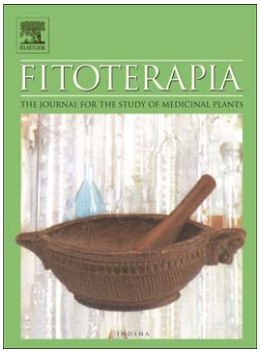

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Four new steroids from the endophytic fungus *Chaetomium* sp. M453 derived of Chinese Herbal Medicine *Huperzia serrata*

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ABSTRACT

An endophytic fungus, *Chaetomium* sp. M453, was isolated from *Huperzia serrata* (Thunb. ex Murray) Trev. and subjected to phytochemical investigation. Three unusual C_{25} steroids, neocyclocitrinols E-G (**1**-**3**), and 3β-hydroxy-5,9-epoxy-(22E,24*R*)-ergosta-7,22-dien-6-one (**4**) together with three known steroids were isolated from solid fermentation products of the fungus, which were elucidated by extensive spectroscopic analyses, including 1D-, 2D-NMR, and HR-ESI-MS experiments. The absolute configuration of **1** was determined by X-ray crystallographic analysis and CD analyses. The acetylcholinesterase inhibitory activities of compounds **1**-**4** were tested *in vitro*. Compound **4** showed weak acetylcholinesterase inhibitory activity.

Keywords: *Huperzia serrata*; Endophytic fungus; *Chaetomium* sp. M453; C25 Steroids; X-ray; Structure identification

1. Introduction

Fungi were considered a great potential value source which could produce diverse structural and active compounds. Endophytic fungi can be found in almost all plants, and most of them belong to the Ascomycota phylum and its anamorphic stages [1]. Fungal endophytes may produce many active secondary metabolites that have a potential use in agriculture, medicine, and industry, and some particular endophytic fungi can produce antitumor substances, for example, vinblastine and vincristine [2], paclitaxel [3,4], podophyllotoxin [5], camptothecin [6]. Some studies have demonstrated that endophytic fungi living in medicinal plants exert many biological activities [7-9]. As part of our ongoing search on active compounds from fungal endophytes, an endophytic fungus (*Chaetomium* sp. M453) was isolated from *Huperzia serrata* (Thunb. ex Murray) Trev., the traditional Chinese medicine Qian Ceng Ta. Here we reported the isolation, structural identification and their activities of four new steroids including three unusual C25 steroids (Fig. 1).

2. Experimental

2.1. General methods

UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. NMR experiments were obtained using Bruker Avance III 600 NMR spectrometers with tetramethylsilane (TMS) as an

internal standard. EI-MS was recorded on a Waters AutoSpec Premier P776 spectrometer electrospray ionization; ESI and HR-ESI-MS were recorded on a Finnigan LCQ-Advantage mass spectrometer and an API QSTAR time-of-flight spectrometer, respectively. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. CD spectra were performed on an Applied Photophysics Chirascan spectrometer (Agilent, Palo Alto, USA). Column chromatography was performed using silica gel (G, 200-300 mesh and GF_{254}) (Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia). Precoated silica gel GF_{254} plates (Qingdao Marine Chemical Factory, Qingdao, China) were used for thin layer chromatography (TLC). Some fractions were purified by the LC3000 Semi-preparation Gradient HPLC (Beijing Chuangxintongheng Science & Technology Co., Ltd, Beijing, China).

2.2. Material

The plant *H. serrata* (Thunb. ex Murray) Trev. was collected in Xichou County, Yunnan Province, People's Republic of China, in July 2013. A voucher specimen (No. 20130710ZPJ) was deposited at the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences. The detail of isolation endophytic fungi was described in previous work [10]. A strain, designated as M453, appeared after culturing for about two weeks and was isolated from the sterilized branch.

The strain, identified to be *Chaetomium* sp., was deposited in State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming, China. The *Colletotrichum* sp. M453 (30 L) was cultured on petri dishes (about 1000 dishes) containing modified Fries medium (1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 130 mg $CaCl_2 \cdot 2H_2O$, 1.0 g NH_4NO_3, 20.0 g sucrose, 5.0 g ammonium tartrate, 1.0 g yeast extract, 20 g agar, distilled water 1 L, pH is natural) at 25 °C for 20 days.

2.3. Extraction and isolation

The solid fermentation products was cut into small pieces and extracted exhaustively with an EtOAc/MeOH/AcOH mixture solution (80:15:5, v/v/v) by four times to obtain the crude extracts. The extracts was suspended in water and extracted three times by EtOAc. The ethyl acetate extract's residue (11.6 g) was separated on a column of silica gel G (200-300 mesh) eluting by petroleum ether-EtOAc (100: 4→6:4) and then $CHCl_3$-MeOH (100:6→0:100) gradient solvent system to produce eight fractions (Fr.1-Fr.8). The fraction Fr.5 (791 mg) was separated on a column of silica gel G (200-300 mesh) eluted with $CHCl_3$-acetone (100:2→0:100) and then chromatographed on Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) to produce six fractions (Fr.5.1-Fr.5.6). The Fr.5.2 (115 mg) was subjected on a column of silica gel G (200-300 mesh) eluted with a gradient solvent of $CHCl_3$-acetone (100:0→0:100) and then

