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Strophasterols E and F: Rearranged ergostane-type sterols from *Pleurotus eryngii*

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ABSTRACT

Strophasterols E (**1**) and F (**2**) were isolated from the fruiting bodies of *Pleurotus eryngii,* together with four new ergostane-type sterols (**3–6**). Single-crystal X-ray diffraction analysis performed on the tris-*p*-bromobenzoate derivatives of compounds **1** and **2** allowed these two compounds to be identified as the structurally rare (22*S*,23*R*)- and (22*S*,23*S*)-5α,6α-epoxy-3β,7β,23-trihydroxy-15(14 → 22)-*abeo*-ergost-8-en-14-one, respectively. The inhibitory effects on nitric oxide production of the six new steroids thus isolated from the fruiting bodies of *P. eryngii* were also evaluated.

1. Introduction

In recent years, several new rearranged ergostane-type steroids have been isolated from corals and fungi [1]. Among them, unprecedented 15(14 → 22)-*abeo*-ergostane sterols named strophasterols A–D were isolated from the mushroom *Stropharia rugosoannulata* by Kawagishi et al. [2]. The structures of strophasterols A and B were determined by single-crystal X-ray diffraction analysis [2,3]. It was also revealed that strophasterol A exhibited an inhibitory effect on endoplasmic reticulum stress cell death caused by thapsigargin. Strophasterol A was synthesized by Heretsch et al. in 2016 [4], and strophasterols A and B were synthesized by Kuwahara et al. in 2017 [5]. These studies will contribute to the development of research on strophasterols from viewpoints such as the preparation of analogues and investigation for their biological activity. Further, in 2017, Aung et al. also reported a 15(14 → 22)-*abeo*-ergostane sterol, named glaucoposterol A, having a hydroxy group at C-23 [6].

The mushroom *Pleurotus eryngii* (pleurotaceae), which is called “eringi” in Japanese and “king oyster mushroom” or “oyster mushroom” in English, is native to Europe, North Africa, and Asia [7]. In Japan, where *P. eryngii* is commonly regarded as an edible produce, 29,000 t of this mushroom were produced in 2003 [7]. *P. eryngii* contains a protein showing cytotoxicity with respect to tumor cells [8], a polypeptide exhibiting antioxidant, antitumor, and immunostimulatory activities [9], polysaccharides exhibiting inhibitory effects on lipid accumulation [10,11] and antitumor activity [12], pleurone, 4*H*-1,3-dioxine-2,4-dione showing an inhibitory effect on human neutrophil elastase [13], and eryngiolide A, a macrocyclic diterpenoid with cytotoxic properties toward tumor cells [14]. In our ongoing search for structurally interesting and bioactive compounds extracted from *P. eryngii*, we isolated ergostane sterols exhibiting inhibitory properties with respect to nitric oxide production [15] and human recombinant aromatase activity [16]; these compounds include structurally interesting sterols like eringiacetals A [17] and B [18] and pleurocins A and B [18]. (Pleurocins A and B were synthesized from ergosterol by Heinze and Heretsch in 2019 [19].) In this paper, we describe the isolation and structural elucidation of new 15(14 → 22)-*abeo*-ergostane sterols (**1**, **2**), named strophasterols E and F, respectively, and four new ergostane-type sterols (**3–6**).

2. Experimental

2.1. General methods

Dulbecco’s modified Eagle’s medium (DMEM), antibiotics, and lipopolysaccaride from *Escherichia coli* O157 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan); fetal bovine serum (FBS) was purchased from Invitrogen Co. (Carlsbad, CA, USA); sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan); N^G-monomethyl-L-arginine acetate (L-NMMA) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All other chemicals and

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reagents were of analytical grade. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter (Jasco, Tokyo, Japan). UV spectra were recorded using a HITACHI U-2000 spectrometer (Hitachi, Tokyo, Japan). IR spectra were recorded on an FT/IR-680 Plus spectrometer from JASCO Corp.. Electron ionization mass spectrometry (EI MS) spectra were recorded using a Hitachi 4000H double-focusing mass spectrometer (70 eV) (Hitachi), and a JEOL JMS-700 (JEOL, Tokyo, Japan) for fast atomic bombardment mass spectrometry (FAB MS). ^{1}H and ^{13}C NMR spectra were recorded using an Agilent-NMR-vnmrs600 (^{1}H: 600 MHz; ^{13}C: 150 MHz) spectrometer (Agilent Technologies, CA, USA), using $CDCl_3$, C_5D_5N, or $(CD_3)_2SO$ as solvents, and tetramethylsilane as an internal standard. Silica gel (70–230 mesh, Merck, Darmstadt, Germany) and silica gel 60 (230–400 mesh, Nacalai Tesque, Inc.) were used for column chromatography purifications. HPLC was performed using an octadecylsilane column [Cosmosil $5C_{18}$-MS-II column (Nacalai Tesque, Inc.) (25 cm × 20 mm i.d.) and using as eluents methanol (MeOH)/H_2O (85:15, System I), MeOH/H_2O (80:20, System II), MeOH/H_2O (75:25, System III) at 35 °C at a flowrate of 4.0 mL/min.

2.2. Materials

The fruiting bodies of *P. eryngii*, produced in Kagawa, Japan, were purchased from HOKUTO Corp. in 2014 (Sample 1) and 2015 (Sample 2). A voucher specimen was deposited at the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

2.3. Extraction and isolation

2.3.1. Sample 1

The fruiting bodies of *P. eryngii* (dry weight: 11 kg) were subjected to extraction with MeOH under reflux conditions over 3 consecutive days for a total of four times. The MeOH extract (2625 g) was then partitioned between ethyl acetate (AcOEt) and H_2O. The AcOEt-soluble fraction (240 g) was subjected to column chromatography (CC) over silica [SiO_2 (2.8 kg); $CHCl_3$/AcOEt (1:0, 5:1, 1:1, and 0:1) and MeOH as eluents, used in increasing order of polarity] resulting in 37 fractions: Fr. *S1*-A–*S1*-Z and Fr. *S1*-a–*S1*-i.

Fr. *S1*-X (3158.27 mg), which was eluted using $CHCl_3$/AcOEt (1:1), was itself subjected to SiO_2 CC to yield 17 fractions: *S1*-X1–*S1*-X17. Fr. *S1*-X7 (225.66 mg), which had been eluted with hexane/AcOEt (1:1), was subjected to preparative HPLC (System II) to afford compound **5** (1.33 mg; t_R 74.1 min). Fr. *S1*-X9 (3158.27 mg), which had been eluted with hexane/AcOEt (1:1), was subjected to preparative HPLC (System II) to give compound **1** (0.74 mg; t_R 36.3 min). Preparative HPLC (System II) of Fr. *S1*-X10 (218.10 mg), which had been eluted with hexane/AcOEt (1:1), also gave compound **1** (0.35 mg).

Fr. *S1*-YZ (2667.67 mg), consisting of the combination of Fr. *S1*-Y and Fr. *S1*-Z, which had been eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 12 fractions: *S1*-YZ1–*S1*-YZ12. Fr. *S1*-YZ5 (169.58 mg), which had been eluted with pure AcOEt, was subjected to preparative HPLC (System I) to give compound **3** (1.77 mg; t_R 61.5 min). Preparative HPLC (System II) performed on Fr. *S1*-YZ6 (136.92 mg), which had been eluted with hexane/AcOEt (1:1), also gave compound **4** (1.15 mg; t_R 97.9 min).

Fr. *S1*-c (2631.02 mg), which had been eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 22 fractions: *S1*-c1–*S1*-c22. Preparative HPLC (System III) performed on Fr. *S1*-c12 (102.11 mg), which had been eluted with AcOEt, gave compound **2** (2.74 mg; t_R 29.7 min). Notably, compound **2** (4.43 mg, 2.70 mg, and 1.23 mg) was also isolated from Fr. *S1*-c13 (66.83 mg), Fr. *S1*-c14 (62.22 mg), and Fr. *S1*-c15 (152.25 mg), all of which had been eluted with pure AcOEt.

