Chinese Journal of Natural Medicines

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Citation: Mengsha WEI, Weiguang SUN, Linlin LIU, Yongqi LI, Lanqin LI, Qin LI, Yu CHEN, Jieru GUO, Lianghu GU, Hucheng ZHU, Chunmei CHEN, Yonghui ZHANG, Ten ring-B aromatized ergosterols from *Aspergillus spectabilis*, Chinese Journal of Natural Medicines, 2024, 22(7), 654-662. doi: 10.1016/S1875-5364(24)60671-0.

View online: https://doi.org/10.1016/S1875-5364(24)60671-0

Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2024, **22**(7): 654-662
doi: 10.1016/S1875-5364(24)60671-0
Chinese Journal of Natural Medicines

•Original article•

Ten ring-B aromatized ergosterols from *Aspergillus spectabilis*

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Available online 20 Jul., 2024

[ABSTRACT] Spectasterols F−O (**1**−**10**), ten interesting ergosterols with an aromatized B ring, were obtained from *Aspergillus spectabilis*. Their structures and absolute configurations were determined using a combination of high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), nuclear magnetic resonance (NMR) spectroscopy, single-crystal X-ray diffraction analyses, and electronic circular dichroism (ECD) calculations. Structurally, these aromatic ergosterols feature versatile side chains. Notably, compound aromatic ergosterols featured versatile side chains, and compound **4** is an unusual C23 ergosterol characterized by a shorter side chain due to oxidative cleavage between C-23 and C-24. All compounds were evaluated for their neuroprotective activities, with compound **8** showing a dose-dependent ability to reduce apoptosis and protect mitochondrial function in glutamate-induced SH-SY5Y cells.

[KEY WORDS] Steroids; *Aspergillus spectabilis*; Aromatic ergosterols; Neuroprotective activities; Structure elucidation
[CLC Number] R284.1 **[Document code]** A **[Article ID]** 2095-6975(2024)07-0654-09

Introduction

Aromatic steroids are a subclass of steroids that contain one or more aromatic rings in their steroid skeleton. These lipids are produced by bacteria, plants, fungi, and animals [1]. Aromatization can occur at rings A, B, C, or D [2, 3]. Most aromatic steroids possess an aromatized A ring (more than 220 known compounds), while only a minority exhibit an aromatized B ring (about 20 known compounds) [1]. Naturally occurring ergosterols with a monoaromatic B ring mainly include three types: 19-norergostane, 19(10→6)-*abeo*-ergostane, and l(10→6)-*abeo*-ergostane. Anthrasteroid hydrocarbons, the first examples of ergosterols belonging to the l(10→6)-*abeo*-ergostane type, were isolated from deep-sea sediments in 1983 [4]. The 19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3*β*-ol was discovered in Amoeba *Acanthamoeba polyphaga* in 1987 [5]. Phycomysterols A and B and neoergosterol, possessing a 19-norergostane skeleton, were isolated from the fungus *Phycomyces blakesleeanus* in 1998 [6]. In recent years, only a few members of this family have been discovered from fungi, such as *Aspergillus ustus* [7], *Colletotrichum* sp. [8], *Fusarium* sp. [9], and *Penicillium citreo-viride* [10]. These compounds exhibit various biological activities, including the regulation of liver X receptor activity, antibacterial, antifungal, and anti-HIV activities [1, 11].

Our previous investigation on *Aspergillus spectabilis* demonstrated the presence of five aromatic ergosterols with unusual ring systems [12]. Continuing this work, we isolated ten ergosterols with an aromatic B ring, named spectasterols F−O (**1**−**10**). Herein, we report the isolation, structural elucidation, and bioactivity evaluation of these novel aromatic ergosterols.

Results

Spectasterol F (**1**) was isolated as colorless crystals with a molecular formula of $C_{28}H_{42}O_3$, as disclosed by the $[M + Na]^+$ ion peak at *m/z* 449.3024 in the high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (Calcd. for $C_{28}H_{42}O_3Na^+$, 449.3032), requiring eight degrees of unsaturation. The IR spectrum of **1** showed characteristic

[Received on] 14-Jan.-2024
[Research funding] This work was supported by the National Key Research and Development Program of China (No. 2021YFA0910500), the National Natural Science Foundation of China (No. U22A20380 and 82104043), the Innovative Research Groups of the National Natural Science Foundation of China (No. 81721005), and the Science and Technology Major Project of Hubei Province (No. 2021ACA012).
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These authors have no conflict of interest to declare.

absorptions for hydroxy groups (3421 cm^{-1}) and an aromatic ring (1635 and 1463 cm^{-1}). The ^1H nuclear magnetic resonance (NMR) data of **1** (Table S1), combined with HSQC spectra, revealed the presence of six methyl groups (δ_H 0.76, s; 0.86, d, J = 6.5 Hz; 0.87, d, J = 6.5 Hz; 0.97, d, J = 6.8 Hz; 1.43, s; and 2.20, s), two olefinic and one aromatic protons (δ_H 5.72, d, J = 15.7 Hz; 5.59, dd, J = 15.7, 8.0 Hz; 6.75, s), an oxygenated methine (δ_H 4.12, m), and a series of methylenes and methines protons ranging from δ_H 1.56 to 3.40. The ^{13}C NMR and DEPT spectra of **1** exhibited a total of 28 carbon resonances, including six methyls (δ_C 16.3, 17.5, 19.8, 20.1, 20.2, and 26.4), seven methylenes (δ_C 23.5, 24.9, 25.3, 30.1, 31.6, 33.0, and 36.5), seven methines groups including an oxygenated one (δ_C 67.7) and three sp^2 hybrid ones (δ_C 125.7, 133.2, and 134.5), and eight non-protonated carbons including two oxygenated ones (δ_C 78.6 and 87.4) and five aromatic or olefinic ones (δ_C 130.0, 131.0, 133.2, 134.0, and 137.8). These characteristic data (Tables S1 and S2) implied that **1** should be an aromatic ergosterol resembled (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3*β*-ol [8] (Fig. 1).

