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Six new ergostane-type steroids from king trumpet mushroom (*Pleurotus eryngii*) and their inhibitory effects on nitric oxide production

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ABSTRACT

Six new ergostane-type steroids; (22*E*)-3β,5α,6α,11-tetrahydroxy-9(11)-*seco*-ergosta-7,22-dien-9-one (**1**), (22*E*)-8,14-epoxyergosta-6,22-diene-3β,5α,9α-triol (**2**), (22*E*)-4α,5α-epoxyergosta-7,22-diene-3β,6β-diol (**3**), (22*E*)-3β,4β,5α-trihydroxyergosta-7,22-dien-6-one (**4**), (22*E*)-ergosta-7,22-diene-3β,5β,6α-triol (**5**), and (22*E*)-6β-methoxyergosta-7,22-diene-3β,5α-diol 3-*O*-β-D-glucopyranoside (**6**) were isolated from the fruiting bodies of king trumpet mushroom (*Pleurotus eryngii*), along with fourteen known compounds (**7**–**20**). All isolated compounds were evaluated for their inhibitory effects on macrophage activation using a nitric oxide production inhibition assay.

1. Introduction

Pleurotus eryngii (English name: king trumpet mushroom, Japanese name: eringi) is an edible mushroom, indigenous to Europe, Asia, and North Africa [1], which it is produced in Japan, China, and the US [2]. It is known as a health food containing amino acids, vitamins, and dietary fiber [3]. In a previous investigation, *P. eryngii* extracts were shown to have the inhibitory effects on human neutrophil elastase (HNE) [4], antioxidant and antimutagenic activities [5], and inhibitory effects on allergic mediators [6]. In addition, there have been reports detailing the chemical constituents from *P. eryngii*, and their biological activities, including the antioxidant [7] and antitumor activities [8] of a polysaccharide extracted from *P. eryngii*, structural elucidation of pleurone and its HNE-inhibitory effects [4], isolation of 8 ergostane-type steroids (such as 5α,9α-epidioxy-8α,14α-epoxy-(22*E*)-ergosta-6,22-dien-3β-ol and 3β,5α-dihydroxyergost-7-en-6-one [9], and structural elucidation of eryngiolide A, and its cytotoxicity against human cancer cell lines [10]. We recently isolated a new 5,6-*seco*-ergostane-type steroid, named eringiacetal A, with a cage-shaped structure from the fruiting bodies of *P. eryngii* [11]. In this study, we isolated twenty ergostane-type steroids, and elucidated the structures of six new compounds; (22*E*)-3β,5α,6α,11-tetrahydroxy-9(11)-*seco*-ergosta-7,22-dien-9-one (**1**), (22*E*)-8,14-epoxyergosta-6,22-diene-3β,5α,9α-triol (**2**), (22*E*)-4α,5α-epoxyergosta-7,22-diene-3β,6β-diol (**3**), (22*E*)-3β,4β,5α-trihydroxyergosta-7,22-dien-6-one (**4**), (22*E*)-ergosta-7,22-diene-3β,5β,6α-triol (**5**), and (22*E*)-6β-methoxyergosta-7,22-diene-3β,5α-diol 3-*O*-β-D-glucopyranoside (**6**). In addition, the isolated constituents were evaluated for inhibitory effects on nitric oxide (NO) production.

2. Experimental section

2.1. General methods

Chemicals and reagents were purchased as follows: fetal bovine serum (FBS) from *Invitrogen Co.* (Carlsbad, CA, USA); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) from *Sigma-Aldrich Japan Co.* (Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM), antibiotics, and lipopolysaccharide (LPS) from *Escherichia coli* O157, from *Nacalai Tesque, Inc.* (Kyoto, Japan); sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride from *Tokyo Chemical Industry Co., Ltd.* (Tokyo, Japan); and N^G-monomethyl-L-arginine acetate (L-NMMA) from *Dojindo Molecular Technologies, Inc.* (Kumamoto, Japan). All other chemicals and reagents were of analytical grade. Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1720X FTIR spectrophotometer. The ^1H and ^{13}C NMR spectra were ran using an Agilent-NMR-vnmrs600 (^1H:

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600 MHz; ^{13}C: 150 MHz) and an Agilent-NMR-vnmrs400 (^{1}H: 400 MHz; ^{13}C: 100 MHz) in $CDCl_3$, C_5D_5N, and DMSO-d_6 with tetramethylsilane as the internal standard. The conformations for NOESY experiments correspond to energy-minimized conformation. Calculation was performed using Chem3D with MM2 force field (Chem3D Pro version 12.0.2.1076; CambridgeSoft, MA, USA). EIMS was recorded using a Hitachi 4000H double-focusing mass spectrometer (70 eV), and FABMS was recorded using a JEOL JMS-7000 mass spectrometer. Silica gel (70–230 mesh, Merck) and silica gel 60 (230–400 mesh, *Nacalai Tesque, Inc.*) were used for column chromatography. HPLC was carried out using an SiO_2 column [*Cosmosil 5SL-II column* (*Nacalai Tesque, Inc.*), 25 cm × 20 mm i.d.] with hexane/AcOEt [1:1 (System *I*), 3:7 (System *II*), and 1:4 (System *III*), and 0:1 (System *IV*)], and on ODS column [*Cosmosil 5C$_{18}$-MS-II column* (*Nacalai Tesque, Inc.*) (25 cm × 20 mm i.d.) with MeOH/H_2O (95:5) (System *V*), MeOH/H_2O (9:1) (System *VI*), MeCN/H_2O (8:2) (System *VII*), *Cosmosil 5C$_{18}$-PAQ column* (*Nacalai Tesque, Inc.*) (25 cm × 20 mm i.d.) with MeOH/H_2O (9:1) (System *VIII*)] at 35 °C flow rate 4.0 mL/min.

2.2. Materials

The fruiting bodies of *P. eryngii*, produced in Nagano, Japan (sample 1) and Kagawa, Japan (sample 2), were purchased from Japan agricultural cooperatives (JA) Zennou Nagano in 2013, and HOKUTO Corp. in 2014. A voucher specimen was deposited in the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

2.3. Extraction and isolation

2.3.1. Sample 1

Sample 1 [the fruiting bodies of *P. eryngii* (fresh weight 40 kg), produced in Nagano, Japan] were subjected to extraction with MeOH under reflux (1 week, 3 times). The MeOH extract (2035 g) was then partitioned between AcOEt and H_2O (5 L/5 L, 4 times). The AcOEt-soluble fraction (115 g) was subjected to SiO_2 column chromatography (CC) [SiO_2 (3.5 kg); $CHCl_3$/AcOEt (1:0, 10:1, 5:1, 1:1, and 0:1), and AcOEt:MeOH (5:1, and 0:1) in increasing order of polarity] resulting in 18 fractions (Fr. *S1*-A–*S1*-R).

Fr. *S1*-H (490.9 mg), eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 14 fractions, *S1*-H1–*S1*-H14. Preparative HPLC (System *III*) of *S1*-H11 (65.0 mg), eluted with hexane/AcOEt (1:1), gave *S1*-H11-1–*S1*-H11-8, and then re-preparative HPLC of *S1*-H-11-6 (4.0 mg; t_R 47.8 min) gave **18** (1.2 mg; t_R 19.2 min) (System *V*).

Fr. *S1*-I (397.1 mg), eluted with $CHCl_3$/AcOEt (1:1 and 0:1), was subjected to SiO_2 CC to yield 13 fractions, *S1*-I1–*S1*-I13. Preparative HPLC of *S1*-I8 (54.8 mg) (System *III*), eluted with hexane/AcOEt (1:1), gave 9 fractions, *S1*-I8-1–*S1*-I8-9. Fr. *S1*-I8-8 was **3** (1.1 mg; t_R 54.6 min). Purification of *S1*-I8-3 (4.2 mg; t_R 38.0 min) with HPLC (System *IV*) gave **15** (0.9 mg; t_R 32.6 min), *S1*-I8-6 (13.0 mg; t_R 43.3 min) gave **5** (2.9 mg; t_R 67.8 min). Preparative HPLC of *S1*-I9 (System *IV*) gave **2** (4.2 mg; t_R 30.9 min).

Fr. *S1*-J (548.3 mg), eluted with AcOEt, was subjected to SiO_2 CC to yield 16 fractions, *S1*-J1–*S1*-J16. Preparative HPLC (System *II*) of *S1*-J8 (83.6 mg), eluted with hexane/AcOEt (1:1), gave 14 fractions; *S1*-J8-1–*S1*-J8-16, and then re-preparative HPLC (System *V*) of *S1*-J8-14 (18.5 mg; t_R 84.8 min) gave **8** (11.4 mg; t_R 36.5 min).