purified by LC3000 semi-preparation gradient HPLC to produce **2** (2.9 mg). The Fr.5.3 (334 mg) was subjected on a column of silica gel (GF_{254}) using $CHCl_3$-MeOH, 100:1→6:4) gradient solvent system to produce three fractions (Fr.5.3.1-Fr.5.3.3). The Fr.5.3.1 (152 mg) was separated on Sephadex LH-20 (Acetone) and chromatographed by LC3000 semi-preparation gradient HPLC and then purified by preparational TLC separating plates (GF_{254}) to produce **1** (2.0 mg). The Fr.5.3.2 (112 mg) was subjected on Sephadex LH-20 (Acetone) and purified by LC3000 semi-preparation gradient HPLC to produce **4** (1.0 mg) and **5** (1.4 mg). The fraction Fr.6 (336 mg) was separated on a column of silica gel (GF_{254}) eluted with $CHCl_3$-MeOH (100: 3→0 : 100) to give six fractions of Fr.6.1 to Fr.6.6. The Fr.6.2 (101 mg) was chromatographed by Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) to produce four fractions (Fr.6.2.1-Fr.6.2.4). The Fr.6.2.2 (73 mg) was subjected on a column of silica gel (GF_{254}) using $CHCl_3$-MeOH (100:3→0:100) gradient solvent system and then purified by LC3000 semi-preparation gradient HPLC to produce **3** (1.1 mg). The Fr.6.5 (106 mg) was subjected on Sephadex LH-20 ($CHCl_3$:MeOH, 1:1) and then separated by LC3000 semi-preparation gradient HPLC, and purified by preparational TLC [sample (7.9 mg) was dissolved in MeOH and repeated subjected to TLC separating plates and then performed by $CHCl_3$-acetone (100:8) (100 mL eluent containing 0.3 mL CH_3COOH)] to produce **6** (2.0 mg). The Fr.7 (117 mg) was chromatographed on a

column of silica gel (GF_{254}) eluted with a gradient eluent ($CHCl_3$-MeOH, 100:3→0:100) to give six fractions (Fr.7.1 to Fr.7.6). The Fr.7.5 (92 mg) was separated on Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) and then purified by LC3000 semi-preparation gradient HPLC to produce **7** (3.2 mg).

Neocyclocitrinol A (**1**): Colorless needle; $[\alpha]_D^{25}$ = +147 (*c* = 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 202 (4.14), 246 (4.19); NMR data see Table 1; ESI-MS: 439 $[M + Na]^+$; HR-ESI-MS: 439.2456 ($[M + Na]^+$, calc. 439.2460).

Crystal data: All single-crystal X-ray diffraction data were collected at 100 (2) K on Bruker APEX DUO with Cu Kα radiation (λ = 1.54178 Å). The structures were solved by direct methods (SHELXS-97) and refined using full-matrix least-squares difference Fourier techniques. $C_{25}H_{36}O_5$•H_2O, M = 434.55, a = 11.9797(17) Å, b = 7.4355(10) Å, c = 13.6934(18) Å, α = 90°, β = 113.605(6)°, γ = 90°, V = 1117.7(3) Å^3, T = 100(2) K, space group $P21$, Z = 2, μ(CuKα) = 0.733 mm^{-1}, 8725 reflections measured, 3367 independent reflections (R_{int} = 0.0604). The final R_1 values were 0.0967 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.2654 ($I > 2\sigma(I)$). The final R_1 values were 0.0987 (all data). The final $wR(F^2)$ values were 0.2699 (all data). The goodness of fit on F^2 was 1.227. Flack parameter = 0.2(2).

Cyclocitrinol B (**2**): Colorless amorphism; $[\alpha]_D^{24}$ = +93 (*c* = 0.13,

MeOH); UV (MeOH) λ_{max} (log ε): 202 (4.12), 240 (4.06), 275 (3.65); NMR data see Table 1; ESI-MS: 439 $[M + Na]^+$; HR-ESI-MS: 439.2405 ($[M + Na]^+$, calc. 439.2460).

Cyclocitrinol C (**3**): Colorless amorphism; $[\alpha]_D^{23}$ = +71 (*c* = 0.11, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.14), 229 (4.22), 270 (3.75); NMR data see Table 2; ESI-MS: 437 $[M + Na]^+$; HR-ESI-MS: 437.2288 ($[M + Na]^+$, calc. 437.2304).

3β-hydroxy-5,9-epoxy-(22E,24R)-ergosta-7,22-dien-6-one (**4**): Colorless amorphism; $[\alpha]_D^{24}$ = -34 (*c* = 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 200 (3.78), 238 (3.95); NMR data see Table 2; EI-MS: 426 $[M]^+$; HR-ESI-MS: 427.3195 ($[M + H]^+$, calc. 427.3207).

2.4. Assay activities

AChE inhibitory activities of the compounds were assayed by the spectrophotometric method developed by Ellman with modification [11]. S-Acetylthiocholine iodide, S-butyrylthiocholine iodide, 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), acetylcholinesterase derived from human erythrocytes were purchased from Sigma Chemical. Compounds (**1-4**) dissolved in DMSO. The reaction mixture (totally 200 μL) containing phosphate buffer (pH 8.0), test compound (50 μM), and acetyl cholinesterase (0.02 U/mL), was incubated for 20 min at 37 °C. Then, the reaction initiated by the addition of 40 μL of solution

containing DTNB (0.625 mM) and acetylthiocholine iodide (0.625 mM) for AChE inhibitory activity assay, respectively. The hydrolysis of acetylthiocholine monitored at 405 nm every 30 seconds for one hour. Tacrine was using as positive control with final concentration of 0.333 μM. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows: % inhibition = (E - S)/E × 100 (E is the activity of the enzyme without test compounds and S is the activity of enzyme with test compound) [11].

Antitumor activity was measured by the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazalium bromide, MTT, Sigma] assay [12]. Five cell lines were selected for testing (leukemia cell line HL-60, hepatocarcinoma cell line SMMC-7721, lung adenocarcinoma cell line A-549, breast cancer cell line MCF-7 and colon cancer cell line SW480), and MW300 used as control.

3. Results and discussion

The nucleotide sequences of the ITS5-5.8S rDNA-ITS4 region of the endophytic fungus M453 was registered in the GenBank database [accession number KX756618], and the strain was determined to be *Colletotrichum* sp. based on ITS analysis.