The ^1H–^1H correlation spectroscopy (COSY) cross-peaks of H_2-1/H_2-2/H-3/H_2-4, H_2-11/H_2-12, and H-14/H_2-15/H_2-16 along with key heteronuclear multiple bond correlations (HMBCs) (Fig. 2) from Me-19 to C-5, C-6, and C-7, from H_2-4 to C-5, C-6, and C-10, from H_2-1 to C-5, C-9, and C-10, from H-7 to C-5 and C-9, from H_2-11 to C-8, C-9, and C-10, from H-14 to C-7 and C-9, and from Me-18 to C-12, C-13, C-14, and C-17 suggested that **1** shared the same rings A–D as well as the same substitution pattern as (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3*β*-ol. The HMBCs from Me-21 to C-17, C-20, and C-22, and ^1H–^1H COSY cross-peaks of H-22/H-23/H-24/H-25/Me-26 (Me-27), and H-24/Me-28 elucidated the hydroxylation of C-17 and C-20, which were different from (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,22-tet-

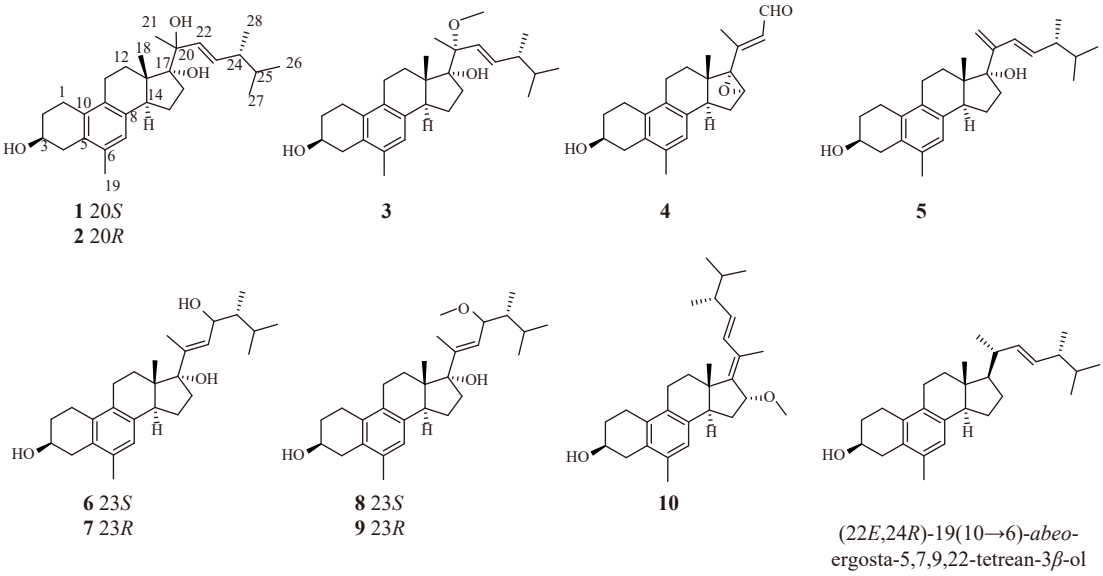

Fig. 1 Structures of 1–10.

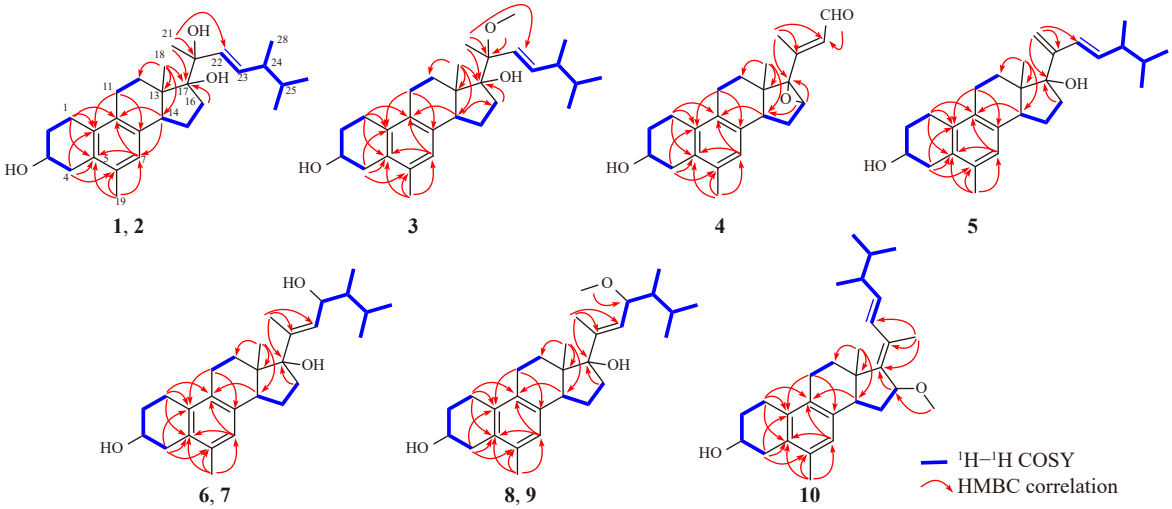

Fig. 2 Key ^1H–^1H COSY and HMBCs of compounds 1–10.

raen-3β-ol. Thus, the planar structure of **1** was determined.

The *E* configuration of double bond $\Delta^{22,23}$ was deduced from the large coupling constant (J = 15.7 Hz) between H-22 and H-23. Additionally, the nuclear Overhauser effect spectroscopy (NOESY) cross-peaks of H_3-18/H-15 and H_3-18/H_3-21 supported the *trans*-fused C/D rings and the β-orientation of C-17−C-20 bond (Fig. 3), consistent with normal ergosta-type steroids. Finally, the absolute configuration of **1** was determined to be 3*S*,13*S*,14*S*,17*R*,20*S*,24*R* (Fig. 4, CCDC 2261300) by single-crystal X-ray diffraction analysis using Cu Kα radiation, with a Flack parameter of −0.06(3). Thus, the structure of compound **1** was established and named as (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3β,17α,20β-triol.

Spectasterol G (**2**) shared the same molecular formula, $C_{28}H_{42}O_3$ as **1**. The ^{1}H and ^{13}C NMR data of **2** (Tables S1 and S2) were almost identical to those of **1** except for minor shifts in the resonances assigned to C-17 ($\Delta\delta_C$ −0.1), C-20 ($\Delta\delta_C$ −0.2), and C-21 ($\Delta\delta_C$ −0.6), suggesting that compound **2** could be a C-17 or C-20 epimer of compound **1**. The NOESY cross-peak of H-14/OH-17 confirmed the α-orientation of OH-17 (Fig. 3). Therefore, compound **2** was deduced to be the C-20 epimer of compound **1**, named (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3β,17α,20α-triol.