2.3.2. Sample 2

The fruiting bodies of *P. eryngii* (dry weight: 13 kg) were extracted with MeOH under reflux conditions over 3 consecutive days for a total of four times. The MeOH extract (1920 g) was partitioned between AcOEt and H_2O. The AcOEt-soluble fraction (385 g) was subjected to CC over silica [SiO_2 (3.5 kg); hexane/AcOEt (5:1, 1:1, and 0:1) and AcOEt/MeOH (10:1, and 0:1) as eluents, used in increasing order of polarity] resulting in 14 fractions: Fr. *S2*-A–*S2*-N.

Fr. *S2*-F (33 g), which had been eluted with hexane/AcOEt (1:1), was itself subjected to SiO_2 CC to produce 16 fractions: Fr. *S2*-F1–*S2*-F16. Fr. *S2*-F5 (33 g) was then eluted with hexane:AcOEt 5:1 and subjected once more to SiO_2 CC to yield 18 fractions: Fr. *S2*-F5-1–*S2*-F5-18.

Preparative HPLC using System II and applied to Fr. *S2*-F5-9 (153.70 mg), which had been eluted with pure AcOEt, gave compound **5** (0.92 mg).

Preparative HPLC using System I and applied to Fr. *S2*-F5-10 (219.51 mg), which had been eluted with pure AcOEt, gave product **6** (1.49 mg; t_R 76.7 min). Preparative HPLC using System II and applied to Fr. *S2*-F5-11 (175.44 mg), which had been eluted with pure AcOEt, gave compounds **3** (1.84 mg; t_R 83.0 min) and **6** (1.26 mg; t_R 86.6 min).

Performing SiO_2 CC on Fr. *S2*-F6 (5 g), which had been eluted with hexane/AcOEt 5:1, resulted in the 35 fractions: Fr. *S2*-F6-1–*S2*-F6-35. Preparative HPLC using System I and applied to Fr. *S2*-F6-11 (183.03 mg) with AcOEt as eluent gave compound **5** (0.73 mg; t_R 45.5 min); preparative HPLC using System II and applied to Fr. *S2*-F6-12 (127.67 mg) with AcOEt as eluent gave product **6** (0.83 mg).

2.3.3. Strophasterol E (1)

White solid, $[\alpha]_D^{20}$ −5.0 (*c* 0.20, EtOH); UV (EtOH) λ_{max} (log ε): 241.5 (3.86) nm; IR (KBr) ν_{max}: 3446, 2956, 2869, 1650, 1460, 1377, 1075, 1042 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z*: 461.3268 $[M + H]^+$ (calcd for $C_{28}H_{45}O_5$, 461.3267).

2.3.4. Strophasterol F (2)

Amorphous solid; $[\alpha]_D^{20}$ −16.0 (*c* 0.058, EtOH); UV (EtOH) λ_{max} (logε): 241.0 (3.87) nm; IR (KBr) ν_{max}: 3451, 2957, 2876, 1651, 1461, 1377, 1061, 1027 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z*: 461.3268 $[M + H]^+$ (calcd for $C_{28}H_{45}O_5$, 461.3267).

2.3.5. (22E)-9α,11α-epoxyergosta-7,22-diene-3β,5α,6β-triol (3)

Amorphous solid; $[\alpha]_D^{20}$ −45.6 (*c* 0.022, EtOH); IR (KBr) ν_{max}: 3433, 1558, 1384 cm^{-1}; ^{1}H and ^{13}C NMR in $CDCl_3$ data, see Table 2; ^{1}H NMR in C_5D_5N (400 MHz) δ_H: 0.71 (3H, s), 0.83 (3H, d, 6.8 Hz), 0.84 (3H, d, 6.4 Hz), 0.93 (3H, d, 6.8 Hz), 0.98 (3H, d, 6.8 Hz), 1.74 (3H, s), 2.94 (1H, t, 12.4 Hz), 3.28 (1H, d, 5.6 Hz), 4.54 (1H, br s), 4.83 (1H, m, $W_{1/2}$ = ca. 21 Hz), 5.12 (m, overlapped), 5.20 (1H, m), 6.09 (br d, 3.2), 6.16 (1H, d, 3.6 Hz), 6.88 (1H, d, 6.0 Hz) ppm; HREIMS *m/z*: 444.3241 $[M]^+$ (calcd for $C_{28}H_{44}O_4$, 444.3240).

2.3.6. (22E)-5α,6α-epoxyergosta-8(14),22-diene-3β,7α,15α-triol (4)

Colorless crystal (MeOH); mp 212–215 °C; $[\alpha]_D^{20}$ −135.0 (*c* 0.029, EtOH); IR (KBr) ν_{max}: 3429, 1455, 1384 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HREIMS *m/z*: 444.3237 $[M]^+$ (calcd for $C_{28}H_{44}O_4$, 444.3240).

2.3.7. (22E)-5α,6α-epoxy-3β,7α,14β-trihydroxyergosta-8,22,dien-15-one (5)

Amorphous solid; $[\alpha]_D^{20}$ −87.4 (*c* 0.097, EtOH); UV (EtOH) λ_{max} (logε): 214.5 (3.62) nm; IR (KBr) ν_{max}: 3443, 2957, 2923, 2851, 1741, 1384 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HRFABMS *m/z*: 481.2928 $[M + Na]^+$ (calcd for $C_{28}H_{42}O_5Na$, 481.2929).

2.3.8. (22E)-3β,5α,6β-trihydroxy-9,11-seco-ergosta-7,22,dien-9-one (6)

Amorphous solid; $[\alpha]_D^{20}$ + 0.2 (c 0.11, EtOH) ; UV (EtOH) λ_{max} (logε) 201.0 (3.73), 227.5 (3.56) nm; IR (KBr) ν_{max}: 3433, 2958, 1742, 1473, 1389, 1268 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3; HREIMS *m/z*: 446.3395 $[M]^+$ (calcd for $C_{28}H_{46}O_4$, 446.3396).

Table 1
^{1}H and ^{13}C NMR data for compounds **1** and **2** in $CDCl_3$ (δ in ppm, J in Hz).

	1					**2**			
position	δ_H		δ_C			δ_H		δ_C	
1α	1.74	(1H, m)	29.4	t		1.74[b]		29.3	t
1β	1.88	(1H, m)				1.88	(1H, m)		
2α	2.04	(1H, m)	30.40[a]	t		2.04	(1H, m)	30.4	t
2β	1.69	(1H, m)				1.70[b]			
3	3.96	(1H, m)	68.3	d		3.96	(1H, tt, 11.2, 5.3)	68.3	d
4α	1.52[b]		38.8	t		1.52[b]		38.8	t
4β	2.22	(1H, dd, 12.9, 11.4)				2.22	(1H, m)		
5			62.5	s				62.5	s
6	3.24	(1H, d, 2.6)	59.3	d		3.24	(1H, d, 2.6)	59.4	d
7	4.91	(1H, br s)	62.9	d		4.88	(1H, br s)	63.0	d
8			128.3	s				128.2	s
9			159.9	s				159.7	s
10			39.6	s				39.6	s
11α	2.42	(1H, dddd, 19.2, 7.6, 5.3, 1.2)	22.1	t	11A	2.24	(1H, dt, 19.1, 4.9)	22.0	t
11β	2.26	(1H, dt, 19.2, 5.3)			11B	2.42	(1H, dddd, 19.1, 9.1, 5.2, 1.1)		
12A	1.64	(1H, m)	32.3	t		1.66	(1H, m)	31.6	t
12B	2.09[b]					2.06	(1H, dt, 13.8, 5.0)		
13			45.8	s				45.6	s
14			205.6	s				205.3	s
15	1.42[a, b]		27.7[a]	t		1.50[b]		23.9	t
16	1.41[a, b]		30.44[a]	t		1.40	(1H, m)	26.4	t
17	1.85	(1H, m)	50.8	d		1.94	(1H, dd, 16.4, 8.5)	48.1	d
18	1.03	(3H, s)	19.9	q		0.99	(3H, s)	19.4	q
19	1.33	(3H, s)	22.5	q		1.33	(3H, s)	22.4	q
20	1.99	(1H, m)	36.0	d		1.73[b]		36.7	d
21	1.11	(3H, d, 6.8)	23.9	q		0.99	(1H, d, 5.6)	20.3	q
22	1.80	(1H, m)	50.4	d		1.61	(1H, m)	50.9	d
23	3.36	(1H, m)	79.0	d		3.44	(1H, dd, 7.0, 3.8)	73.2	d
24	1.51[b]		41.6	d		1.35[b]		42.6	t
25	2.09[b]		26.4	d		1.74[b]		28.7	d
26	0.92	(3H, d, 7.0)	22.2	q		0.91	(1H, d, 6.8)	21.6	q
27	0.81	(3H, d, 6.7)	16.2	q		0.78	(1H, d, 6.7)	16.8	q
28	0.79	(3H, d, 7.0)	10.5	q		0.89	(1H, d, 7.0)	9.9	q

