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Phomopsterones A and B, Two Functionalized Ergostane-Type Steroids from the Endophytic Fungus *Phomopsis* sp. TJ507A

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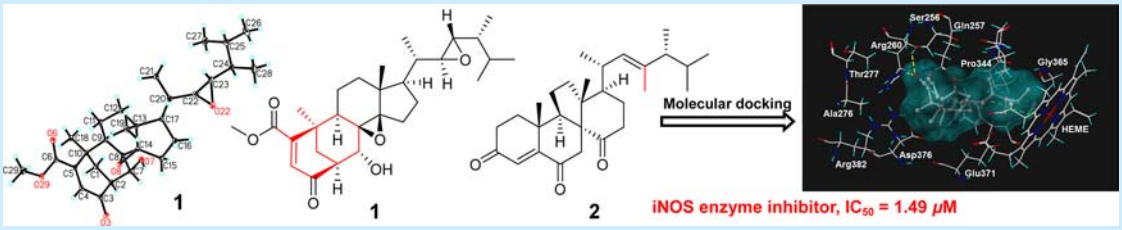
Supporting Information



ABSTRACT: Two new functionalized ergostane-type steroids, phomopsterones A (**1**) and B (**2**), were isolated from the plant-derived *Phomopsis* sp. TJ507A. Their structures were determined on the basis of spectroscopic data, a modified Mosher's method, X-ray crystallographic analysis, and quantum chemical calculations. Compound **1** is an unprecedented ergosteroid featuring a rearranged bicyclo[3.3.1]nonane motif resulting from B-ring scission and a subsequent 180° rotation of the ring A during biosynthesis. Compound **2** exhibited anti-inflammatory activity.

Steroids are a remarkable group of the most important small molecules in biology since they are evolutionarily conserved lipid biomolecules in the plasma membranes of eukaryotes, have low toxicity, low vulnerability to multidrug resistance (MDR), and high bioavailability, and play diversiform cellular roles related to membrane structure and signaling.[1] Over the past few decades, as the second largest marketed drug category, steroid-based drugs have been widely applied in clinical practice for the therapy of a wide range of diseases, largely due to their anti-inflammatory activity.[2] It is worth noting that different cleavage patterns within the tetracyclic steroidal systems of natural origin have given rise to the generation of many skeletally diverse steroids, as exemplified by 3,4-, 5,6-, 8,9-, 8,14-, 9,10-, 9,11-, 11,12-, 13,14-, and 13,17-secosterioids.[3−6] Therefore, the chemical and pharmacological diversities of steroids have resulted in continuous interest from chemists and biologists.[7]

The terrestrial- and marine-derived fungi that belong to the genus *Phomopsis* are known for their powerful biosynthetic capabilities to produce structurally diverse secondary metabolites, such as steroids,[8] terpenoids,[9] cytochalasins,[10] and polyketides,[8b,11] which display a broad spectrum of bioactivities, such as antimicrobial, antitumor, anti-inflammatory, antiangiogenesis, and antiviral effects.

In our continuous endeavor to search for structurally fascinating and bioactive natural products from fungi,[12] the fungus *Phomopsis* sp. TJ507A, which was originally isolated from the medically important plant *Phyllanthus glaucus*, was analyzed. Two functionalized ergostane-type steroids, phomopsterones A (**1**) and B (**2**), and a biosynthetically related known steroid (**3**)[13] were isolated (Figure 1). Structurally, phomopsterone A (**1**), a highly oxygenated and unprecedented rearranged ergostane-type steroid, features a unique bicyclo[3.3.1]nonane motif with an α-oriented Me-19 group, which results from B-ring scission and a rotation of 180° by ring A during biosynthesis[14] and is confirmed by X-ray crystallographic analysis, shedding new light on steroid biosynthesis. Phomopsterone B (**2**) represents the first example of a C_{29}-steroid possessing a 13(14 → 8)-*abeo*-8-ergostane skeleton. This paper addresses the details of the chemical and biological characterization and possible biogenetic pathway of these compounds.