Spectasterol H (**3**) had a molecular formula of $C_{29}H_{44}O_3$, as disclosed by the $[M + Na]^+$ ion peak at *m/z* 463.3187 in the HR-ESI-MS spectrum. The ^{1}H and ^{13}C NMR data of **3** (Tables S1 and S2) closely resembled those of **1** except for the presence of an additional methoxy group (δ_H 3.15, δ_C 49.9), further confirmed by the HMBC from OMe-20 to C-20. The similar coupling constant ($^3J_{H\text{-}22,H\text{-}23}$ = 16.2 Hz) and key NOESY cross-peaks, as those of **1**, determined the relative configurations of steroid nuclear except for C-3. The configuration of C-3 was assigned by comprising the NMR data of **3** with those of co-occurring **1** and related compounds [6, 8]. Additionally, NOESY correlations were used to determine the relative configuration of C-20 by analyzing the Newman projection of C-17−C-20 (Fig. 5). The observed NOESY correlations of H-16/H-22, H-16/H_3-21, and H_3-18/H_3-21 suggested the R^* configuration of C-20. Further comparison of the ^{1}H and ^{13}C NMR data of the side chain in **3** with those of **1** and **2**, as well as literature data, confirmed the 24R^* configuration of **3** [13-15]. The absolute configuration of compound **3** was further determined by comparison of its electronic circular dichroism (ECD) spectrum with that of **1** (Fig. 6). Therefore, the structure of **3** was established as (20*R*,22*E*,24*R*)-20-methoxyl-19(10→6)-*abeo*-ergosta-5,7,9,22-tetraen-3β,17α-diol.

Spectasterol I (**4**) was obtained as a white amorphous powder, with a molecular formula of $C_{23}H_{30}O_4$ based on HR-ESI-MS data, implying nine degrees of unsaturation. The ^{1}H and ^{13}C NMR (Tables S1 and S2) data indicated that **4** had the same steroid nuclear as **1**, but the side chain was significantly different. The HMBC cross-peaks from Me-21 (δ_H 2.29) to C-17 (δ_C 73.3), C-20 (δ_C 155.0), and C-22 (δ_C 130.6), and from H-23 (δ_H 10.1) to C-22, located an aldehyde group at C-22 and the double bond between C-20 and C-22. Additionally, the HMBCs from H-16 to C-14 and C-17 and the ^{1}H−^{1}H COSY cross-peaks of H-14/H_2-15/H-16 indicated the oxidation of C-16. The chemical shift of upfield

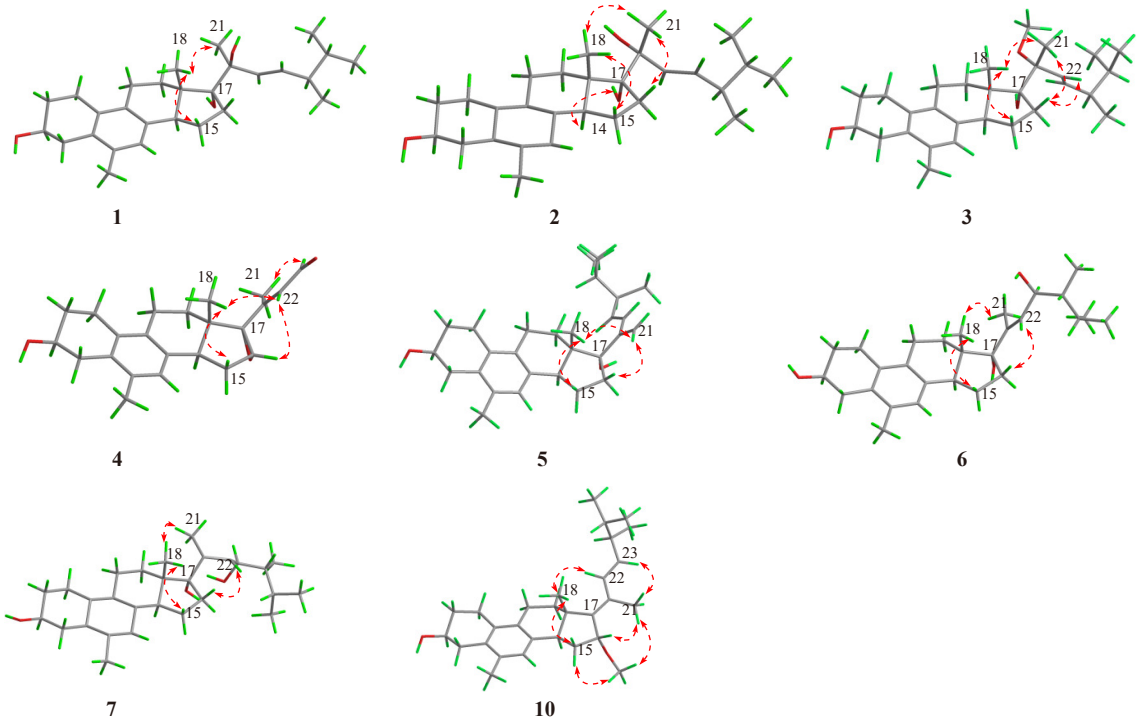

Fig. 3 Selected key NOESY correlations of compounds 1−7 and 10.

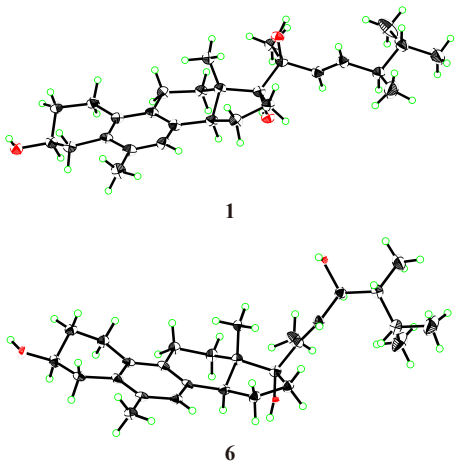

Fig. 4 X-ray crystallographic structures of 1 and 6.

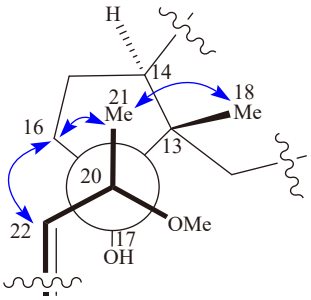

Fig. 5 Newman projection with a view along the C-17−C-20 bond of 3.

of C-16 (δ_C 62.4) and C-17 (δ_C 73.3), combined with the HR-ESI-MS data, suggested the presence of a ternary oxygen ring. Thus, the planar structure of compound **4** was determined. The NOESY cross-peaks of H_3-18/H-22 and H-22/H-16 suggested that the 16,17-epoxy group was *α*-oriented. Based on the biosynthetic relationship of compounds **1**−**3** and the closely related chemical shift of H-3 in **4** (δ_H 4.13) compared to **1** (δ_H 4.12), the OH-3 was assigned a *β*-orientation. Furthermore, the absolute configuration of **4** was determined by ECD calculations (Fig. 6). Thus, the structure of compound **4** was established as (20*E*)-16*α*,17*α*-epoxy-22-aldehyde-19(10→6)-*abeo*-24−28-pentanorergosta-5,7,9,20-tetraen-3*β*-ol.