[a] Interchangeable.
[b] Overlapped with other signals.

2.4. Preparation of the tris-p-bromobenzoate derivative of compound 1 (1a)

p-Bromobenzoyl chloride (10.00 mg) was added to a solution of compound **1** (0.31 mg) in 1 mL of anhydrous pyridine. The resulting mixture was then stirred at room temperature for 15 h. The solvent was then evaporated *in vacuo* to give the crude product. The crude product was then purified by HPLC [*Cosmosil 5C$_{18}$-MS-II column* (*Nacalai Tesque, Inc.*)(25 cm × 20 mm i.d.) with MeOH] to give **1a** (0.54 mg; t_R 47.7 min).

2.4.1. Strophasterol E tris-p-bromobenzoate (1a)

Colorless needles (acetone); ^{1}H NMR in $CDCl_3$ (400 MHz) δ_H: 0.83 (3H, d, 7.2 Hz), 0.86 (3H, d, 6.4 Hz), 0.95 (3H, s), 1.06 (3H, d, 6.4 Hz), 1.46 (3H, s), 3.41 (1H, d, 2.8 Hz), 5.19 (1H, t, 6.4 Hz), 5.22 (1H, m), 6.40 (1H, d, 2.8 Hz), 7.54 (2H, d, 8.0 Hz), 7.57 (2H, d, 8.4 Hz), 7.61 (2H, d, 8.4 Hz), 7.80 (2H, d, 8.4 Hz), 7.86 (2H, d, 8.4 Hz), 7.91 (2H, d, 8.0 Hz) ppm; FAB MS *m/z*: 1007 [M + H (^{79}Br × 3)]$^+$, 1009 [M + H (^{79}Br × 2, ^{81}Br × 1)]$^+$, 1011 [M + H (^{79}Br × 1, ^{81}Br × 2)]$^+$, and 1013 [M + H (^{81}Br × 3)]$^+$; HR FAB MS *m/z*: 1007.1360 [M + H]$^+$ (calcd for $C_{49}H_{54}{}^{79}Br_3O_8$, 1007.1369).

2.4.2. X-Ray crystallographic analysis of 1a

$C_{49}H_{53}Br_3O_8$, Mr 1009.64, monoclinic, space group: *C*2, a = 38.9257(9) Å, b = 6.62720(10) Å, c = 19.7922(5) Å, β = 103.423(2)°, V = 4966.29(19) Å^3, Z = 4, F(0 0 0) = 2064, μ(Cu Kα) = 1.54184 mm^{-1}, measured independent reflections = 8512, number of reflections used for refinement = 7674 (I > 2σ(I)), parameters used for refinement = 540, final R_1 = 0.0890 (for I > 2σ(I)) and wR = 0.2409 (for all data), (δ/σ) max = 0.001, $\Delta\rho_{max}$ = 2.694 eÅ^{-3}, and $\Delta\rho_{min}$ = −0.905 eÅ^{-3}, Flack parameter = 0.011(19). X-ray diffraction data were collected on a Rigaku XtaLAB Pro P200 Hybrid Photon Counting diffractometer using the CuKα radiation. The structure of **1a** was solved employing direct methods (SHELXT Version 2014/4 [20]), and it was refined by the full-matrix least-squares on F^2 for all reflections (SHELXL Version 2014/7 [21]). The positions of all hydrogen atoms were determined using AFIX instructions, whereas the positions of all other atoms were refined anisotropically. CCDC 1902287 (for **1a**) contains the supplementary crystallographic data for this study. These data may be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.5. Preparation of the tris-p-bromobenzoate derivative of compound 2 (2a)

The tris-*p*-bromobenzoate derivative of compound **2** (**2a**, 2.96 mg), which was purified by HPLC (t_R 38.7 min), was prepared from **2** (2.31 mg) by applying the same procedure utilized for the preparation of **1a** (see details above).

2.5.1. Strophasterol F tris-p-bromobenzoate (2a)

Colorless needles (acetone–MeOH); ^{1}H NMR in $CDCl_3$ (400 MHz) δ_H: 0.79 (3H, s), 0.80 (3H, d, 6.8 Hz), 0.84 (3H, d, 6.8 Hz), 0.95 (3H, d, 6.8 Hz), 1.10 (3H, d, 6.4 Hz), 1.46 (3H, s), 3.41 (1H, d, 2.8 Hz), 5.21 (1H, m), 5.23 (1H, dd, 8.4 Hz, 2.8 Hz), 6.37 (1H, d, 2.8 Hz), 7.54 (2H, d, 8.8 Hz), 7.57 (2H, d, 8.8 Hz), 7.58 (2H, d, 8.4 Hz), 7.80 (2H, d, 8.8 Hz), 7.86 (2H, d, 8.8 Hz), 7.89 (2H, d, 8.4 Hz) ppm; FAB MS *m/z*: 1007

Table 2
^{1}H and ^{13}C NMR data for compounds **3–5** in $CDCl_3$ (δ in ppm, J in Hz).