Compound **1** was obtained as colorless needle. The HR-ESI-MS data indicated a molecular formula of $C_{25}H_{34}O_5$ based on the $[M + Na]^+$ ion

signal at *m/z* 439.2456. The ^{13}C-NMR and DEPT spectra (Table 1) revealed six quaternary carbons, nine methines, seven methylenes and three methyls. According to the ^{1}H-, ^{13}C-, and DEPT-NMR (Table 1), compound **1** was an unusual C25 steroid skeleton [13-15]. Further, comparison the ^{1}H and ^{13}C NMR data (Table 1) with those of isocyclocitrinol A [14], a hydroxyl group was located at C-5 in compound **1** based on the following HMBC correlations (Fig. 1): one proton of methylene at δ_H 2.88 (H-4) correlated with the carbons at δ_C 36.30 (C-2) and 76.5 (C-5); the olefinic proton at δ_H 5.56 (H-7) with carbons at δ_C 160.0 (C-8), 76.5 (C-5), 57.2 (C-14) and 54.8 (C-9); the methylene protons at δ_H 2.51 and 2.76 (H-18) with carbons at δ_C 141.7 (C-10), 125.4 (C-1), 76.5 (C-5) and 50.1 (C-4). In the COSY spectrum (Fig. 2), the three branches were deduced to be –C-1−C-2−C-3−C-4− (−branch), –C-9−C-11−C-12− (−branch) and −C-22–C-23–C-24−C-25− (−branch) from a complete interpretation of the key cross-peaks (H-1/H-2/H-3/H-4; H-9/H-11/H-12 and H-22/H-23/H-24/H-25). These, together with other correlations (Fig. 2), determined the planar structures. The relative stereochemistry of C-9 and C-14 were same with isocyclocitrinol on the basis of NOESY correlations: H-9 (δ_H 2.83) correlated with H-14 (δ_H 2.21) (Fig. 2). The total relative configuration of compound **1** was determinated by X-ray crystallographic analysis (Fig. 3). Its absolute configuration was determined on the basis of the CD spectral analyses (Fig. 4). The CD

curves of **1** showed very similar with this type compound positive CE around 250 nm and negative CE around 325 nm [15], indicating the same absolute configuration as shown in Fig. 1, and named as neocyclocitrinol E.

Compound **2** was obtained as colorless amorphism. The HR-ESI-MS data indicated a molecular formula of $C_{25}H_{34}O_5$ based on the $[M + Na]^+$ ion signal at *m/z* 439.2405. The MS and NMR spectroscopic data (Table 1) of compound **2** were very similar to those of compound **1**, but the hydroxyl group was located at C-14 in compound **2** in stead of at C-5 in compound **1**. The HMBC experiment (Table 1) confirmed the structure: the methyl protons at δ_H 0.89 (H-19) correlated with the carbons at δ_C 32.4 (C-12), 49.7 (C-13), 86.7 (C-14) and 50.8 (C-17); the olefinic proton at δ_H 5.66 (H-7) correlated with carbons at δ_C 50.3 (C-5), 161.1 (C-8), 50.4 (C-9) and 86.7 (C-14). The 14-OH configuration was determinated to be α-configuration by comparison of the ^{1}H-NMR data H-7 and H-17 with photo-transformation of 20-hydroxyecdysone and 14-epi-20-hydroxyecdysone [16]. Moreover, the similar specific rotations and CD (Fig. 4) of compounds **2**, **1** and other similar compounds suggested that they had similar configuration [15, 17]. Based on the above data, the configuration of compound **2** was shown in Fig. 1, and named neocyclocitrinol F.

Compound **3** was obtained as colorless amorphism. The HR-ESI-MS data indicated a molecular formula of $C_{25}H_{34}O_5$ based on the $[M + Na]^+$ ion signal at *m/z* 437.2288. The MS and NMR spectroscopic data (Table 2) showed that compound **3** was very similar to compound **2** (Table 1), but one hydroxyl at C-22 of **2** was shifted and replaced by a keto group at the C-24 in **3**, and a double bond (C-23−C-24) of **2** was shifted to C-22−C-23 in **3** (Fig. 1). The HMBC experiment (Table 2) showed that the methyl protons at δ_H 1.28 (H-21) correlated with carbons at δ_C 55.8 (C-17), 76.0 (C-20) and 157.4 (C-22); the olefinic proton at δ_H 6.99 (H-22) correlated with the carbons at δ_C 76.0 (C-20), 28.9 (C-21), 127.7 (C-23) and 202.0 (C-24); the olefinic proton at δ_H 6.16 (H-23) correlated with the carbons at δ_C 76.0 (C-20), 202.0 (C-24) and 27.4 (C-25); the methyl protons at δ_H 2.17 (H-25) correlated with carbons at δ_C 157.4 (C-22), 127.7 (C-23) and 202.2 (C-24). These data together with other correlations (Table 2) established the planar structure. The 14-OH configuration was determinated to be α-configuration by comparison of the NMR data H-7 and H-17 with photo-transformation of 20-hydroxyecdysone and 14-epi-20-hydroxyecdysone [16]. Moreover, the similar specific rotations and CD (Fig. 4) of compound **3** and other similar compounds suggested that they had similar configuration [15]. Based on the above data, the configuration of compound **3** was shown in Fig. 1, and named neocyclocitrinol G.

Compound **4** was obtained as yellow oil. The HR-ESI-MS data indicated a molecular formula of $C_{28}H_{42}O_3$ based on the $[M + H]^+$ ion signal at *m/z* 427.3195. Comparison with 3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one [18], an O-bridge connected between C-5 and C-9 in compound **4** on basis of EI-MS, HR-ESI-MS and HMBC correlations (Table 2). From the ^{1}H-NMR spectrum, the coupling constant of H-22 and H-23 was 15.2 Hz, revealing that the double bond at C-22 was in the *E* configuration. The relative stereochemistry of part carbons were determinated on the basis of NOESY correlations: H-19 (δ_H 0.57) correlated with H-21 (δ_H 0.97); H-19 (δ_H 0.57) with H-18 (δ_H 0.90). In addition, the similar physiochemical and specific rotations [3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one, -64 (*c* 0.056, $CHCl_3$); **4**, -34 (*c* = 0.10, MeOH)] suggested their configurations are the same [18]. Based on the above data, the configuration of compound **4** was shown in Fig. 1, and named 3β-hydroxy-5,9-epoxy-(22*E*,24*R*)-ergosta-7,22-dien-6-one.

Compounds **1-4** were assayed for cytotoxicity and acetylcholinesterase (AChE) inhibitory activities. No new compounds showed any cytotoxicity at 40 μM; then compound **4** showed weak AChE inhibitory activity (The percentage inhibition was at 20%~60% in 50 μM).