Spectasterol J (**5**) was obtained as an amorphous powder. Its molecular formula, $C_{28}H_{40}O_2$, was deduced from the HR-ESI-MS (*m/z* 431.2920 $[M + Na]^+$), indicating nine degrees of unsaturation. The ^1H and ^{13}C NMR data (Tables S1 and S2) of **5** closely resembled those of compound **1**, with the main differences being the replacement of a methyl group and an oxygenated non-protonated carbon in **1** with a terminal double bond (δ_C 110.7 and 150.3). The $^1H-^1H$ COSY correlations of H-22/H-23/H-24/H-25/Me-26 (Me-27) and H-24/Me-28, combined with the HMBCs from H_2-21 to C-17, C-20, and C-22, revealed the position of the terminal double bond at $\Delta^{20,21}$. Additionally, the similar experimental ECD spectra (Fig. 6) of **5** and **1** assigned the absolute configuration of **5** to be 3*S*,13*S*,14*S*,17*S*,24*R*. Thus, the structure of compound **5** was defined as (22*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,20(21),22-pentaen-3*β*,17*α*-diol.

The molecular formula of spectasterol K (**6**) was determined to be $C_{28}H_{42}O_3$ by HR-ESI-MS (*m/z* 449.3026 $[M + Na]^+$) and NMR data. The ^1H and ^{13}C NMR data (Tables S2 and S3) of **6** resembled that of **1**. The key differences were the absence of one olefinic proton and an oxygenated non-protonated carbon and the presence of an oxygenated methine (δ_H 4.39, dd, *J* = 8.5, 6.3 Hz) in **6**. The HMBCs from Me-21 (δ_H 1.85) to C-17 (δ_C 86.6), C-20 (δ_C 140.4), and C-22 (δ_C 129.8), and from Me-28 (δ_H 0.95) to C-23 (δ_C 71.2), C-24 (δ_C 45.3), and C-25 (δ_C 29.3), combined with the $^1H-^1H$ COSY correlations of H-22/H-23/H-24/H-25/Me-26 (Me-27) and H-24/Me-28, revealed that compound **6** differed from **1** due to migration of the double bond from $\Delta^{22,23}$ to $\Delta^{20,22}$, and the relocation of the hydroxy group from C-20 to C-23. The X-ray experiment using Cu K*α* radiation (Fig. 4, CCDC, 2261771) determined the absolute configuration of **6** as 3*S*,13*S*,14*S*,17*S*,23*S*,24*R* based on the Flack parameter of −0.04(3). Hence, **6** was named as (20*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,20-tetraen-3*β*,17*α*,23*β*-triol.

Spectasterol L (**7**) was obtained as an amorphous powder. Its molecular formula, $C_{28}H_{42}O_3$, was determined to be the same as compound **6**. The HR-ESI-MS and ^{13}C NMR data (Table S2) of **7** were almost identical to those of **6,** with the main differences observed at C-23 ($\Delta\delta_C$ −0.8), C-25 ($\Delta\delta_C$ −2.0), and C-27 ($\Delta\delta_C$ −1.6). Considering the biosynthetic pathway, naturally occurring ergosterols typically share the same 24*R** configuration [13-15]. Therefore, compound **7** was deduced to be the C-23 epimer (23*R**) of compound **6**. Based on the similar ECD spectra of **7** and **6** (Fig. 6), the absolute configuration of compound **7** was established as (20*E*,24*R*)-19(10→6)-*abeo*-ergosta-5,7,9,20-tetraen-3*β*,17*α*,23*α*-triol.

Spectasterols M (**8**) and N (**9**) were obtained as amorph-

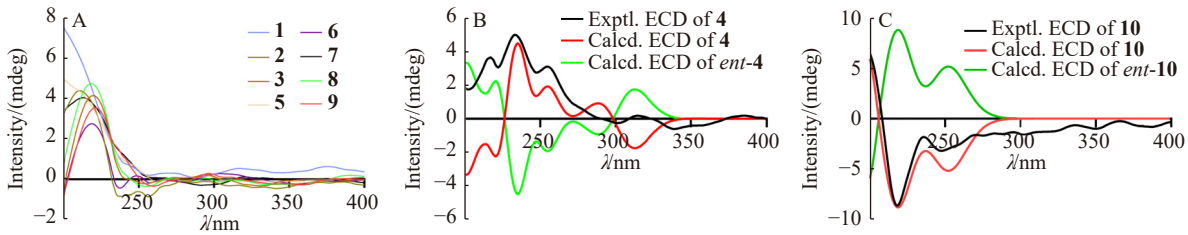

Fig. 6 (A) Experimental ECD spectra of 1−3 and 5−9. (B) Experimental ECD curve of 4 and calculated ECD spectra of 4 and *ent*-4. (C) Experimental ECD curve of 10 and calculated ECD spectra of 10 and *ent*-10.

ous powder. Their molecular formula were both determined to be $C_{29}H_{44}O_3$ by HR-ESI-MS *(m/z* 463.3199 and 463.3193 $[M + Na]^+$). The overall NMR data (Tables S2 and S3) of **8** and **9** closely resembled those of **6** and **7** except for the presence of an additional methoxyl group (δ_H 3.23, δ_C 56.4 in **8**; δ_H 3.28, δ_C 56.1 in **9**), which was further confirmed by the HMBC from OMe-23 to C-23. The discrepancy in ^{13}C NMR data of C-23 ($\Delta\delta_C$ 1.0), C-25 ($\Delta\delta_C$ 1.9), and C-27 ($\Delta\delta_C$ 1.5) between **8** and **9** demonstrated that these compounds were a pair of C-23 epimers. Thus, the planar structures of **8** and **9** were elucidated.