	3					**4**					**5**			
position	δ_H		δ_C			δ_H		δ_C			δ_H		δ_C	
1α	1.94	(1H, m)	25.9	t		1.48[a]		31.9	t		1.66	(1H, m)	29.4	t
1β	0.99	(1H, m)				1.69[a]					1.89[a]			
2α	1.90	(1H, m)	30.2	t		1.97[a]		31.0	t		1.67	(1H, m)	30.4	t
2β	1.53[a]					1.58[a]					2.00	(1H, m)		
3	4.11	(1H, m)	67.2	d		3.94	(1H, tt, 11.4, 5.0)	68.6	d		3.93	(1H, tt, 10.9, 6.7)	68.5	d
4α	1.87[a]		38.4	t		1.45[a]		39.3	t		1.46[a]		38.8	t
4β	2.00[a]					2.15	(1H, d, 13.3, 11.4)				2.21	(1H, dd, 13.2, 11.5)		
5			76.1	s				67.5	s				62.2	s
6	3.91	(1H, m)	72.9	d		3.20	(1H, d, 3.9)	60.4	d		3.13	(1H, d, 3.2)	59.4	d
7	5.73	(1H, dd, 5.0, 2.4)	125.0	d		4.55	(1H, dd, 3.9, 0.9)	65.0	d		4.34	(1H, d, 3.2)	65.1	d
8			138.7	s				131.7	s				122.4	s
9			62.8	s		2.52	(1H, t, 9.1)	38.4	d				144.7	s
10			38.3	s				36.1	s				39.2	s
11	3.17	(1H, d, 5.6)	54.6	d	11α	1.57[a]		19.0	t		2.17	(1H, m)	22.1	t
					11β	1.47[a]								
12α	1.87[a]		39.9	t		1.35	(1H, m)	36.8	t	12A	1.47[a]		28.4	t
12β	2.17	(1H, dd, 14.9, 5.7)				1.97[a]				12B	1.88[a]			
13			43.3	s				43.7	s				43.6	s
14	2.35	(1H, ddt, 11.7, 7.6, 2.0)	46.8	d				156.0	s				85.8	s
15α	1.64	(1H, m)	22.4	t	15	4.72	(1H, m)	69.9	d				217.8	s
15β	1.52[a]													
16α	1.79	(1H, m)	28.3	t		1.66[a]		38.8	t		2.40	(1H, dd, 10.8, 9.6)	37.6	t
16β	1.29	(1H, m)				1.73	(1H, m)				1.82	(1H, t, 10.8)		
17	1.30[a]		56.3	d		1.66[a]		53.2	d		1.95	(1H, dd, 19.3, 9.6)	37.3	d
18	0.65	(3H, s)	13.9	q		0.84	(3H, s)	19.3	q		0.80	(3H, s)	15.8	q
19	1.31	(3H, s)	21.5	q		0.93	(3H, s)	16.8	q		1.33	(3H, s)	22.1	q
20	1.99[a]		40.1	d		2.10	(1H, m)	38.5	d		2.04	(1H, m)	40.4	d
21	1.00	(1H, d, 6.4)	20.8	q		1.04	(3H, d, 6.7)	21.4	q		1.09	(3H, d, 6.4)	21.0	q
22	5.14	(1H, dd, 15.3, 8.5)	134.9	d		5.21	(1H, m)	135.2	d		5.07	(1H, dd, 15.3, 9.1)	133.6	d
23	5.23	(1H, dd, 15.3, 7.5)	132.5	d		5.22	(1H, m)	132.6	d		5.25	(1H, dd, 15.3, 7.9)	134.3	d
24	1.86[a]		42.8	d		1.86	(1H, m)	42.8	d		1.85[a]		42.8	d
25	1.47	(1H, m)	33.0	d		1.47[a]		33.1	d		1.47[a]		32.9	d
26	0.82	(3H, d, 6.7)	19.6	q		0.84	(3H, d, 6.8)	19.7	q		0.81	(3H, d, 6.8)	19.6	q
27	0.84	(3H, d, 6.7)	19.9	q		0.82	(3H, d, 6.8)	20.0	q		0.83	(3H, d, 6.7)	19.9	q
28	0.91	(3H, d, 6.8)	17.6	q		0.92	(3H, d, 7.6)	17.6	q		0.90	(3H, d, 7.0)	17.5	q
										7-OH	2.91	(1H, br s)		
										14-OH	3.20	(1H, s)		

[a] Overlapped with other signals.

$[M + H\ (^{79}Br \times 3)]^+$, 1009 $[M + H\ (^{79}Br \times 2,\ ^{81}Br \times 1)]^+$, 1011 $[M + H\ (^{79}Br \times 1,\ ^{81}Br \times 2)]^+$, and 1013 $[M + H\ (^{81}Br \times 3)]^+$; HR FAB MS *m/z*: 1007.1373 $[M + H]^+$ (Calcd for $C_{49}H_{54}{}^{79}Br_3O_8$, 1007.1368).

2.5.2. X-Ray crystallographic analysis of *2a*

$C_{49}H_{53}Br_3O_8$, Mr 1009.64, monoclinic, space group: $P2_1$, a = 7.0048(2) Å, b = 15.9151(6) Å, c = 22.9432(8) Å, β = 95.959(3)°, V = 2543.94(15) $Å^3$, Z = 2, F(0 0 0) = 1032, μ(Cu Kα) = 1.54184 mm^{-1}, measured independent reflections = 10,037, number of reflections used for refinement = 7358 ($I > 2\sigma(I)$), parameters used for refinement = 540, final R_1 = 0.0815 (for $I > 2\sigma(I)$) and wR = 0.2144 (for all data), (δ/σ) max = 0.001, $\Delta\rho_{max}$ = 1.313 e$Å^{-3}$, and $\Delta\rho_{min}$ = −1.004 e$Å^{-3}$, Flack parameter = 0.016(18). CCDC 1850820 (for **2a**) contains the supplementary crystallographic data for this study.

2.6. Cell cultures.

RAW264.7 cells (mouse macrophages) [obtained from *DS Pharma Biomedical Co., Ltd.* (Osaka, Japan)] were cultured in DMEM. The medium was supplemented with 10% FBS and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). Cells were incubated at 37 °C in a 5% CO_2 humidified incubator.

2.7. Assessment of RAW264.7 cell proliferation

Assessment of RAW264.7 cell proliferation was performed as described previously [18].

2.8. Assay for the inhibition of nitric oxide production

The assay for the inhibition of nitric oxide production was performed as described previously [18].

3. Results and discussion

Samples 1 and 2 of the *P. eryngii* fruiting bodies were extracted with MeOH, and liquid–liquid partition of the extracts resulted in the isolation of AcOEt-soluble and H_2O-soluble fractions. The AcOEt-soluble fractions of samples 1 and 2 were purified by silica gel CC and reverse-phase HPLC. Compounds **1–5** were isolated from sample 1, and compounds **3**, **5**, and **6** were isolated from sample 2 (Fig. 1).

Compound **1** exhibited an $[M + H]^+$ ion peak observed by HR FAB MS at 461.3268, which was compatible with the molecular formula $C_{28}H_{45}O_5$ (calcd 461.3267). Compound **1**'s IR spectrum was characterized by absorption maxima that indicated the presence of the hydroxy (ν_{max} 3446 cm^{-1}) and carbonyl (ν_{max} 1650 cm^{-1}) groups; moreover, the characteristics of this compound's UV spectrum suggested the presence of a conjugated enone system (λ_{max} 241.5 nm). The features of the ^{1}H and ^{13}C NMR spectra of **1** in $CDCl_3$ were indicative of

Table 3
^{1}H and ^{13}C NMR data for compound **6** (δ in ppm, J in Hz).

	6							
position	δ_H[a]		δ_C[a]		δ_H[b]		δ_C[b]	
1α	1.83[c]		27.5	t	2.00[c]		27.7	t
1β	1.30[c]				1.68[c]			
2α	1.71	(1H, m)	30.6	t	1.99[c]		30.5	t
2β	1.29[c]				1.52[c]			
3	3.76	(1H, dtt, 5.6, 10.8, 5.3)	65.2	d	4.06	(1H, m)	67.1	d
4α	1.56[c]		39.3	t	1.78[c]		39.2	t
4β	1.86[c]				2.16[c]			
5			75.2	s			76.9	s
6	3.80	(1H, t, 5.6)	70.5	d	4.03	(1H, m)	72.3	d
7	6.29	(1H, d, 5.6)	140.0	d	6.39	(1H, d, 5.0)	137.3	d
8			134.3	s			138.5	s
9			203.7	s			203.1	s
10			47.1	s			48.2	s
11	0.75	(3H, t, 7.0)	7.5	q	0.84	(3H, t, 6.2)	7.8	q
12A	0.85	(1H, m)	29.9	t	0.92[c]		30.8	t
12B	1.23	(1H, m)			1.33[c]			
13			46.1	s			46.9	s
14	3.14	(1H, dd, 11.7, 9.1)	40.1	d	3.32	(1H, dd, 11.5, 8.8)	41.1	d
15α	1.48[c]		26.4	t	1.66[c]		27.3	t
15β	1.56[c]				1.56[c]			
16α	1.63	(1H, m)	25.8	t	1.71[c]		26.2	t
16β	1.39	(1H, m)			1.45[c]			
17	1.68	(1H, m)	47.8	d	1.78[c]		48.6	d
18	0.67	(3H, s)	17.1	q	0.72	(3H, s)	17.7	q
19	1.15	(3H, s)	20.9	q	1.35[c]		21.8	q
20	2.09	(dd-like, 14.1, 7.0)	38.3	d	2.13[c]		38.8	d
21	0.98	(3H, d, 6.8)	20.7	q	1.02	(3H, d, 6.8)	21.1	q
22	5.26	(1H, dd, 15.3, 7.9)	134.7	d	5.25	(1H, dd, 15.3, 7.9)	134.9	d
23	5.22	(1H, dd, 15.3, 6.8)	131.6	d	5.21	(1H, dd, 15.3, 7.3)	132.5	d
24	1.87[c]		42.0	d	1.86	(1H, m)	43.0	d
25	1.47[c]		32.3	d	1.47[c]		33.1	d
26	0.80	(3H, d, 6.7)	19.3	q	0.82	(3H, d, 6.8)	19.7	q
27	0.82	(3H, d, 6.7)	19.7	q	0.84	(3H, d, 6.7)	20.0	q
28	0.90	(3H, d, 7.0)	17.2	q	0.92	(3H, d, 6.8)	17.6	q
3-OH	4.34	(1H, d, 5.6)						
5-OH	4.32	(1H, s)						
6-OH	5.40	(1H, d, 5.6)						