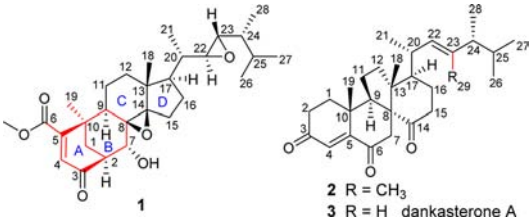


Figure 1. Structures of compounds **1**−**3**.

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Phomopsterone A (**1**) was obtained as colorless crystals with the molecular formula $C_{29}H_{42}O_6$, as inferred from its ^{13}C NMR and HRESIMS ($[M + Na]^+$, *m/z* 509.2879, calcd for 509.2879) data, corresponding to nine sites of unsaturation. The IR spectrum of **1** revealed the presence of hydroxyl (3499 cm^{-1}) and carbonyl (1728 and 1668 cm^{-1}) functionalities. The ^1H NMR data (Table S1) showed characteristic resonances of three singlet methyl groups at δ_H 0.79 (H_3-18), 1.36 (H_3-19), and 3.75 (OCH_3); three oxygenated methine groups at δ_H 3.51 (br s, H-7), 2.54 (dd, *J* = 2.2, 8.0 Hz, H-22), and 2.43 (dd, *J* = 2.2, 7.8 Hz, H-23); and an olefinic proton at δ_H 6.59 (s, H-4). The ^{13}C NMR and DEPT data of **1** (Table S1) revealed 29 carbon resonances that were ascribed to a methoxy group, six methyl groups, five methylene groups, ten methine groups (including three oxygenated and one olefinic), and seven quaternary carbons (including two oxygenated, one olefinic, one ketone, and one ester carbonyl). Among them, two olefinic carbons and two carbonyl carbons accounted for three out of nine sites of unsaturation, suggesting that compound **1** possessed a hexacyclic ring system.

All proton resonances were assigned to their respective carbons via the HSQC spectrum. Referring to the NMR and HRESIMS data of previously reported ergostane-type steroids[8] from the genus *Phomopsis* prompted us to consider that compound **1** was an unusual rearranged ergosteroid comprising two subunits, A and B, which were finally ascertained as follows (Figure 2).

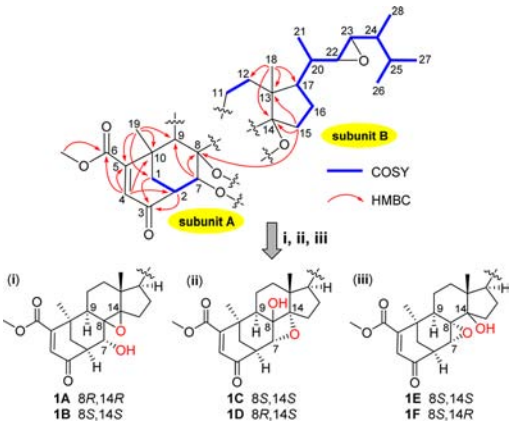

Figure 2. Structural elucidation of **1** based on NMR analysis.

The HMBC correlations from H-1 and H-2 to C-3; from H-7 to C-8 and C-9; from H-4 to C-2, C-5, C-6, and C-10; from H_3-19 to C-1, C-5, C-9, and C-10; and from the OCH_3 group to C-6, coupled with $^1H-^1H$ COSY cross-peaks of H_2-1/H-2/H-7, confirmed the presence of subunit A, which constructed a unique bicyclo[3.3.1]nonane motif. In addition, subunit B was confirmed by the $^1H-^1H$ COSY spectrum of two independent spin systems, incorporating H_2-11/H_2-12 and H_2-15/H_2-16/H-17/H-20 (H-20/H_3-21)/H-22/H-23/H-24 (H-24/H_3-28)/H-25 (H-25/H_3-27)/H_3-26 and the HMBC correlations from H_3-18 to C-12, C-13, C-14, and C-17 and from H_2-15 to C-13 and C-14. The fusion of subunits A and B was identified at C-8 and C-9 based on the $^1H-^1H$ COSY correlation between H-9 (δ_H 2.13) and H_2-11 (δ_H 1.34 and 1.63) and the HMBC correlation from H-15 (δ_H 1.95) to C-8 (δ_C 64.5).