In general opinion, the steroids are modified triterpenes containing the tetracyclic ring system of lanosterol [19], but lacking the three methyl groups at C-4 and C-14 [20]. Base on the unusual C25 skeleton structure, this type compounds belong to steroid. According to the ^{13}C-isotope labeled [13] and chemical synthesis results [21] , the biosynthetic origin of this framework (**1**-**3**, **5**) most likely produces via a 1,2 migration of the C-5−C-10 bond to give a new C-5−C-18 bond [22]: assuming the participation of enzymes able to make C-19 in ergosterol-type precursor, C-5 can form a C-5−C-18 bond to producing the framework (Scheme 1). The side chain of this framework can also originate from ergosterol-type precursor (Scheme 1): oxidations at C-22, C-23 and C-25 of ergosterol-type precursor and then cleavage of C-24–C-25 bond by oxidoreductase [23].

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments.

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References

[1] Y. Huang, J. Wang, G. Li, Z. Zheng, W. Su, FEMS Immunol. Med. Microbiol. 31 (2001) 163.

[2] N. Neuss, M.N. Neuss, In The Alkaloids. New York: Academic Press, 37 (1990) 229.

[3] A. Stierle, G. Strobel, D. Stierle, P. Grothaus, G. Bignami, J. Nat. Prod. 58 (1995) 1315.

[4] K. Shrestha, G.A. Strobel, S.P. Shrivastava, M.B. Gewali, Planta Med. 67 (2001) 374.

[5] A.L. Eyberger, R. Dondapati, J.R. Porter, J. Nat. Prod. 69 (2006) 1121.

[6] S.C. Puri, V. Verma, T. Amna, G.N. Qazi, M. Spiteller, J. Nat. Prod. 68 (2005) 1717.

[7] S. Phongpaichit, N. Rungjindamai, V. Rukachaisirikul, J. Sakayaroj,

FEMS Immunol. Med. Microbiol. 48 (2006) 367.

[8] W.Y. Huang, Y.Z. Cai, K.D. Hyde, H. Corke, M. Sun, Fungal Divers. 33 (2008) 61.

[9] L.H. Rosa, V.N. Gonçalves, R.B. Caligiorne, T.M.A. Alves, A. Rabello, P.A. Sales, Braz, J. Microbiol. 41 (2010) 420.

[10] Y.M. Chen, Y.H. Yang, X.N. Li, C. Zou, P.J. Zhao, Molecules 20 (2015) 16924.

[11] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.

[12] J. Su, P.J. Zhao, L.M. Kong, X.Y. Li, J.M. Yan, Y. Zeng, Y. Li, PloS one 8 (2013) e71333.

[13] L. Du, T. Zhu, Y. Fang, Q. Gu, W. Zhu, J. Nat. Prod. 71 (2008) 1343.

[14] T. Amagata, A. Amagata, K. Tenney, F.A. Valeriote, E. Lobkovsky, J. Clardy, P. Crews, Org. Lett. 5 (2003) 4393.

[15] M.W. Xia, C.B. Cui, C.W. Li, C.J. Wu, Mar. drugs 12 (2014) 1545.

[16] J. Harmatha, M. Budesinodot, K. Vokac, Steroids 67 (2002) 127.

[17] W.C. Lai, B. Danko, J. Csabi, Z. Kele, F.R. Chang, M.L. Pascu, A. Hunyadi, Steroids 89 (2014) 56.

[18] J. Valisolalao, B. Luu, G. Ourisson, Tetrahedron 39 (1983) 2779.

[19] P. M. Dewick. Medicinal Natural Products A Biosynthetic Approach. 3rd Ed. Wiley: Chichester; 2009.

[20] J. Acimovic, S. Goyal, R. Kosir, M. Golicnik, M. Perse, A. Belic, D.

Rozman, . Scientific reports 6(2016).

[21] S. El Sheikh, A.M. zu Greffen, J. Lex, J.M. Neudorfl, H.G. Schmalz, Synlett 2007 (2007) 1881.

[22] A. M. Marinho, E. Rodrigues-Filho, A. G. Ferreira, L. S. Santos, J. Brazil. Chem. Soc. 16 (2005) 1342.

[23] S. Sugano, R. Miura, N. Morishima, J. Biochem. 120 (1996) 780.