[a] Measured in $(CD_3)_2SO$.
[b] Measured in $CDCl_3$.
[c] Overlapped with other signals.

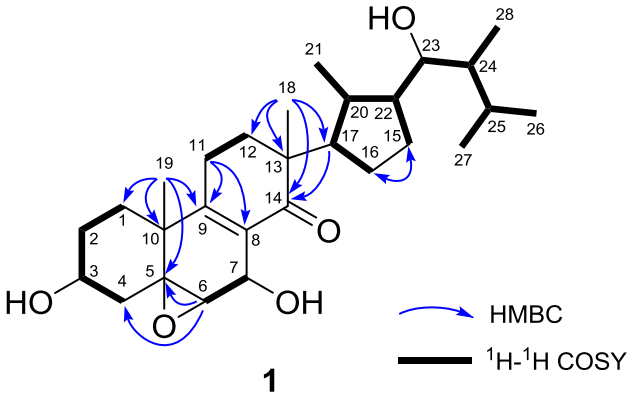

Fig. 2. HMBC and ^{1}H–^{1}H COSY correlations observed for compound **1**.

the presence of two tertiary methyl groups [δ_H 1.03 (s), 1.33 (s)], four secondary methyl groups [δ_H 0.79 (d, J = 7.0 Hz), 0.81 (d, J = 6.7 Hz), 0.92 (d, J = 7.0 Hz), 1.11 (d, J = 6.8 Hz)], three sp^3 oxygenated methine groups [δ_H 3.36 (m), 3.96 (m), 4.91 (br s); δ_C 62.9 (d), 68.3 (d), 79.0 (d)], a trisubstituted epoxy group [δ_H 3.24 (d, J = 2.6 Hz); δ_C 59.3 (d), 62.5 (s)], a tetrasubstituted olefin group [δ_C 128.3 (s), 159.9 (s)], and a ketone carbonyl group [δ_C 205.6 (s)] (Table 1). The heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations (Fig. 2) of Me-19 [δ_H 1.33 (s)]/C-1, C-5 [δ_C 62.5 (s)], C-9 [δ_C 159.9 (s)], and C-10; H_2-11/C-8 [δ_C 128.3 (s)], and C-9; Me-18 [δ_H 1.03 (s)]/C-12 and C-13; and ^{1}H–^{1}H correlation spectroscopy (COSY) correlations (Fig. 2) of H_2-1–H_2-2–H-3 [δ_H 3.96 (m)]–H_2-4; H-6 [δ_H 3.24 (d, J = 2.6 Hz)]–H-7 [δ_H 4.91 (br s)]; H_2-11–H_2-12 indicated that compound **1** possesses the A, B, and C rings of a typical ergostane-type sterol. However, the correlation from Me-18 to a ketone carbonyl group (δ_C 205.6, C-14) suggested cleavage of the C-14–C-15 bond. In addition, the ^{1}H–^{1}H COSY correlations of H_2-16–H-17–H-20–H-22–H-15, and the HMBC correlation of H-15/C-16 suggested that a cyclopentane ring was formed via a linkage between C-15 and C-22. The other HMBC and ^{1}H–^{1}H COSY correlations were observed as reported in Fig. 2, and they led to the conclusion that **1** is characterized by a planar structure. Compound **1** has a similar structure to strophasterol A [2], except for the fact that it has a hydroxy group at C-23.

In order to determine compound **1**'s absolute structure, a single-crystal X-ray diffraction analysis of **1**'s tris-*p*-bromobenzoate derivative (**1a**) was successfully conducted. The absolute configurations at C-17, C-20, C-22, C-23, and C-24 were found to be 17*R*, 20*S*, 22*S*, 23*R*, and

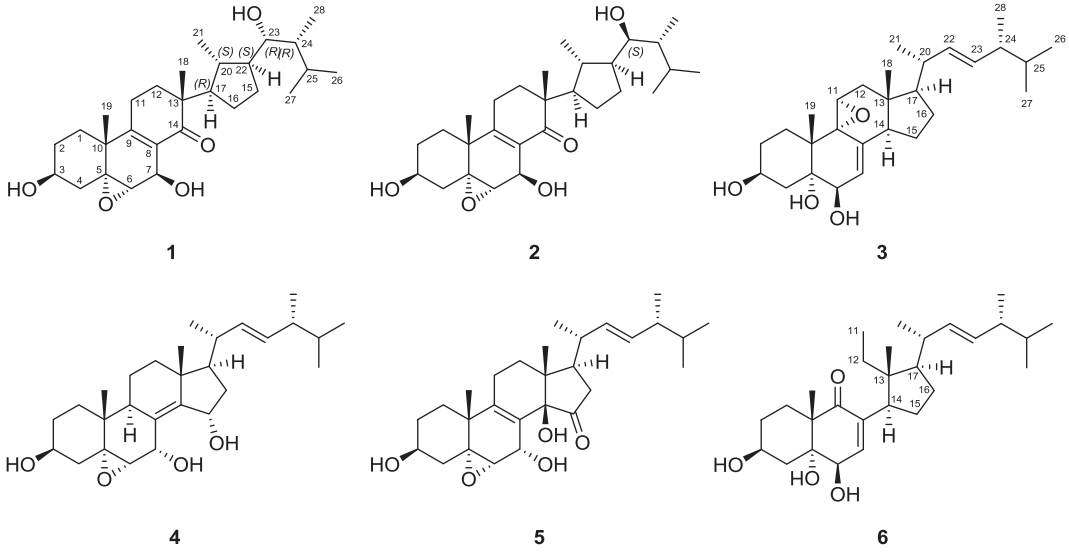

Fig. 1. Structures of steroids isolated from the fruiting bodies of *Pleurotus eryngii*.