In addition to the A/B/C/D-ring systems, the molecular formula $C_{29}H_{42}O_6$ and two additional rings of **1** suggest the presence of two epoxy groups, wherein one should be generated between C-22 and C-23 on account of a similar spin−spin coupling ($^3J_{H\text{-}22,23}$ = 2.2 Hz) to the reported values,[15] indicating a *trans* relationship between H-22 and H-23. However, how two of the oxygenated carbons at C-7, C-8, and C-14 constructed the remaining one-ring system was puzzling. Theoretically, three possible planar structures (i, ii, and iii, as shown in Figure 2) were proposed because it was difficult to determine the connections of these carbons by only interpreting the NMR data.

A NOESY experiment (Figure S11) was conducted to reveal the partial relative configuration of **1**, in which the cross-peaks of H-9 (δ_H 2.13)/Me-19 (δ_H 1.36), H-1α (δ_H 2.32)/H-9/H-12α (δ_H 1.07)/H-17 (δ_H 1.54), H-12β (δ_H 1.07)/Me-18 (δ_H 0.79), and H-1α/H-2 (δ_H 2.75)/H-1β (δ_H 2.10) demonstrated that H-2, H-9, H-17, and Me-19 are cofacial and α-directional, whereas Me-18 has an opposite β-orientation. The singlet of H-7 (δ_H 3.51, br s) with no recognizable vicinal coupling constant (~0 Hz) to H-2 suggests that H-2 and H-7 possess a *trans*-relationship with a dihedral angle of approximately 90°. Thus, H-7 was determined to be β-directional.

The relative configurations of C-20 to C-24 on the side chain were ascertained to be 20*S**/22*S**/23*S**/24*R** via conformational analysis (Figure S32) based on the NOESY correlations, the coupling constants, and the *trans*-relationship between H-22 and H-23.

Taking an overall consideration, only the quaternary carbons C-8 and C-14 could not be assigned a relative configuration. Therefore, a total of six possible structures (**1A**−**F**) were deduced to exist (Figure S30), whose relative configurations were determined using computer-generated 3D drawing and molecular structure model based on ball-and-stick methods. Furthermore, a combination of NOESY interpretations and calculated ^{13}C NMR chemical shifts at the B3LYP/6-311++G(d,p) level of structures **1A**−**F** confirmed that the planar structure and relative configuration of **1** were determined to be the same as those of structure **1A** (for detailed analysis, see the Supporting Information).

To define its absolute configuration, the (*S*)- and (*R*)-MTPA esters of **1** were prepared, and significant $\Delta\delta_H$ values ($\Delta\delta_H = \delta_{S\text{-MTPA-ester}} - \delta_{R\text{-MTPA-ester}}$) of the proton signals adjacent to C-7 were observed (Figure S3). Referring to the rule of a modified Mosher's method,[16] the absolute configuration of C-7 was deduced to be *R*. Thus, the absolute structure (2*S*,7*R*,8*R*,9*R*,10*R*,13*R*,14*R*,17*R*,20*S*,22*S*,23*S*,24*R*) of **1** was determined.

The highly oxygenated and rearranged architecture and multiplex chiral centers of **1** prompted us to attempt to confirm the above deduction using crystallography. After repeated attempts, a suitable crystal of **1** was obtained from chloroform, which was subsequently subjected to a single-crystal X-ray diffraction experiment with Cu Kα radiation (Figure 3), supporting our conclusion regarding its absolute structure on the basis of a Flack parameter of −0.04(8) (CCDC 1503043).[17] These assignments were identical to the result obtained using a combined ECD strategy (Figure S4), in which the calculated ECD spectrum using a time-dependent density functional theory (TDDFT) method at the B3LYP/6-311++G** level with PCM in methanol showed a good fit with the experimental plot of **1**, further confirming its absolute configuration.