Legend Figures

Figure 1. The structures from *Chaetomium* sp. M453

Figure 2. Observed COSY and key HMBC correlations for compound **1**

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

Figure 4. CD spectra of **1-3** in MeOH solution

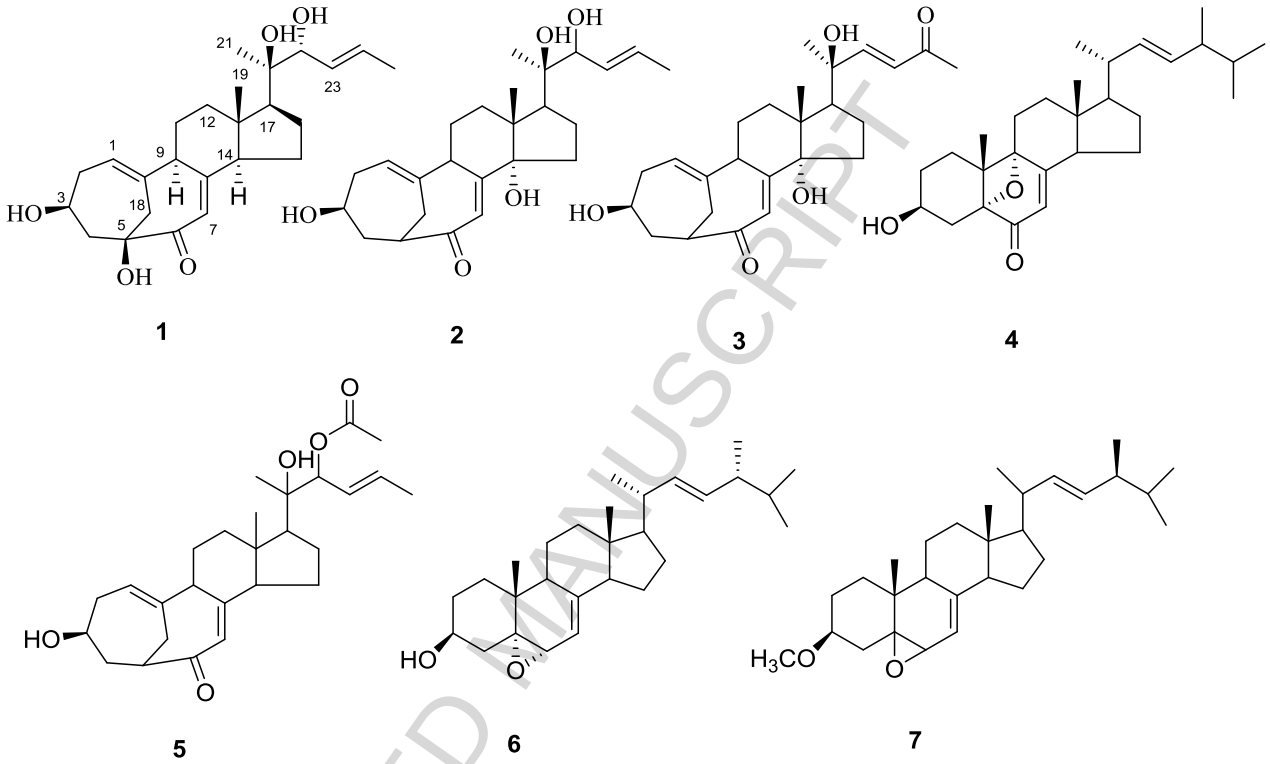