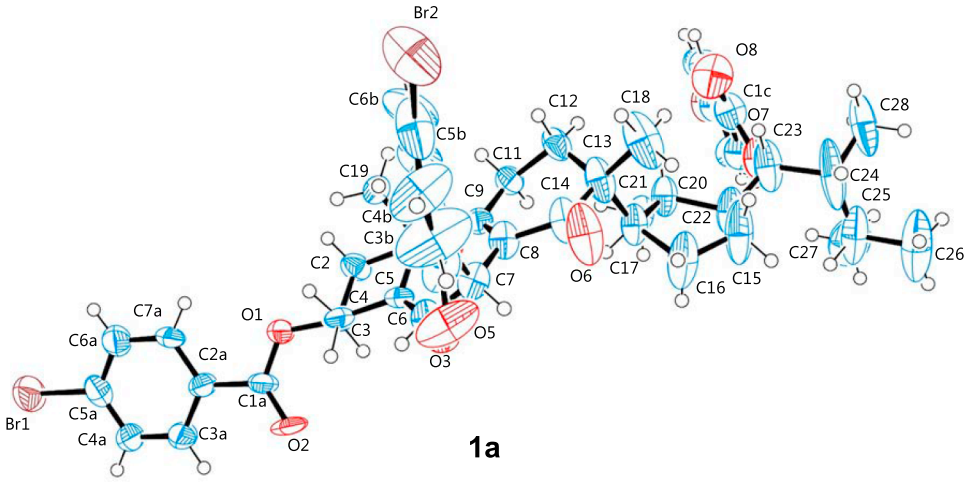

Fig. 3. ORTEP drawing of compound **1a**.

24*R*, which are the same as those of strophasterol A, except for C-23 (Fig. 3). Therefore, the structure of compound **1** was established as (22*S*,23*R*)-5α,6α-epoxy-3β,7β,23-trihydroxy-15(14 → 22)-*abeo*-ergost-8-en-14-one. Notably, 15(14 → 22)-*abeo*-ergostane sterols are rare compounds, with only six having been reported to be present in nature, including strophasterols A-D, reported by Wu et al. [2], glaucoposterol A, reported by Aung et al. [6], and tricholumin A, reported by Song et al. [22].

Compound **2** exhibited an $[M + H]^+$ ion peak observed by HR FAB MS at 461.3268, which was compatible with the molecular formula $C_{28}H_{45}O_5$ (calcd 461.3267). Compound **2**'s IR spectrum indicated the presence of hydroxy (ν_{max} 3451 cm^{-1}) and carbonyl (ν_{max} 1651 cm^{-1}) groups. The features of the ^1H and ^{13}C NMR spectra of **2** in $CDCl_3$ were indicative of the presence of two tertiary methyl groups [δ_H 0.99 (s), 1.33 (s)], four secondary methyl groups [δ_H 0.78 (d, J = 6.7 Hz), 0.89 (d, J = 7.0 Hz), 0.91 (d, J = 6.8 Hz), 0.99 (d, J = 5.6 Hz)], three sp^3oxygenated methine groups [δ_H 3.44 (dd, J = 7.0, 3.8 Hz), 3.96 (tt, J = 11.2, 5.3 Hz), 4.88 (br s); δ_C 63.0 (d), 68.3 (d), 73.2 (d)], a trisubstituted epoxy group [δ_H 3.24 (d, J = 2.6 Hz); δ_C 59.4 (d), 62.5 (s)], a tetrasubstituted olefin group [δ_C 128.2 (s), 159.7 (s)], and a ketone carbonyl group [δ_C 205.3 (s)] (Table 1). The HMBC and ^1H–^1H COSY correlations indicated that compound **2** has the same planar structure as compound **1**. However, given the differences in chemical shifts observed in the ^1H and ^{13}C NMR spectra, particularly when it came to atoms located between C-15 and C-18 and between C-20 and C-28, it was presumed that compound **2** was characterized by a different stereochemistry with respect to compound **1** at C-17, C-22, or C-23. The tris-*p*-bromobenzoate derivative of compound **2** (**2a**) was analyzed by single-crystal X-ray diffraction to confirm compound **2**'s absolute structure. Results from this analysis revealed that compound **2** was the 23-epimer of compound **1** (Fig. 4). Recently, Aung, et al. reported the isolation and structure elucidation of glaucoposterol A [6] extracted from the Basidiomycete *Cortinarius glaucopus*. For this compound, the stereochemistry at C-22 and C-23 [6] was determined mainly based on the computed geometries of glaucoposterol A's conformers calculated by means of the density functional theory using the B3LYP functional at 6–31 + G(d,p) level and of the nuclear Overhauser effect spectroscopy (NOESY) correlations. The spectral data obtained for glaucoposterol A are quite similar to those obtained for compound **2**, except for $[\alpha]_D^{20}$ (**2**: −16.0; glaucoposterol A: +1.67) and δ_C for C-28 (**2**: 9.9 ppm; glaucoposterol A: 14.1 ppm). Their ^1H NMR chemical shifts are identical. The structural differences between compound **2** and glaucoposterol A are limited to the stereochemistry at C-22 and C-23 [**2**: (22*R*, 23*R*); glaucoposterol A: (22*S*, 23*S*)]. It is also possible that the true structure of glaucoposterol A is that of compound **2**. However, we could not confirm the structure because the NMR spectra of glaucoposterol A was not provided in the supplementary information of the Aung et al. report.

Compound **3** exhibited an $[M]^+$ ion peak observed by HR EI MS at 444.3241, which was compatible with the molecular formula $C_{28}H_{44}O_4$ (calcd 444.3240). This compound's IR spectrum indicated the presence of hydroxy groups (ν_{max} 3433 cm^{-1}). The features of the ^1H and ^{13}C NMR spectra of **3** in $CDCl_3$ were indicative of the presence of two tertiary methyl groups [δ_H 0.65 (s), 1.31 (s)], four secondary methyl groups [δ_H 0.82 (d, J = 6.7 Hz), 0.84 (d, J = 6.7 Hz), 0.91 (d, J = 6.8 Hz), 1.00 (d, J = 6.4 Hz)], two sp^3hydroxy methine groups [δ_H 3.91 (m), 4.11 (m); δ_C 67.2 (d), 72.9 (d)], a trisubstituted epoxy group [δ_H 3.17 (d, J = 5.6 Hz); δ_C 54.6 (d), 62.8 (s)], a disubstituted olefin group [δ_H 5.14 (dd, J = 15.3, 8.5 Hz), 5.23 (dd, J = 15.3, 7.5 Hz); δ_C 132.5 (d), 134.9 (d)], and a trisubstituted olefin group [δ_H 5.73 (dd, J = 5.0, 2.4 Hz); δ_C 125.0 (d), 138.7 (s)] (Table 2). HMBC and ^1H–^1H COSY experiments revealed that compound **3** possesses a similar structure to ergosta-7,22-diene-3β,5α,6β-triol [23], except for the presence of an epoxy group between C-9 and C-11. The NOESY correlation between the Me-19 [δ_H 1.31 (s)] and H-11 [δ_H 3.17 (d, J = 5.6 Hz)] suggested that the epoxy group between C-9 and C-11 has an α-face orientation. The 20*R* configuration was indicated by the NOESY correlations between H-20 and Me-18; H-22 and H-16β [24]; and Me-21/H-12β [25]. The configurations of 5-OH and 6-OH were determined to be α and β, respectively, based on the pyridine-induced de-shielding effect ($\Delta\delta$: $\delta C_5D_5N - \delta CDCl_3$) [26] were observed at H-3 (+0.72) and Me-19 (+0.43). Therefore, compound **3** was determined to be (22*E*)-9α,11α-epoxyergosta-7,22-diene-3β,5α,6β-triol.