Fr. *S1*-K (777.3 mg), eluted with AcOEt, was subjected to SiO_2 CC to yield 22 fractions, *S1*-K1–*S1*-K22. Preparative HPLC (System *III*) of *S1*-K15 (93.4 mg), eluted with hexane/AcOEt (1:1), gave 7 fractions; *S1*-K15-1–*S1*-K15-8, and then re-preparative HPLC (System *V*) of *S1*-K15-7 (24.9 mg; t_R 70.5 min) gave **7** (3.7 mg; t_R 35.6 min).

Fr. *S1*-M (690.8 mg), eluted with AcOEt, was fractionated using SiO_2 CC to give *S1*-M1–*S1*-M15. Preparative HPLC (System *IV*) of *S1*-M8 (117.2 mg), eluted with AcOEt, gave 5 fractions; *S1*-M8-1–*S1*-M8-5. Purification of *S1*-M8-3 (3.9 mg; t_R 53.8 min) with HPLC (System *VIII*) gave **12** (2.4 mg; t_R 36.7 min), Fr. *S1*-M8-4 (38.9 mg; 56.2 min) (System *VIII*) gave **14** (1.1 mg; t_R 38.6 min), and **13** (1.7 mg; t_R 40.2 min).

2.3.2. Sample 2

Sample 2 [the fruiting bodies of *P. eryngii* (fresh weight 120 kg), produced in Kagawa, Japan] were subjected to extraction with MeOH under reflux (3 days, 4 times). The MeOH extract (2625 g) was then partitioned between AcOEt and H_2O (10 L/10 L, 4 times). The AcOEt-soluble fraction (240 g) was subjected to SiO_2 column chromatography (CC) [SiO_2 (2.8 kg); $CHCl_3$/AcOEt (1:0, 5:1, 1:1, and 0:1), and MeOH in increasing order of polarity] resulting in 37 fractions (Fr. *S2*-A–*S2*-Z, *S2*-a–*S2*-k). Fr. *S2*-T (2874.5 mg), eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 8 fractions, *S2*-T1–*S2*-T8. Fr. *S2*-T6 (961.2 mg), eluted with hexane/AcOEt (1:1 → 0:1), was subjected to SiO_2 CC to yield 8 fractions, *S2*-T6-1–*S2*-T6-8. Preparative HPLC (System *V*) of Fr. *S2*-T6-4 (140.3 mg) gave **4** (6.28 mg; t_R 41.5 min).

Fr. *S2*-U (2004.7 mg), eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 23 fractions, *S2*-U1–*S2*-U23. Fr. *S2*-U11 (168.6 mg), eluted with hexane/AcOEt (1:1), was subjected to SiO_2 CC to yield 10 fractions, *S2*-U11-1–*S2*-U11-10. Preparative HPLC (System *V*) of Fr. *S2*-U11-7 (125.3 mg), eluted with hexane/AcOEt (1:1), gave **16** (2.6 mg; t_R 43.0 min).

Fr. *S2*-W (2626.2 mg), eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 11 fractions, *S2*-W1–*S2*-W11. Fr. *S2*-W7 (369.9 mg), eluted with AcOEt, was subjected to ODS CC to yield 9 fractions, *S2*-W7-1–*S2*-W7-9. Preparative HPLC (System *VI*) of Fr. *S2*-W7-3 (154.0 mg), eluted with MeOH, gave **9** (7.0 mg; t_R 67.2 min).

Fr. *S2*-a (911.6 mg), eluted with $CHCl_3$/AcOEt (1:1), was subjected to SiO_2 CC to yield 13 fractions, *S2*-a1–*S2*-a13. Preparative HPLC (System *VI*) of Fr. *S2*-a7 (69.8 mg), eluted with AcOEt, gave **10** (3.0 mg; t_R 82.8 min). Preparative HPLC (System *VI*) of Fr. *S2*-a8 (363.6 mg), eluted with AcOEt, gave **11** (5.9 mg; t_R 36.5 min) and **17** (1.8 mg; t_R 63.6 min).

Fr. *S2*-j (3379.0 mg), eluted with AcOEt, was subjected to SiO_2 CC to yield 10 fractions, *S2*-j1–*S2*-j10. Fr. *S2*-j7 (354.3 mg), eluted with AcOEt, was subjected to ODS CC to yield 4 fractions, *S2*-j7-1–*S2*-j7-4. Preparative HPLC (System *VII*) of Fr. *S2*-j4 (161.4 mg), eluted with MeOH, gave **1** (12.0 mg; t_R 20.6 min) and **20** (37.6 mg; t_R 69.8 min). Fr. *S2*-j8 (900.8 mg), eluted with AcOEt:MeOH (1:0 and 10:1), was subjected to ODS CC to yield 10 fractions, *S2*-j8-1–*S2*-j8-10. Preparative HPLC (System *V*) of Fr. *S2*-j8-7 (129.0 mg), eluted with MeOH, gave 11 fractions, *S2*-j8-7-1–*S2*-j8-7-11. Fr. *S2*-j8-7-9 was identified with **19** (10.9 mg; t_R 65.9 min). Fr. *S2*-j8-7-6 was subjected to re-preparative HPLC (System *VI*), and gave **6** (1.7 mg; t_R 85.4 min).