Fig. 1. The structures from *Chaetomium* sp. M453

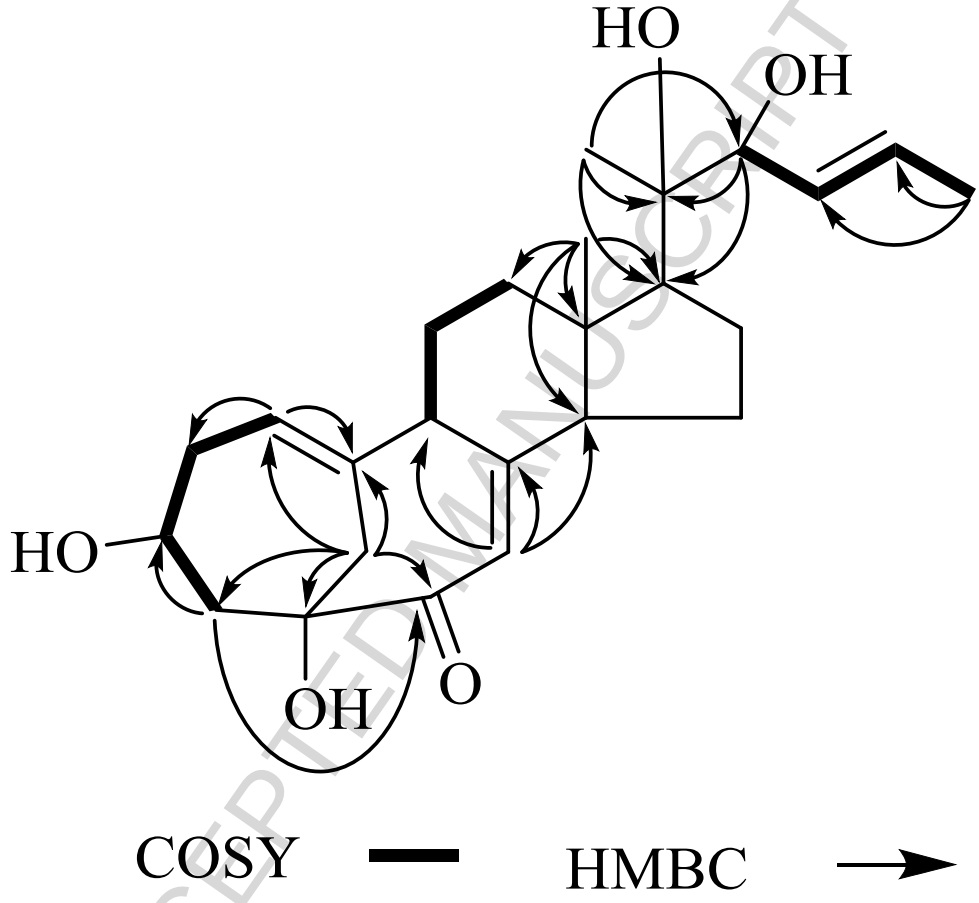


Fig. 2. Observed COSY and key HMBC correlations for compound **1**

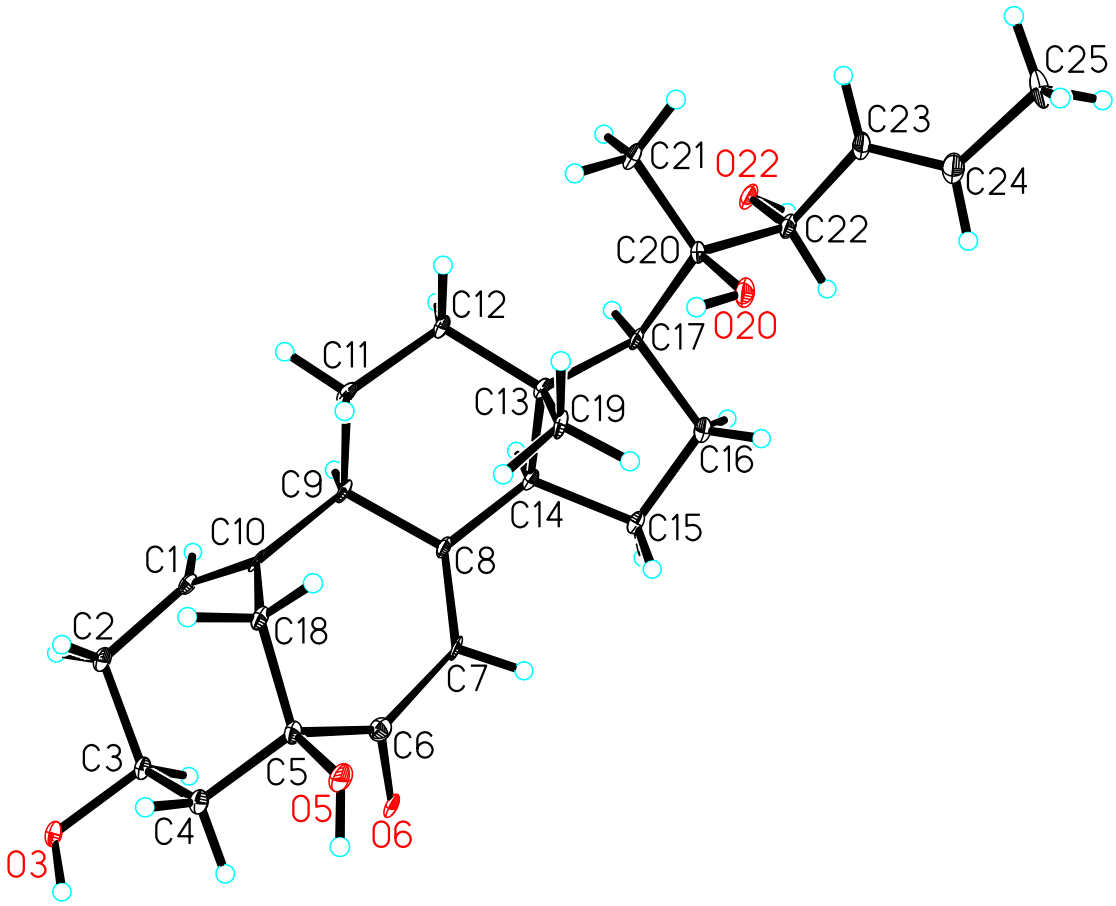


Fig. 3. Crystal X-ray structure of **1**

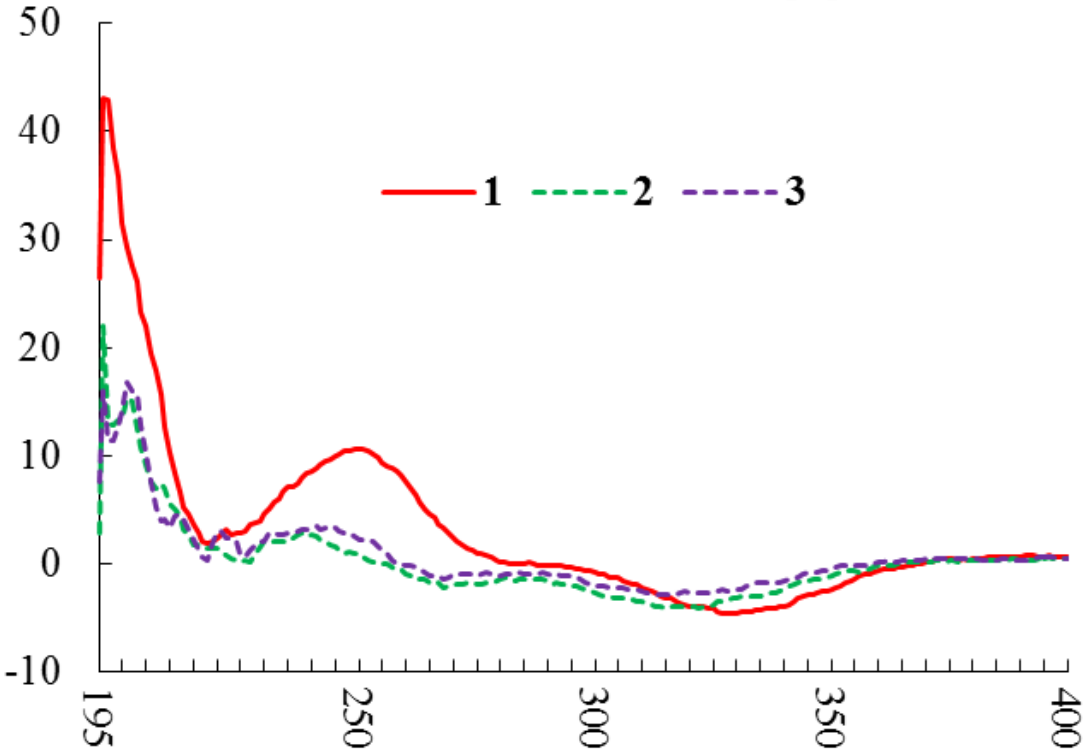
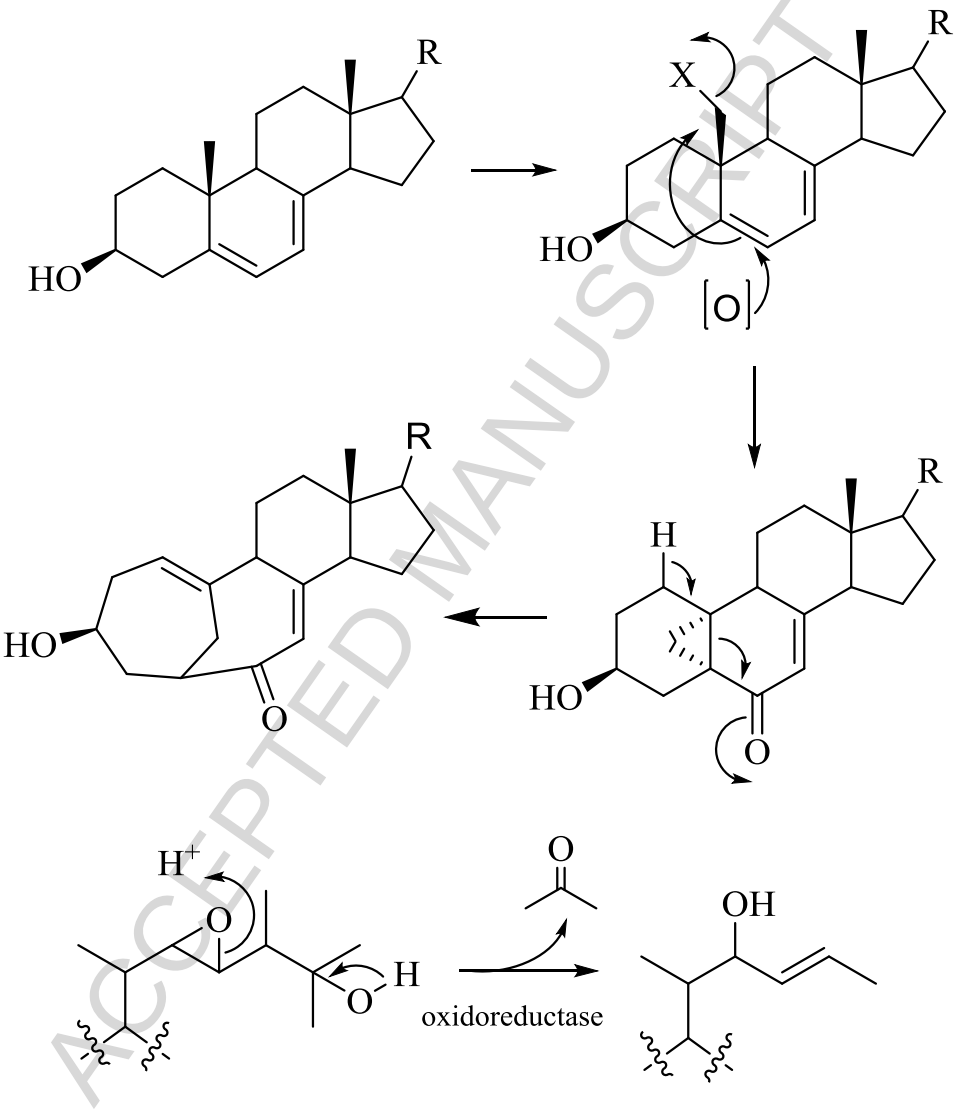