Compound **4** exhibited an $[M]^+$ ion peak observed by HR EI MS at 444.3237, which was compatible with the molecular formula $C_{28}H_{44}O_4$ (calcd 444.3240). Its IR spectrum indicated the presence of hydroxy groups (ν_{max} 3429 cm^{-1}). The features of the ^1H and ^{13}C NMR spectra of compound **4** in $CDCl_3$ were indicative of the presence of two tertiary methyl groups [δ_H 0.84 (s), 0.93 (s)], four secondary methyl groups [δ_H 0.82 (d, J = 6.8 Hz), 0.84 (d, J = 6.8 Hz), 0.92 (d, J = 7.6 Hz), 1.04 (d, J = 6.7 Hz)], three sp^3 hydroxy methine groups [δ_H 3.94 (tt, J = 11.4, 5.0 Hz), 4.55 (dd, J = 3.9, 0.9 Hz), 4.72 (m); δ_C 65.0 (d), 68.6 (d), 69.9 (d)], a trisubstituted epoxy group [δ_H 3.20 (d, J = 3.9 Hz); δ_C 60.4 (d), 67.5 (s)], a disubstituted olefin group [δ_H 5.21 (m), 5.22 (m); δ_C 132.6 (d), 135.2 (d)], and a tetrasubstituted olefin group [δ_C 131.7 (s), 156.0 (s)] (Table 2). Compound **4** was found to have a similar structure to 5α,6α-epoxy-(22*E*, 24*R*)-ergosta-8(14),22-diene-3β,7α-diol [27], except for the presence of a hydroxy group at C-15. The hydroxy groups in C-7 and C-15 were determined to have an α-orientation based on the following NOESY correlations: Me-19/H-7, H-7/H-15, and H-15/Me-18.

Compound **5** exhibited an $[M + Na]^+$ ion peak observed by HR FAB MS at 481.2928, which was compatible with the molecular formula

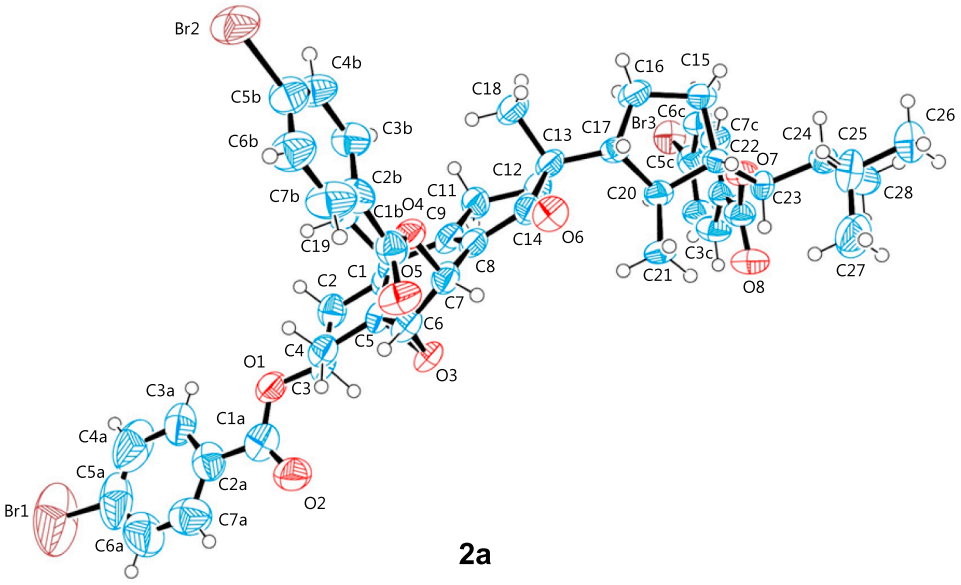

Fig. 4. ORTEP drawing of compound **2a**.

$C_{28}H_{42}O_5Na$ (calcd 481.2929). Its IR spectrum indicated the presence of hydroxy (ν_{max} 3443 cm^{-1}), and carbonyl (ν_{max} 1741 cm^{-1}) groups. The features of the ^1H and ^{13}C NMR spectra of compound **5** in $CDCl_3$ were indicative of the presence of two tertiary methyl groups [δ_H 0.80 (s), 1.33 (s)], four secondary methyl groups [δ_H 0.81 (d, J = 6.8 Hz), 0.83 (d, J = 6.7 Hz), 0.90 (d, J = 7.0 Hz), 1.09 (d, J = 6.4 Hz)], two sp^3hydroxy methine groups [δ_H 3.93 (tt, J = 10.9, 6.7 Hz), 4.34 (d, J = 3.2 Hz); δ_C 65.1 (d), 68.5 (d)], a hydroxy tertiary carbon [δ_C 85.8 (s)], a trisubstituted epoxy group [δ_H 3.13 (d, J = 3.2 Hz); δ_C 59.4 (d), 62.2 (s)], a disubstituted olefin group [δ_H 5.07 (dd, J = 15.3, 9.1 Hz), 5.25 (dd, J = 15.3, 7.9 Hz); δ_C 133.6 (d), 134.3 (d)], and a tetrasubstituted olefin group [δ_C 122.4 (s), 144.7 (s)] (Table 2).

HMBC correlations were identified from Me-18 [δ_H 0.80 (s)] to C-12, C-13, C-14 [δ_C 85.8 (s)], and C-17, and from Me-19 [δ_H 1.33 (s)] to C-1, C-5 [δ_C 62.2 (s)], C-9 [δ_C 144.7 (s)], and C-10. Moreover, the following ^1H–^1H COSY correlations were observed: H_2-1–H_2-2–H-3 [δ_H 3.93 (tt)]–H_2-4; H-6 [δ_H 3.13 (d)]–H-7 [δ_H 4.34 (d)]; H-11–H-12; H_2-16–H-17–H-20(–Me-21 [δ_H 1.09 (d)])–H-22 [δ_H 5.07 (dd)]–H-23 [δ_H 5.25 (dd)]–H-24(–Me-28 [δ_H 0.90 (d)])–H-25(–Me-26 [δ_H 0.81 (d)])–Me-27 [δ_H 0.83 (d)]. These correlations indicate that compound **5** possesses a typical ergost-22-ene structure. The HMBC correlations of H-7/C-5, C-6 [δ_C 59.4 (d)], and C-8 [δ_C 122.4 (s)]; H_2-11/C-8, and C-9; H-16/C-14, and C-15 [δ_C 217.8 (s)] suggested the presence of hydroxy groups existed at C-3, C-7, and C-14, an epoxy group between C-5 and C-6, and a ketone carbonyl group at C-15 (Fig. 5A). In NOESY experiments, the following correlations were observed: Me-19 with H-6 and H-7; Me-18 with 14-OH [δ_H 3.20 (s)]. Therefore, the orientation of the epoxy group between C-5 and C-6 was concluded to be α-face, whereas those of the hydroxy groups at C-7 and C-14 were concluded to be α-face and β-face, respectively. In addition, the pyridine-induced deshielding effects ($\Delta\delta$: $\delta C_5D_5N - \delta CDCl_3$) [26] identified for Me-18 ($\Delta\delta$ + 0.39) supported the β-orientation configuration of the hydroxy group at C-14 (Fig. 5B). Therefore, compound **5** was determined to be (22*E*)-5α,6α-epoxy-3β,7α,14β-trihydroxy-ergosta-8,22,dien-15-one.

Compound **6** was found to have a molecular formula of $C_{28}H_{46}O_4$, as indicated by HR EI MS evidence (m/z 466.3395, calcd 446.3396). Its IR and UV spectra suggested the presence of hydroxy groups (IR ν_{max} 3433 cm^{-1}) and of a conjugated carbonyl group [IR ν_{max} 1742 cm^{-1}; UV λ_{max} 227.5 nm (logε 3.56)].

The features of the ^1H and ^{13}C NMR spectra of compound **6** in DMSO-d_6 were indicative of the presence of two tertiary methyl groups [δ_H 0.67 (s), 1.15 (s)], four secondary methyl groups [δ_H 0.80 (d, J = 6.7 Hz), 0.82 (d, J = 6.7 Hz), 0.90 (d, J = 7.0 Hz), 0.98 (d, J = 6.8 Hz)], a primary methyl group [δ_H 0.75 (t, J = 7.0 Hz)], two sp^3oxygenated methine groups [δ_H 3.76 (dtt, J = 5.6, 10.8, 5.3 Hz), 3.80 (t, J = 5.6 Hz); δ_C 65.2 (d), 70.5 (d)], an sp^3oxygenated tertiary carbon [δ_C 75.2 (s)], a ketone carbonyl carbon [δ_C 203.7 (s)], a disubstituted olefin group [δ_H 5.22 (dd, J = 15.3, 6.8 Hz), 5.26 (dd, J = 15.3, 7.9 Hz); δ_C 131.6 (d), 134.7 (d)], and a trisubstituted olefin group [δ_H 6.29 (d, J = 5.6 Hz); δ_C 134.3 (s), 140.0 (d)].

