., *Colletotrichum orbiculare* Arx., and *Botrytis cinerea* Pers were determined in 96-well microtiter plates using the broth microdilution method reported previously [11,12]. The chemical pesticide carbendazim was used as the positive control.

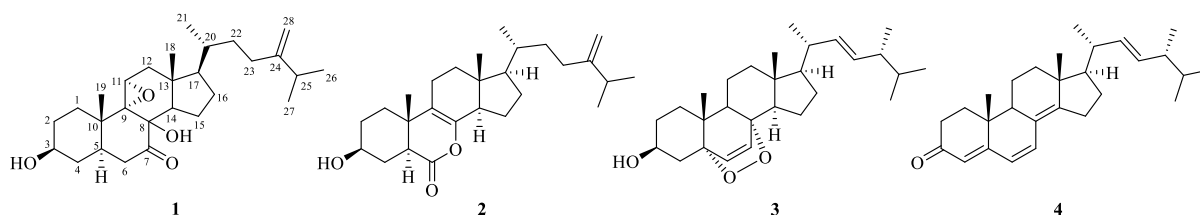


Figure 1. Chemical structures of the isolated steroids

Aspergosterol A (**1**) (Figure 1) was isolated as colorless oil and was assigned the molecular formula of C₂₈H₄₄O₄ by analysis of the negative-mode HRESIMS data at m/z 443.3145 [$M - H$]⁻

(calcd for C₂₈H₄₃O₄, 443.3161). The ¹H NMR and ¹³C NMR data of **1** (Table 1) revealed the presence of discernable signals corresponding to a keto carbonyl group at δ_C 208.1 (C-7), one terminal double bond at δ_H 4.72 (s, H-28 α) and 4.65 (s, H-28 β) as well as at δ_C 106.1 (C-28) and 156.6 (C-24), two oxygenated methines at δ_C 70.0 (C-3) and 60.5 (C-11), two oxygenated unprotonated carbons at δ_C 77.1 (C-8) and 70.2 (C-9), and five methyl groups at δ_H 0.74 (s, H₃-18), 0.94 (s, H₃-19), 0.91 (d, J = 6.5 Hz, H₃-21), 1.03 (d, J = 3.0 Hz, H₃-26), and 1.01 (d, J = 3.0 Hz, H₃-27) and at δ_C 15.6 (C-18), 17.4 (C-19), 18.5 (C-21), 21.9 (C-26), and 21.8 (C-27). These characteristic signals and functionalities resembled those of tennessoid A [7] and **1** was therefore deduced to be an ergosterol-type steroid.

To determine the planar structure of **1**, the ¹H-¹H COSY and HMBC experiments (Figure 2) were further analyzed in detail. ¹H-¹H COSY correlations between H₂-1 and H₂-6, between H-11 and H₂-12, between H-14 and H₂-23, and between H-25 and H₃-27 indicated the presence of four spin systems as shown in Figure 2. Furthermore, distinct HMBC correlations from H₂-1 to C-3 and C-9, from H₂-2 to C-4, from H₂-4 to C-10, from H-5 to C-7, from H₂-6 to C-8, from H₂-12 to C-9, C-14, and C-17, from H-14 to C-7, from H₃-18 to C-14 and C-17, as well as from H₃-19 to C-1 and C-5 resulted in the determination of a 6/6/6/5 tetra-carbocyclic core substituted with two angular methyl groups (C-18 and C-19) (Figure 2). HMBC correlations from H₂-28 to C-23 and C-25 as well as from H₃-26/H₃-27 to C-24 linked the above-mentioned two separate spin systems of H-14 to H₂-23 and H-25 to H₃-26/H₃-27. A C₉ side chain was thus determined to locate at C-17. Finally, on the basis of the noteworthy down-fielded chemical shifts of C-9 (δ_C 70.2) and C-11 (δ_C 60.5), together with the molecular weight of **1**, an oxygen bridge was deduced between C-9 and C-11. Based on these analyses, the planar structure of **1** was determined. Compound **1** was named as aspergosterol A.

The stereochemistry of **1** was established on the basis of its NOESY spectrum. Diagnostic NOE correlations between H-11 and H₃-19/H-1 α and between H₃-18 and H-20 indicated that these protons were oriented at the same side of the 6/6/6/5 tetracyclic skeleton, while in turn, NOE correlations between H-1 β and H-3/H-5, between H-3 and H-5, and between H-14 and H-17 indicated that these protons were oriented at the opposite side (Figure 2). However, the stereochemistry of the OH group at C-8 was undetermined due to the high flexibility of OH group.