Fig. 4. CD spectra of **1**-**3** in MeOH solution



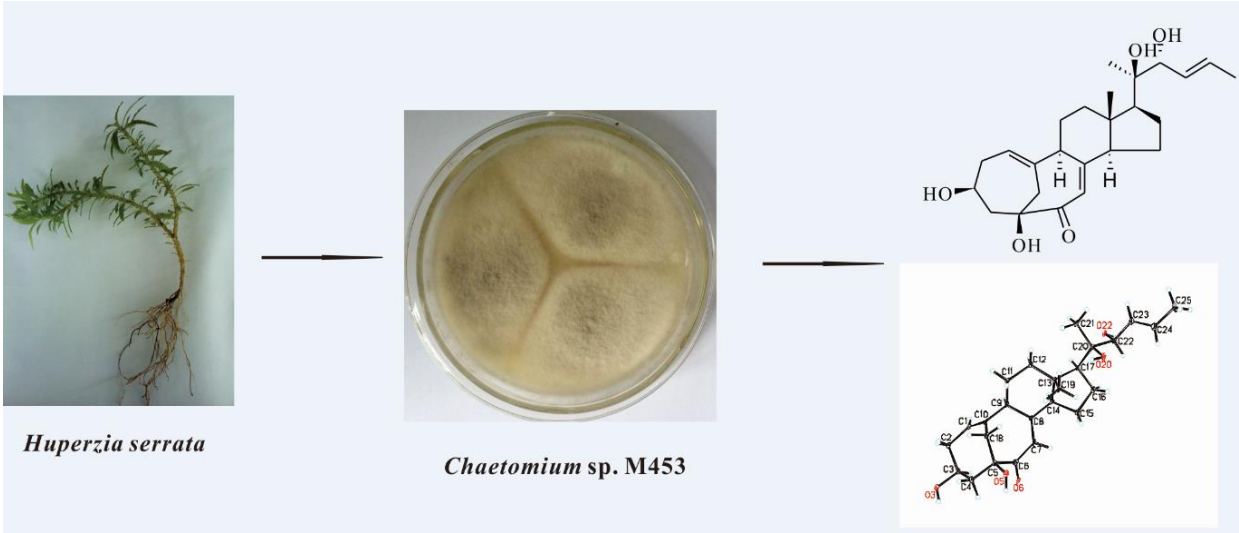
Scheme 1. The proposed biosynthetic pathway to the bicyclo [4:4:1] ring system and the C17 side chain of the unusual C25 steroids

Table 1 NMR data of compounds **1** and **2** (in CD_3OD, *J* in Hz)

Position	**1**			**2**		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1	5.69 (1H, m, overlap)	125.4, d	2, 9	5.53 (1H, t, 7.7)	123.5, d	2, 3, 9, 18
2	2.38 (1H, m)	36.30, t	3	2.38 (1H, m)	37.0, t	1, 3, 4, 10
	2.19 (1H, m)		3, 4, 10	2.10 (1H, m)		1, 3, 4, 10
3	3.29 (1H, s)	67.3, d	-	3.24 (1H, m)	65.3, d	-
4	2.88 (1H, d, 12.8)	50.1, t	2, 5	2.73 (1H, m)	42.1, t	3, 6, 18
	1.63 (1H, m, overlap)		-	1.56 (1H, m)		3, 6
5	-	76.5, s	-	2.71 (1H, m)	50.3, d	3, 4, 6, 10, 18
6	-	205.2, s	-	-	208.3, s	-
7	5.56 (1H, s)	123.6, d	5, 8, 9, 14	5.66 (1H, s)	126.0, d	5, 8, 9, 14
8	-	160.0, s	-	-	161.1, s	-
9	2.83 (1H, dd, 11.9, 5.6)	54.8, d	-	3.41 (1H, q, 5.8)	50.4, d	1, 7, 8, 10, 11
10	-	141.7, s	-	-	147.8, s	-
11	1.92 (1H, m)	28.6, t	13	1.75 (1H, m)	27.8, t	9, 10, 12
	1.63 (1H, m, overlap)		-	1.40 (1H, m)		8, 13
12	2.17 (1H, m)	40.7, t	-	1.44 (1H, m)	32.4, t	14, 17
	1.54 (1H, m)		11	1.87 (1H, m)		14, 16
13	-	47.5, s	-	-	49.7, s	-
14	2.21 (1H, m)	57.2, d	7, 8, 10, 13, 16	-	86.7, s	-
15	1.93 (1H, m)	22.3, t	-	2.00 (1H, m)	33.1, t	13
				1.67 (1H, m)		14
16	1.61 (2H, m, overlap)	23.7, t	-	1.82 (2H, m)	21.5, t	12, 17
17	2.10 (1H, t, 9.8)	56.2, d	12, 13, 15, 19	2.59 (1H, m)	50.8, d	15, 16, 19, 20
18	2.76 (1H, d, 13.3)	36.33, t	1, 4, 5, 6, 10	2.59 (1H, m)	28.5, t	1, 4, 5, 9, 10
	2.51 (1H, d, 13.3)		1, 4, 5, 10	2.51 (1H, m)		1, 4, 5, 6, 9, 10
19	0.90 (3H, s)	14.5, q	12, 13, 14, 17	0.89 (3H, s)	18.35, q	12, 13, 14, 17
20	-	77.2, s	-	-	77.7, s	-
21	1.20 (3H, s)	21.6, q	17, 20, 22	1.09 (3H, s)	22.0, q	17, 20, 22
22	3.83 (1H, d, 5.6)	78.5, d	17, 20	3.75 (1H, d, 5.5)	79.2, d	17, 20, 21, 23, 24
23	5.65 (1H, m)	131.3, d	25	5.57 (1H, m)	131.6, d	21, 22
24	5.71 (1H, m, overlap)	128.8, d	-	5.60 (1H, m)	128.9, d	21, 22
25	1.73 (3H, d, 6.1)	18.2, q	23, 24	1.64 (3H, d, 5.8)	18.40, q	23, 24