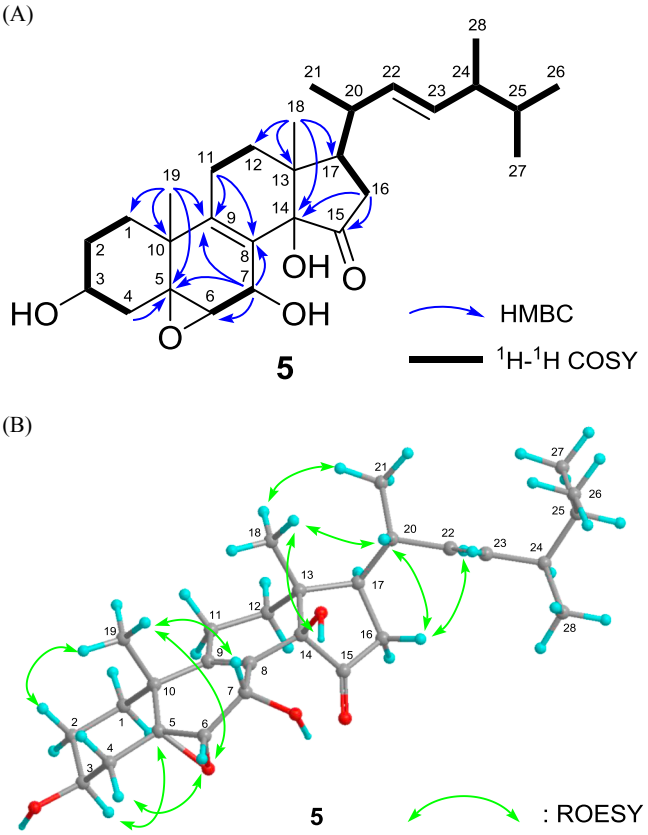

Fig. 5. HMBC, ^1H–^1H COSY, and ROESY correlations observed for compound **5**. (A) Key HMBC and ^1H–^1H COSY correlations. (B) Key ROESY correlations.

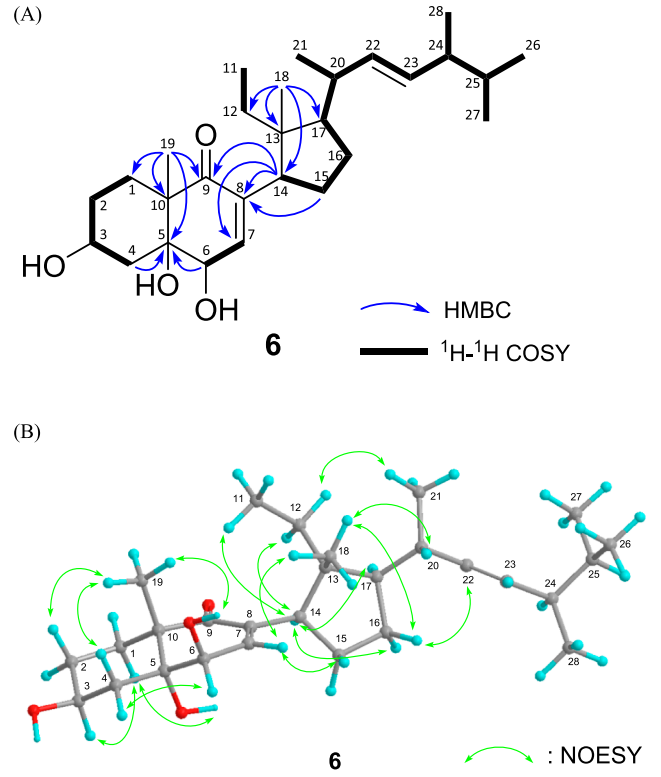

Fig. 6. HMBC, ^{1}H–^{1}H COSY, and NOESY correlations of compound **6**. (A) Key HMBC and ^{1}H–^{1}H COSY correlations. (B) Key NOESY correlations.

A HMBC correlation was determined to exist from Me-18 [δ_H 0.67 (s)] to C-12 [δ_C 29.9 (t)], and ^{1}H–^{1}H COSY correlations were observed between H_2-12 [δ_H A 0.85 (m), B 1.23 (m)] and a primary methyl group [δ_H 0.75 (t)] (Fig. 6A). These data indicate that a primary methyl exists at the molecule's C-11 position. The presence of a primary methyl group at C-11 in conjunction with evidence of a HMBC correlation between Me-19 [δ_H 1.15 (s)]/C-9 [δ_C 203.7 (s)] (Fig. 6A) suggested the lack of a carbon–carbon bond between C-9 and C-11. The following rotating frame nuclear Overhauser effect spectroscopy correlations were observed: Me-19 [δ_H 1.15 (s)]/6-OH [δ_H 5.40 (d)]; H-3 [δ_H 3.76 (dtt)]/H-1α/5-OH [δ_H 4.32 (s)]; H-14 [δ_H 3.14 (dd)]/Me-11, H-12A, H-17; Me-18/H-16β, and H-20 [δ_H 2.09 (dd-like)] (Fig. 6B). Therefore, it was determined the orientation of 5-OH to be α, that of 6-OH to be β, and that of 14-H to be α. The stereochemistry of C-24 was established to be *R* by comparing the ^{13}C NMR chemical shifts in $CDCl_3$ of the resonance peaks attributed to compound **6**'s C-24 [δ_C 43.0] and C-28 [δ_C 17.6] with those of 24*R* [δ_C 42.9 (C-24) and 17.7 (C-28)] and 24*S* [43.2 (C-24) and 18.1 (C-28)] methylcholestane-type steroids [28,29]. The structure of **6** was thus determined to be (22*E*)-3β,5α,6β-trihydroxy-9,11-*seco*-ergosta-7,22-dien-9-one. Numerous proposed unusual natural product structures, which were based mainly on spectroscopic evidence, were later been shown to be incorrect [1,30]. When determining the structures of **3–6**, 2D-NMR was used to provide structural evidence. However, the absolutely certain structures can be obtained by single crystal X-ray diffraction analysis [1], which could not be performed in their structural determination.

Pro-inflammatory mediators, such as NO, tumor necrosis factor-α, interleukin-1β, reactive oxygen species, and other inflammatory mediators, all of which are released by activated macrophages, play important roles in biological defense. However, the overexpression of these mediators induces severe or chronic inflammation, two conditions that have been linked to the onset of diseases like osteoarthritis, rheumatoid arthritis, and diabetes [31]. Macrophages are potential therapeutic targets in patients with the inflammatory diseases mentioned above [31]. The inhibitory effects of compounds **1–6**, and of a positive control, L-NMMA, on NO production were examined on lipopolysaccharide-stimulated mouse macrophage (RAW264.7) cells. Moreover, the cytotoxicities of compounds **1–6** were evaluated via the MTT assay. In detail, compounds **1–6** did not exhibit cytotoxicity in the 1–30 μM concentration range on RAW 264.7 cells. Compound **5** exhibited a superior inhibitory effect on NO production (IC_{50} 13.2 μM) with respect to L-NMMA (IC_{50} 47.6 μM) (see Table S8 in supplementary data). Compound **6** displayed a comparable inhibition of NO production at 30 μM (NO produced 57.8%) as the reference product, L-NMMA (NO produced: 60.8% at 30 μM).

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103011.

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