Table 1. ¹H and ¹³C NMR data for compound **1** (measured in CDCl₃, δ in ppm)

No.	δ_H (mult., J in Hz)	δ_C , type	No.	δ_H (mult., J in Hz)	δ_C , type
1	1.46, m; 1.13, m	30.2, CH ₂	15	2.34, m; 1.54, m	18.4, CH ₂
2	1.84, m; 1.42, m	30.2, CH ₂	16	1.86, m; 1.34, m	27.8, CH ₂
3	3.65, m	70.0, CH	17	1.13, m	56.3, CH
4	1.85, m; 1.25, m	37.4, CH ₂	18	0.74, s	15.6, CH ₃
5	3.01, ddd (14.4, 7.2, 3.6)	34.7, CH	19	0.94, s	17.4, CH ₃
6	2.82, dd (17.7, 7.2); 1.99, dd (17.7, 10.9)	43.7, CH ₂	20	1.39, m	34.9, CH
7		208.1, C	21	0.91, d (6.5)	18.5, CH ₃
8		77.1, C	22	1.54, m; 1.15, m	34.2, CH ₂
9		70.2, C	23	2.06, m; 1.88, m	30.8, CH ₂
10		36.3, C	24		156.6, C
11	3.26, br d (6.0)	60.5, CH	25	2.21, m	33.7, CH
12	2.12, m; 1.66, m	40.1, CH ₂	26	1.03, d (3.0)	21.9, CH ₃
13		42.9, C	27	1.01, d (3.0)	21.8, CH ₃
14	1.60, m	52.7, CH	28	4.72, s; 4.65, s	106.1, CH ₂

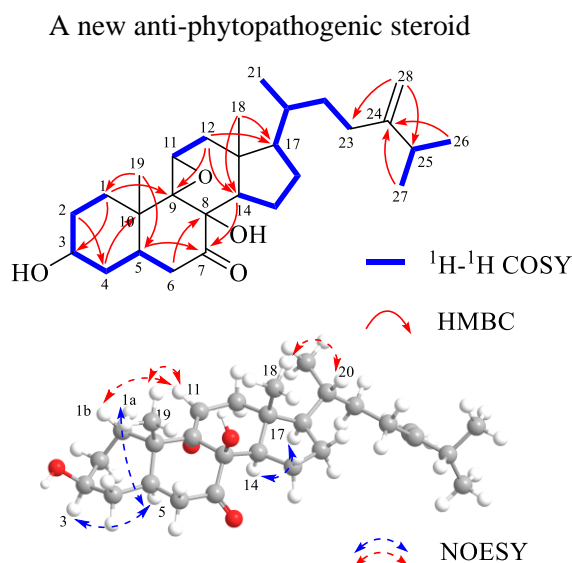


Figure 2. Key $^1\text{H}-^1\text{H}$ COSY, HMBC, and NOE correlations for **1**

The antifungal activities against six phytopathogenic fungi—*Fusarium oxysporum* (Schl.) F.sp *cucumerinum* Owen, *Alternaria alternate* (Fries) Keisslar, *Fusarium graminearum* schw., *Alternaria mali* rob., *Colletotrichum orbiculare* Arx., and *Botrytis cinerea* Pers were determined in 96-well microtiter plates using the broth microdilution method. As shown in Table 2, **1** was found to possess potent anti-phytopathogenic activities against *Alternaria alternate*, *Botrytis cinerea*, and *Fusarium oxysporum*, with MIC values of 4, 8, and 16 $\mu\text{g}/\text{mL}$, respectively, when compared with the positive control carbendazim.

Table 2. Results of antifungal activities (MIC, $\mu\text{g}/\text{mL}$)

Compounds	<i>F. oxysporum</i>	<i>A. alternate</i>	<i>F. graminearum</i>	<i>A. mali</i>	<i>C. orbiculare</i>	<i>B. cinerea</i>
1	16	4	>64	>64	>64	8
carbendazim	16	8	8	8	4	8

In conclusion, four steroids including a new ergosterol-type steroid aspergosterol A (**1**) were isolated and identified from the endophytic fungus *Aspergillus* sp. TE-65L derived from *Nicotiana tabacum* L. Following detailed spectroscopic analysis, their structures were finally determined. Previous studies indicated that steroids possessed various biological activities. In this study, the new steroid **1** was found to possess certain anti-phytopathogenic activities against plant phytopathogenic fungi, suggesting high potential for the development of biopesticides.

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Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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