The chemical shifts of C-25 and C-27 in compound **8** (δ_C 29.3, C-25; δ_C 18.4, C-27) were more concordant with those of compound **6** (δ_C 29.3, C-25; δ_C 18.2, C-27), while those in compound **9** (δ_C 27.4, C-25; δ_C 16.9, C-27) were more similar to those in compound **7** (δ_C 27.3, C-25; δ_C 16.6, C-27). Additionally, the paired coupling constants and splitting patterns of H-23 in **6**–**9** (Fig. 7) suggested the same configurations at C-23 in compounds **6**/**8** and **7**/**9**, respectively. The similar ECD spectra of compounds **8** and **9** to those of **6** established their absolute configurations as (20*E*,23*S*,24*R*)-23-methoxyl-19(10→6)-*abeo*-ergosta-5,7,9,20-tetraen-3β,17α-diol and (20*E*,23*R*,24*R*)-23-methoxyl-19(10→6)-*abeo*-ergosta-5,7,9,20-tetraen-3β,17α-diol, respectively.

Spectasterol O (**10**) was obtained as an amorphous powder. Its molecular formula was determined to be $C_{29}H_{42}O_2$ from HR-ESI-MS (*m/z* 445.3062 $[M + Na]^+$), indicating nine degrees of unsaturation. The ^1H and ^{13}C NMR data of **10** closely resembled those of **1**, except for the lack of a methylene group and two non-protonated oxygenated sp^3 carbon, and the presence of two non-protonated sp^2 carbon, an additional oxygenated methine, and a methoxy group. The HMBCs from Me-21 to C-17, C-20, and C-22, as well as $^1H-^1H$ COSY correlations of H-22/H-23/H-24/H-25/Me-26 (Me-27), and H-24/Me-28, explained the presence of double bonds at $\Delta^{17,20}$ and $\Delta^{22,23}$. Furthermore, the HMBC correlation from OMe-16 (δ_H 3.39) to C-16 (δ_C 83.2) located the methoxy group at C-16. NOESY correlations of H_3-18/H-15β, OCH_3-16/H-15α, H-22/H_3-18, H-23/H_3-21, H_3-21/H-16, and H_3-21/OCH_3-16 determined the relative configurations of ring-D and the *E* configuration of the double bond $\Delta^{17,20}$. The *E* configuration of the double bond at $\Delta^{22,23}$ was determined by the large coupling constant between H-22 and H-23 (J = 15.5 Hz). Additionally, the ECD calculations (Fig. 6) established compound **10** as (16*R*,17*E*,22*E*,24*R*)-16-methoxy-19(10→6)-*abeo*-ergosta-5,7,9,17,22-pentaen-3β-ol.

Compounds **1**–**10** were evaluated for their neuroprotective activities against glutamate-induced SH-SY5Y cell death. Initially, all compounds were pre-examined for their cytotoxicity at a concentration of 40 μmol·L^{-1}. No obvious cytotoxicities were observed, indicating IC_{50} values greater than 40 μmol·L^{-1}. Subsequently, compounds **1**–**10** were tested at concentrations of 5, 10, 20, and 40 μmol·L^{-1} and incubated with SH-SY5Y cells for 4 h before exposure to 20 mmol·L^{-1} glutamate. Cell viability was measured after an additional 24 h using the CCK-8 assay. As shown in Fig. 8A, **8** exhibited a remarkable protective effect on SH-SY5Y cells at concentrations of 10 and 5 μmol·L^{-1}. When exposed to glutamate, which decreased cell viability to approximately 50% of control cell lines, compound **8** significantly enhanced cell survival. This protective effect was further confirmed by Annexin V-FITC/PI double staining. Compared to glutamate treatment alone, pretreatment with compound **8** reduced apoptosis rates to 6.6% and 15.3% at concentrations of 10 and 5 μmol·L^{-1}, respectively, consistent with the CCK-8 assay results (Figs. 8B and 8C). Furthermore, as shown in Fig. 9A, treatment with 20 mmol·L^{-1} glutamate for 24 h resulted in marked cell shrinkage compared to control conditions. In contrast, compound **8** protected against this injury and maintained normal cellular morphology.

JC-1, a membrane-permeable dye, was used to assess mitochondrial membrane potential (ΔΨm) and observed under a

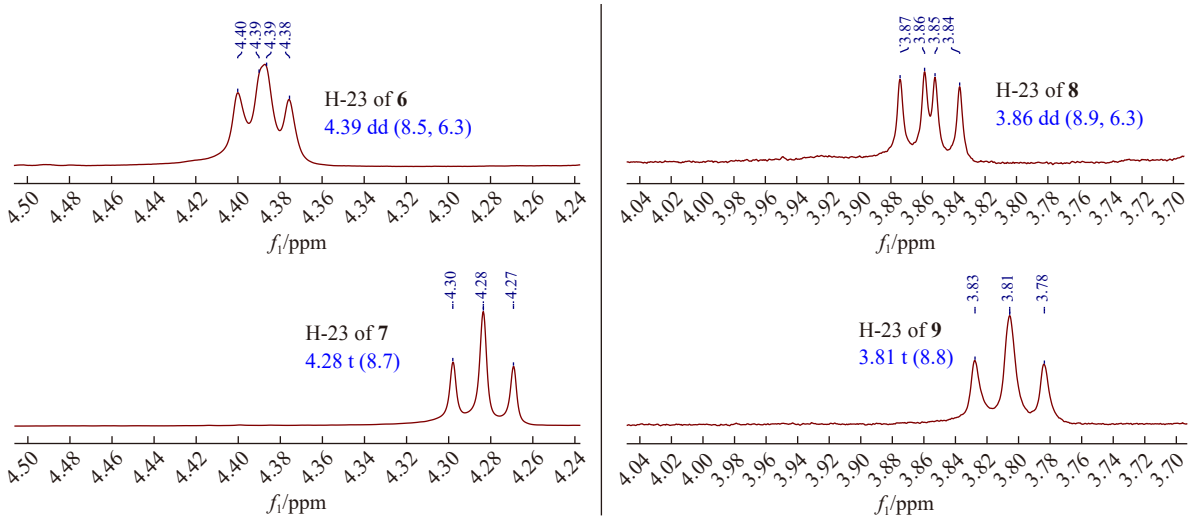

Fig. 7 ^1H NMR spectra of 6–9 showing the coupling patterns of H-23 (*J* in Hz).