Table 2 NMR data of compounds **3** and **4** (in CD_3OD, *J* in Hz)

Position	**3**			**4**		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1	5.53 (1H, t, 7.4)	123.6, d	3, 5, 9, 18	1.39 (1H, m, overlap) 2.20 (1H, dt, 1.5, 13.4)	26.6, t	5, 10 -
2	2.38 (1H, m) 2.10 (1H, m)	37.0, t	1, 3, 4, 10 1, 3, 4, 10	1.36 (1H, m, overlap) 1.78 (1H, m)	31.0, t	- -
3	3.25 (1H, s)	65.3, d	5	3.83 (1H, m)	67.8, d	-
4	2.74 (1H, m) 1.56 (1H, m)	42.1, t	18 3, 6	1.52 (1H, m) 1.92 (1H, m)	37.1, t	- 3, 5
5	2.69 (1H, m)	50.3, d	18	-	80.2, s	-
6	-	208.2, s	-	-	200.1, s	-
7	5.64 (1H, s)	126.2, d	5, 8, 9, 14	5.49 (1H, s)	120.9, d	5, 9, 14
8	-	160.2, s	-	-	165.0, s	-
9	3.40 (1H, m)	50.4, d	1, 7, 8, 10, 11		76.1, s	-
10	-	147.7, s	-	-	42.8, s	-
11	1.43 (1H, m) 1.73 (1H, m, overlap)	27.8, t	8, 9, 12, 13 -	1.70 (1H, m) 1.64 (1H, m)	29.1, t	8, 9, 13 8, 9, 13
12	2.02 (1H, m) 1.73 (1H, m, overlap)	32.8, t	11, 13, 19 -	1.62 (1H, m) 1.80 (1H, m)	36.2, t	- 9, 14
13	-	49.8, s	-	-	46.2, s	-
14	-	86.5, s	-	2.66 (1H, dd, 11.5, 7. 6)	52.8, d	7, 8, 13, 15, 19
15	1.88 (1H, m) 1.46 (1H, m)	32.4, t	16 8, 13, 14	1.42 (1H, m) 1.52 (1H, m)	23.4, t	- -
16	1.68 (2H, m)	22.8, t	15, 17, 20	1.36 (1H, m) 1.89 (1H, m)	29.3, t	- -
17	2.39 (1H, m)	55.8, d	13, 15, 16, 19, 20, 22	1.34 (1H, m)	57.4, d	12, 13, 16, 19, 20, 21, 22
18	2.56 (1H, m) 2.50 (1H, m)	28.5, t	1, 4, 5, 9, 10 1, 4, 5, 6, 9, 10	0.90 (3H, s)	20.6, q	1, 5, 9, 10
19	0.81 (3H, s)	19.0, q	12, 13, 14, 17	0.57 (3H, s)	12.6, q	12, 13, 14, 17
20	—	76.0, s	—	1.97 (1H, m)	41.7, d	17, 22, 23
21	1.28 (3H, s)	28.9, q	17, 20, 22	0.96 (3H, d, 6.6)	21.6, q	17, 20, 22
22	6.99 (1H, d, 15.9)	157.4, d	20, 21, 23, 24	5.13 (1H, dd, 8.2, 15.2)	133.6, d	21, 23, 24
23	6.16 (1H, d, 15.9)	127.7, d	20, 24, 25	5.19 (1H, dd, 7.6, 15.2)	136.7, d	19, 22
24	-	202.0, s	-	1.76 (1H, m)	44.4, d	22, 23
25	2.17 (3H, s)	27.4, q	22 (w), 23, 24	1.39 (1H, m, overlap)	34.4, d	-
26	-	-	-	0.77 (3H, d, 6. 8)	20.5, q	24, 25, 27
27	-	-	-	0.75 (3H, d, 6.8)	20.1, q	24, 25, 26
28	-	-	-	0.85 (3H, d, 6.8)	18.2, q	23, 24, 25



Graphical abstract

Highlights:

>The chemical constituents of the entophytic fungus *Chaetomium* sp. M453 were investigated.

>Three unusual C25 steroids, neocyclocitrinols E-G and 3β-hydroxy-5,9-epoxy-(22*E*,24*R*)-ergosta-7,22-dien-6-one were isolated.

>Their structures were elucidated by extensive 1D- and 2D-NMR, and HR-ESI-MS experiments.

>The absolute configuration of **1** was determined by X-ray crystallographic analysis and CD analyses.

>Compound **4** showed weak acetylcholinesterase inhibitory activity.