Phomopsterone B (**2**) was obtained as colorless prisms. The molecular formula $C_{29}H_{42}O_3$ was determined collectively on the basis of its HRESIMS and NMR data, indicative of nine sites of unsaturation. Comparison of the ^1H and ^{13}C NMR data of **2** (Table S1) with those of **3**,[13] which was confirmed by single-

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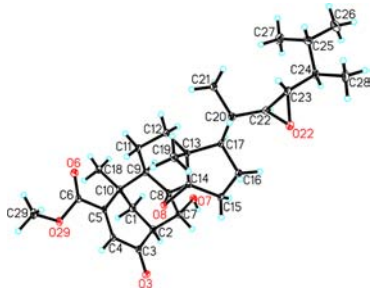


Figure 3. X-ray crystallographic structure of **1**.

crystal X-ray diffraction, revealed structural similarities with regard to the A/B/C/D ring systems and substituent patterns, with the only difference being that a C-23 olefinic proton in **3** is replaced by a singlet methyl in **2**, as confirmed by the HMBC correlations of H_3-29 with C-22, C-23, and C-24. 2D NMR techniques (Figure S33) further verified the planimetric map and relative structure of **2**. The agreement between the experimental ECD spectra (Figure S34) of **2** and **3** revealed that these compounds possess identical absolute configurations of 8*R*,9*R*,10*R*,13*R*,17*R*,20*R*,24*R*.

Biosynthetically, compounds **1** and **2** might plausibly be traced back to the normal ergosteroids since compound **1** possesses an extremely unusual α-oriented Me-19, which sheds new light on the steroid biosynthesis. The possible biogenetic pathway for **1** and **2** was proposed (Scheme 1). Initiated by an oxidative cleavage between C-6 and C-7 of co-isolated compound **4**, intermediate **a** is formed, of which ring A then undergoes a rotation of 180°, providing advantageous conditions for a vital aldol condensation reaction to produce intermediate **b**. Further epoxidation and methyl esterification reactions could create **1**. Similarly, originating from **4**, the biosynthesis routes to **2** involve key protonation, Wagner−Meerwein rearrangement, oxidation, and methylation reactions.

Due to the anti-inflammatory activity of steroids,[2] compounds **1**−**3** were investigated by virtual screening for several significant inflammatory targets, including iNOS, SIRT2, VCAM, ICAM, JAK1, TNF-α, JAK2, IL-5, and IL-17. The calculated results of docking scores (Table S8) predicted that iNOS enzyme showed significantly higher binding affinity for **1**−**3** than other targets. To prove this speculation, an in vitro enzyme-based validation was performed;[18] indeed, compound **2** exhibited inhibitory potency against the iNOS enzyme (IC_{50} = 1.49 μM). Compound **2** was then subjected to computational docking to better understand the hypothetic mechanism. As shown in Figure 4,

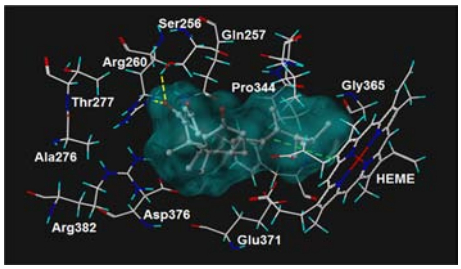


Figure 4. Low-energy binding conformations of **2** bound to murine iNOS (3NW2) generated by virtual ligand docking. Dotted yellow and green lines indicate hydrogen bonds and p−p interactions, respectively.

compound **2** is buried in a pocket between Gln257, Pro344, Glu371 and a Heme moiety, exhibiting two strong hydrogen bonds between compound **2** and Agr260 and Ser256 and a p-p interaction between the C-22−C-23 double bond and Heme moiety. Based on the molecular docking results, the double bond on the side chain of ergosteroids is a worthy target for further structural modification.