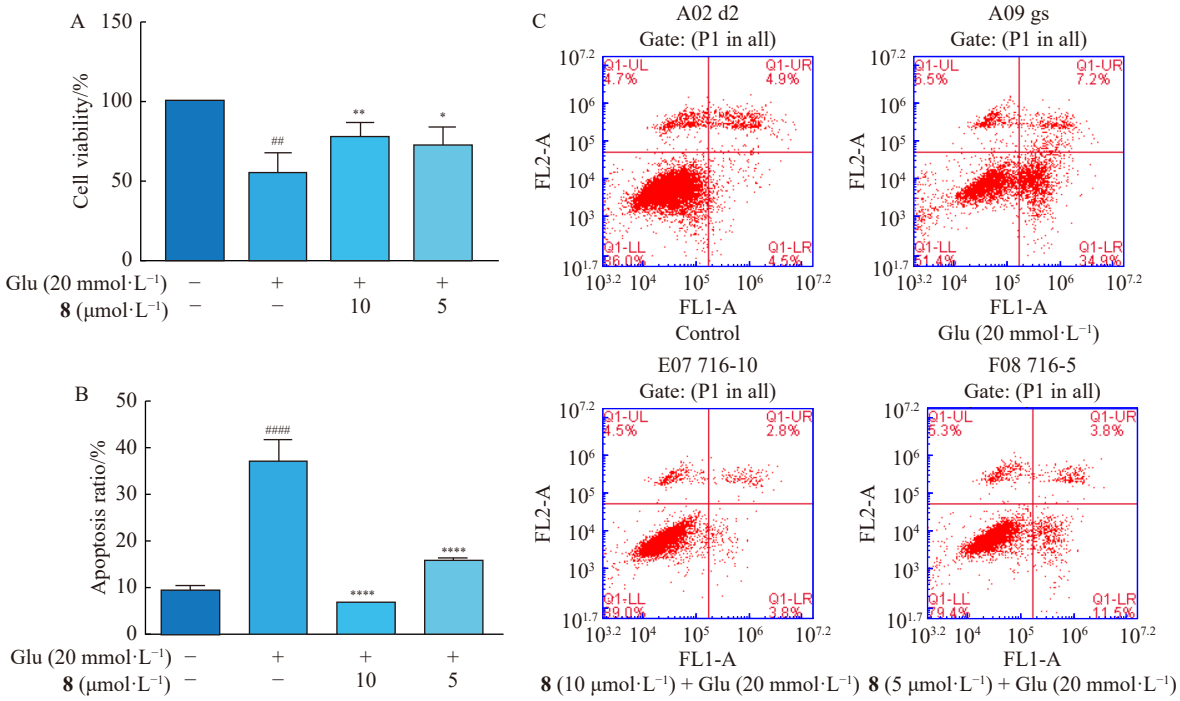

Fig. 8 Protective effects of compound 8 on the viability of glutamate-treated SH-SY5Y cells. (A) CCK-8 results suggested that compound 8 can increase cell viability. (B, C) Compound 8 decreased glutamate-induced cellular apoptosis in SH-SY5Y cells. Flow cytometry was applied to determine the apoptotic ratio after Annexin V-FITC/PI staining. The percentage of apoptotic cells was calculated in the bar chart. Data are presented as the means ± SD (n = 3). $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ *vs* Control (the no glutamate or compound 8-treated group); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ *vs* the glutamate-treated group.

fluorescence microscope. As shown in Figs. 9B and 9C, treatment with glutamate significantly reduced the red fluorescence in SH-SY5Y cell lines compared to the control group, indicating a loss of ΔΨm. Pre-incubation with compound **8** resulted in a noticeable increase in red fluorescence compared to glutamate treatment alone, suggesting that compound **8** helps preserve mitochondrial membrane potential and provides a protective effect against glutamate-induced neurotoxicity.

Experimental

General experimental procedures

Optical rotations were measured in MeOH using a Rudolph Autopol IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). UV spectra were obtained using a PerkinElmer Lambda 35 spectrophotometer (PerkinElmer, Inc., USA). IR spectra were recorded using a Bruker Vertex 70 FT-IR spectrophotometer (Bruker, Karlsruhe, Germany). NMR spectra were acquired on a Bruker AM-400 NMR or a Bruker AM-600 NMR spectrometer. ECD data were collected using a JASCO-810 instrument (JASCO Co., Ltd., Tokyo, Japan). HR-ESI-MS data were recorded on a Bruker micrOTOF II spectrometer. Compounds were purified using an Agilent 1260 HPLC system equipped with an Ultimate XB-C_{18} column (5 μm, 10 mm × 250 mm). Chemical shifts are expressed in ppm with reference to the signals of $CDCl_3$ (δ_H 7.26/δ_C 77.16), CD_3OD (δ_H 3.31/δ_C 49.00), and DMSO-d_6 (δ_H 2.50/δ_C 39.52). Crystallographic data were collected on an XtaLAB PRO MM007HF diffractometer equipped with graphite-monochromatized Cu Kα radiation. Column chromatography packing materials included silica gel (80−120 mesh, 100−200 mesh, and 200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), ODS (50 μm, YMC, Japan), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). The compounds were visualized by spraying silica gel plates with 10% H_2SO_4 in EtOH and heating.

Fungal material

The fungus *Aspergillus spectabilis* M. Christensen & Raper (Aspergillaceae) was originally isolated from Artemisia grassland and coal-spoil soils in Wyoming. The strain used in this work was obtained from the China General Microbiological Culture Collection Center (No. 3.4339). A voucher sample is preserved in the culture collection center of Tongji Medical College, Huazhong University of Science and Technology.

Extraction and isolation

The fungal strain was cultured on potato dextrose agar (PDA) at 28 °C for seven days to prepare the seed culture. Agar plugs were inoculated into 800 Erlenmeyer flasks (1 L) that had been previously sterilized by autoclaving, each containing 250 g rice and 200 mL distilled water. All flasks were incubated at 28 °C. The fermented rice substrate was extracted seven times with 95% aqueous EtOH at room temperature, and the solvent was evaporated under vacuum to yield a residue. The residue was suspended in H_2O and successively

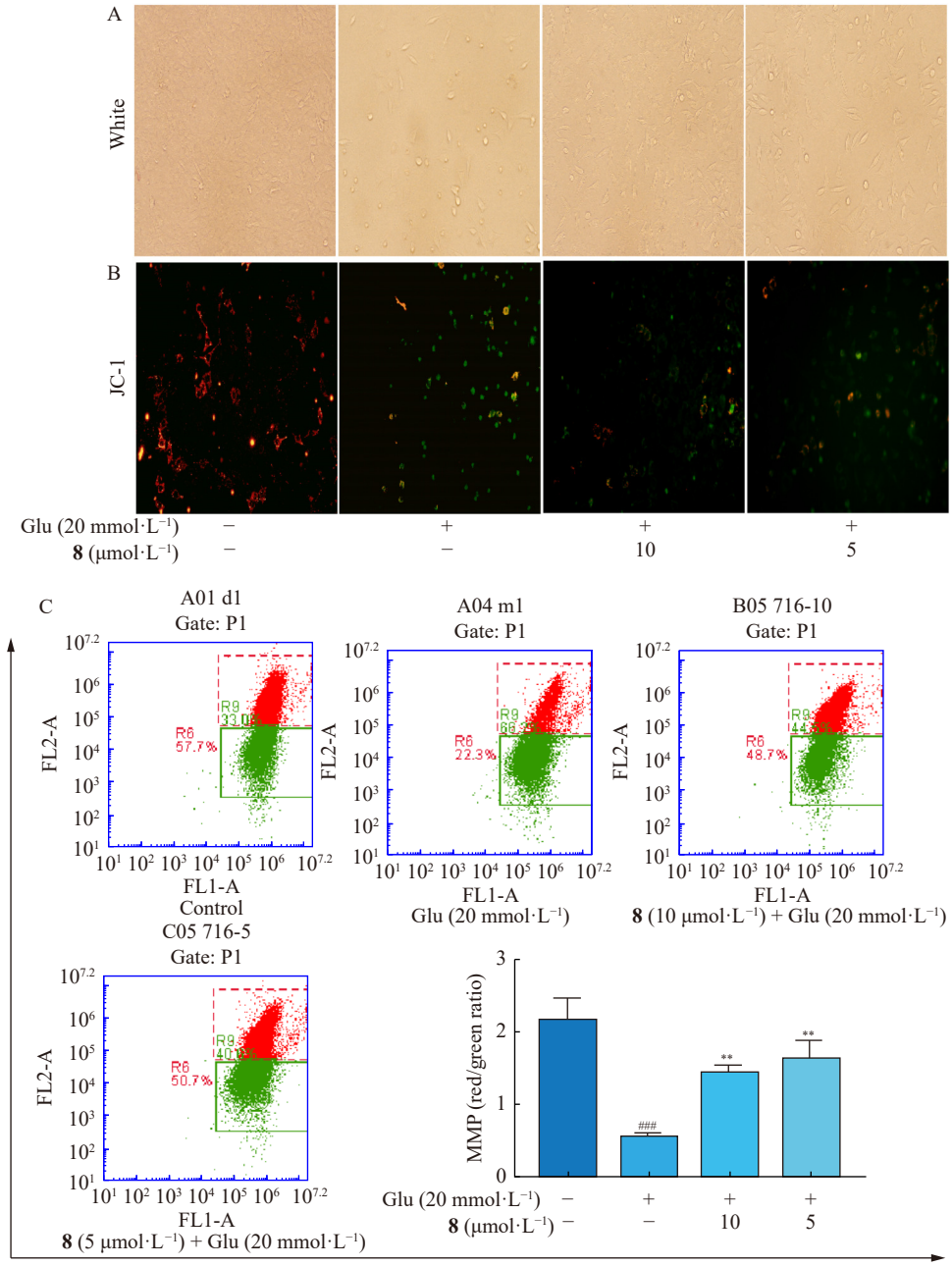

Fig. 9 Compound 8 protects mitochondrial function in glutamate-treated SH-SY5Y cells. (A) Cellular morphological changes were observed by phase contrast microscopy. (B) SH-SY5Y cells were measured using JC-1 staining by an inverted fluorescence microscope. (C) SH-SY5Y cells were measured using JC-1 staining by flow cytometry. Data are presented as the means ± SD (n = 3). $^{###}P < 0.001$ *vs* Control (the no glutamate or compound 8-treated group); $^{**}P < 0.01$ *vs* the glutamate-treated group.

partitioned with ethyl acetate (EtOAc). The EtOAc partition fraction (2.0 kg) was fractionated into eight fractions (Fr. 1−8) using silica gel column chromatography (CC), eluting with a gradient of petroleum ether (PE) : EtOAc (30 : 1−0 : 1, *V/V*) and EtOAc : MeOH (20 : 1−0 : 1, *V/V*). Fr. 3 (160.0 g) was further separated on an ODS column (MeOH−H_2O, 50%, 60%, 70%, 80%, 90%, 100%) to yield eight fractions (Fr. 3.1−3.8). Fr. 3.4 was subjected to silica gel CC (PE : EtOAc, 15 : 1−0 : 1, *V/V*) to obtain seven fractions (Fr. 3.4.1−3.4.9). Fr. 3.4.4 was further purified by repeated semipreparative HPLC (MeOH : H_2O, 92 : 8) to yield compounds **1** (10.2 mg), **2** (4.2 mg), and **3** (36.4 mg). Fr. 3.4.5 was further separated using Sephadex LH-20 (CH_2Cl_2 : MeOH, 1 : 1, *V/V*) to obtain compound **4** (3.0 mg). Fr. 3.4.6 was further separated using Sephadex LH-20 (CH_2Cl_2 : MeOH, 1 : 1, *V/V*) and semipreparative HPLC (MeCN : H_2O, 85 : 15) to obtain compound **5** (5.4 mg). Fr. 3.4.7 was purified by semipreparative HPLC (MeCN : H_2O, 82 : 18) to yield compounds **6** (15.5 mg) and **7** (21.1 mg). Repeated purification of Fr. 3.4.9 by semipreparative HPLC

(MeCN : H_2O, 80 : 20) afforded compounds **8** (4.4 mg) and **9** (4.8 mg). Fr. 3.4.2 was further purified by silica gel CC (PE : CH_2Cl_2, 3 : 1–0 : 1, *V/V*) and semipreparative HPLC (MeOH : H_2O, 75 : 25) to yield compound **10** (2.0 mg).

*Spectasterol F (**1**)*: Colorless crystals; $[\alpha]_D^{20}$ −30 (*c* 0.1, MeOH); IR ν_{max}: 3431, 2955, 1635, 1463 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.68), 225 (3.99) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 200 (+8.68) nm; for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 449.3024 (Calcd. for $C_{28}H_{42}O_3Na^+$, 449.3032).

*Spectasterol G (**2**)*: White power; $[\alpha]_D^{20}$ −128 (*c* 0.1, MeOH); IR ν_{max}: 3422, 2957, 1646, 1460 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.63), 225 (3.98) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 208 (+32.2) nm; for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 449.3036 (Calcd. for $C_{28}H_{42}O_3Na^+$, 449.3032).