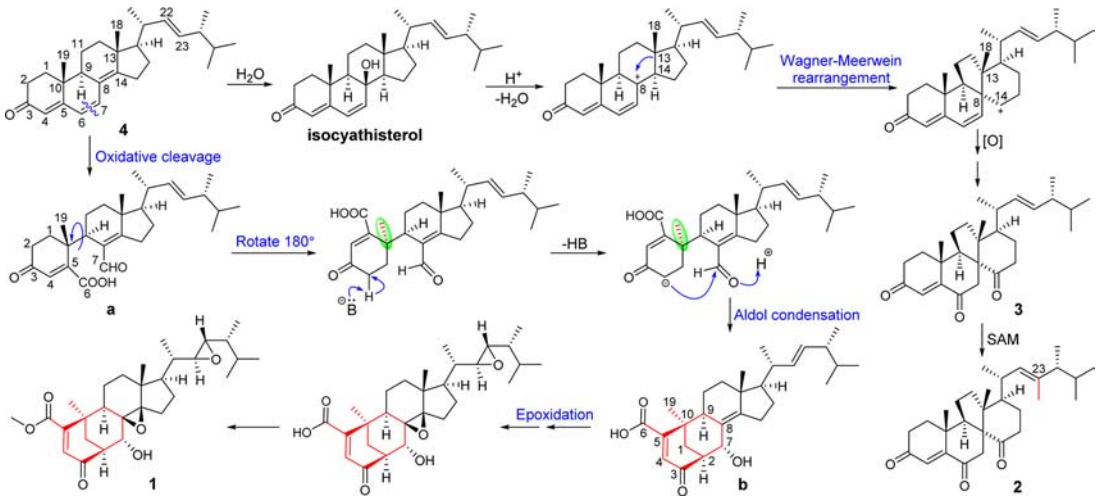
To further test in vitro anti-inflammatory activity, compounds **1**−**3** were evaluated for their inhibitory activities against LPS-induced nitric oxide (NO) production in RAW 264.7 mouse macrophages.[18] As shown in Table 1, compounds **2** and **3**

Table 1. Inhibitory Activities against LPS-Induced NO Production, the iNOS Enzyme, and RAW264.7 Cells (Given as IC_{50})

	IC_{50} (μM)		
compd	NO	iNOS	RAW264.7
1	>25	16.43	>10
2	4.65	1.49	>10
3	13.04	6.58	>10
MG132[a]	0.18	0.11	0.69

[a]Positive control.

Scheme 1. Proposed Biosynthetic Pathway for 1 and 2



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exhibited significant inhibitory activities with IC_{50} values of 4.65 and 13.04 μM, respectively, indicating that the methylation at C-23 could enhance the inhibitory potency. An MTT assay was carried out to determine whether the suppressive effect was related to cell viability, but no cytotoxicity was detected in RAW 264.7 macrophages, suggesting that the inhibitory activities against NO production in LPS-stimulated RAW264.7 cells did not involve general cytotoxicity. Our finding further verified that fungal metabolites are important sources of anti-inflammatory agents, wherein representative cases include vermelhotin,[19] periconianone A,[20] curindolizine,[21] and so on.

In conclusion, the highly oxygenated and rearranged architecture and multiple chiral centers of phomopsterone A (**1**) create a tremendous challenge in determining the absolute structure of this compound by interpreting the NMR data alone. The combination of a modified Mosher's method, X-ray crystallographic analysis, and quantum chemical calculations enabled us to construct **1** as an unprecedented ergosteroid possessing a unique bicyclo[3.3.1]nonane motif with an α-oriented Me-19 group, which provides new insight into steroid biosynthesis. Moreover, phomopsterone B (**2**) represents the first example of a C_{29}-steroid possessing a 13(14 → 8)-*abeo*-8-ergostane skeleton. Molecular docking was used to predict that these compounds are iNOS enzyme inhibitors, and compound **2** indeed exhibited iNOS enzyme inhibitory activity (IC_{50} = 1.49 μM) in vitro. This approach can be helpful in searching for lead compounds from natural products obtained in limited amounts. The discovery of compounds **1** and **2** not only expands the ergostane-type scaffoldings but also provides promising target molecules for further scientific research. More importantly, this unique biosynthetic pathway also warrants further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03557.

X-ray crystallographic data of **1** (CIF)

Experimental details; HRESIMS, IR, UV, and NMR data of **1** and **2**; quantum chemical calculations of **1**; X-ray crystallographic data of compound **1**; CD spectra of **2** and **3** (PDF)

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Author Contributions

‖Z.H. and Y.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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