*Spectasterol H (**3**)*: White power; $[\alpha]_D^{20}$ −25 (*c* 0.1, MeOH); IR ν_{max}: 3350, 2933, 1462 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.79), 225 (4.09) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 206 (+15.1) nm; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 463.3187 (Calcd. for $C_{29}H_{44}O_3Na^+$, 463.3188).

*Spectasterol I (**4**)*: White power; $[\alpha]_D^{20}$ +44 (*c* 0.1, MeOH); IR ν_{max}: 3428, 2929, 1674, 1436 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.75), 225 (4.30) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 224 (+3.95), 234 (+8.07) nm; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 375.1920 (Calcd. for $C_{23}H_{28}O_3Na^+$, 375.1936).

*Spectasterol J (**5**)*: White power; $[\alpha]_D^{20}$ −31 (*c* 0.1, MeOH); IR ν_{max}: 3428, 2957, 1621, 1463 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.65), 225 (4.15) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 208 (+9.54) nm; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + H]$^+$ *m/z* 431.2920 (Calcd. for $C_{28}H_{40}O_2Na^+$, 431.2926).

*Spectasterol K (**6**)*: Colorless crystals; $[\alpha]_D^{20}$ −72 (*c* 0.1, MeOH); IR ν_{max}: 3410, 2930, 1681, 1465 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.42), 225 (3.76) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 267 (+10.17) nm; for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Tables S2 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 449.3026 (Calcd. for $C_{28}H_{42}O_3Na^+$, 449.3032).

*Spectasterol L (**7**)*: White power; $[\alpha]_D^{20}$ −176 (*c* 0.1, MeOH); IR ν_{max}: 3400, 2958, 1646, 1466 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.62), 225 (3.92) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 201 (+5.54) nm; for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Tables S2 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 449.3032 (Calcd. for $C_{28}H_{42}O_3Na^+$, 449.3032).

*Spectasterol M (**8**)*: White power; $[\alpha]_D^{20}$ −48 (*c* 0.1, MeOH); IR ν_{max}: 3431, 2959, 1631, 1465 cm^{-1} UV (MeOH) λ_{max} (log ε) = 203 (4.72), 225 (4.02) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 209 (+14.82) nm; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables S2 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 463.3199 (Calcd. for $C_{29}H_{44}O_3Na^+$, 463.3188).

*Spectasterol N (**9**)*: White power; $[\alpha]_D^{20}$ −23 (*c* 0.1, MeOH); IR ν_{max}: 3412, 2958, 1631, 1465 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.85), 225 (4.13) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+8.05) nm; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables S2 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 463.3193 (Calcd. for $C_{29}H_{44}O_3Na^+$, 463.3188).

*Spectasterol O (**10**)*: White power; $[\alpha]_D^{20}$ −14 (*c* 0.1, MeOH); IR ν_{max}: 3364, 2958, 1678, 1596, 1457 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 203 (4.30), 248 (4.95) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 217 (−13.46) nm; for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Tables S1 and S3; HR-ESI-MS [M + Na]$^+$ *m/z* 445.3062 (Calcd. for $C_{29}H_{42}O_2Na^+$, 445.3083).

X-ray crystal structure analysis

Colorless crystals of compounds **1** and **6** were obtained from a MeOH : CH_2Cl_2 (9 : 1, *V/V*) solution at room temperature. The crystal data were collected using an XtaLAB PRO MM007HF diffractometer equipped with a graphite monochromator and employing Cu Kα radiation. The structures were refined using the SHELXTL refinement package with least squares minimization. The crystallographic data for compounds **1** and **6** were deposited in the Cambridge Crystallographic Data Centre (CCDC) under deposition numbers 2261300 and 2261771, respectively. These data can be freely obtained from the Cambridge Crystallographic Data Centre by visiting www.ccdc.cam.ac.uk/conts/retrieving.html.

*Crystal data for spectasterol F (**1**)*: $C_{28}H_{42}O_3{\cdot}2CH_3OH$, M = 490.70, a = 12.546 10(10) Å, b = 14.859 70(10) Å, c = 15.610 90(10) Å, α = 90°, β = 90°, γ = 90°, V = 2910.36(4) Å^3, T = 293(2) K, space group $P2_12_12_1$, Z = 4, μ(Cu Kα) = 0.584 mm^{-1}, 41 159 reflections measured, 5836 independent reflections (R_{int} = 0.0247). The final R_1 values were 0.0303 [$I > 2\sigma(I)$]. The final wR_2 values were 0.0789 [$I > 2\sigma(I)$]. The final R_1 values were 0.0308 (all data). The final wR_2 values were 0.0793 (all data). The goodness of fit on F^2 was 1.022. Flack parameter = −0.06(3).

*Crystal data for spectasterol K (**6**)*: $C_{28}H_{42}O_3{\cdot}CH_3OH$, M = 458.66, a = 9.036 00(10) Å, b = 7.050 Å, c = 20.694 70(10) Å, α = 90°, β = 98.2630(10)°, γ = 90°, V = 1304.645(16) Å^3, T = 293(2) K, space group $P2_1$, Z = 2, μ(Cu Kα) = 0.591 mm^{-1}, 28 268 reflections measured, 5104 independent reflections (R_{int} = 0.0220). The final R_1 values were 0.0311 [$I > 2\sigma(I)$]. The final wR_2 values were 0.0851 [$I > 2\sigma(I)$]. The final R_1 values were 0.0311 (all data). The final wR_2 values were 0.0851 (all data). The goodness of fit on F^2 was 1.111. Flack parameter = −0.04(3).

Neuroprotective activity assay in vitro

The experimental procedures and methods were referenced as previously reported [16].

Conclusion

It is worth noting that spectasterol I (**4**) is the first reported C23 steroid possessing an aromatic B-ring from a fungus. A literature search revealed that fewer than 20 monoaromatic B-ring ergosterols have been isolated from nature. The discovery of a series of monoaromatic B-ring steroids from *Aspergillus* spectabilis enriches the family of aromatic steroids.

Steroid drugs have become indispensable in hospitals and are also important pharmaceutical intermediates. The neuroprotective activities of these monoaromatic B-ring steroids are yet to be further studied by pharmacologists, offering a promising area for future research.

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Cite this article as: WEI Mengsha, SUN Weiguang, LIU Linlin, *et al*. Ten ring-B aromatized ergosterols from *Aspergillus spectabilis* [J]. *Chin J Nat Med*, 2024, **22**(7): 654-662.