2.3.3. (22E)-3β,5α,6α,11-Tetrahydroxy-9(11)-seco-ergosta-7,22-dien-9-one (**1**)

Colorless crystal; mp 145–146 °C; $[\alpha]_D^{26}$ 35.4 (*c* = 0.098, EtOH); UV λ_{max}^{EtOH} nm (log ε): 205.5 (3.88), 238.0 (3.93); IR ν_{max}^{KBr} cm^{-1}: 3340, 2957, 2872, 1670, 1472, 1455, 1372, 1048, 680, 668; FABMS *m*/*z*: 463 [M+H]$^+$, 485 [M+Na]$^+$, 445 [M−H_2O+H]$^+$; HRFABMS *m*/*z*: 463.3430 [M+H]$^+$ (calcd for 463.3416: $C_{28}H_{47}O_5$).

2.3.4. (22E)-8,14-Epoxyergosta-6,22-diene-3β,5α,9α-triol (**2**)

Colorless crystal; mp 139–140 °C; $[\alpha]_D^{20}$ 116.3 (*c* = 0.091, EtOH); IR ν_{max}^{KBr} cm^{-1}: 3336, 2955, 2869, 1559, 1457, 1155, 1101, 1054; FABMS *m*/*z*: 467 [M+Na]$^+$; HRFABMS *m*/*z*: 467.3142 [M+Na]$^+$ (calcd for 467.3137: $C_{28}H_{44}O_4Na$).

*2.3.5. (22E)-4α,5α-Epoxyergosta-7,22-diene-3β,6β-diol (**3**)*

Colorless crystal; mp 215–218 °C; $[\alpha]_D^{20}$ −13.1 (*c* = 0.065, EtOH); UV λ_{max}^{EtOH} nm (log ε): 201.5 (3.88); IR ν_{max}^{KBr} cm^{-1}: 3450, 2932, 1467, 1456, 1088, 1014; EIMS *m*/*z* (rel. int.): 428 (53) $[M]^+$, 410 (31) $[M-H_2O]^+$, 395 (26), 341 (28), 304 (51), 155 (100); HREIMS *m*/*z*: 428.3295 $[M]^+$ (calcd for 428.3291: $C_{28}H_{44}O_3$).

*2.3.6. (22E)-3β,4β,5α-Trihydroxyergosta-7,22-dien-6-one (**4**)*

Colorless crystal; mp 194–195 °C; $[\alpha]_D^{27}$ 22.4 (*c* = 0.11, EtOH); UV λ_{max}^{EtOH} nm (log ε): 248.5 (3.99); IR ν_{max}^{KBr} cm^{-1}: 3401, 2956, 2872, 1662, 1617, 1458, 1383, 1367, 1247, 1084, 990, 968; EIMS *m*/*z*: 444 $[M]^+$ (94), 426 $[M-H_2O]^+$ (100), 369 (96), 357 (96), 317 (23), 137 (51); HREIMS *m*/*z*: 444.3240 $[M]^+$ (calcd for 444.3239: $C_{28}H_{44}O_4$).

*2.3.7. (22E)-Ergosta-7,22-diene-3β,5β,6α-triol (**5**)*

Colorless crystal; mp 178–180 °C; $[\alpha]_D^{26}$ +60.0 (*c* = 0.037, EtOH); UV λ_{max}^{EtOH} nm (log ε): 206.5 (3.84), 259.5 (2.91); IR ν_{max}^{KBr} cm^{-1}: 3465, 3388, 2955, 2872, 1739, 1459, 1161, 1105, 1063; EIMS *m*/*z* (rel. int.): 430 (86) $[M]^+$, 412 (100) $[M-H_2O]^+$, 394 (20), 355 (14), 314 (16), 287 (14), 269 (12); HREIMS *m*/*z*: 430.3450 $[M]^+$ (calcd for 430.3453: $C_{28}H_{46}O_3$).

*2.3.8. (22E)-6β-Methoxyergosta-7,22-diene-3β,5α-diol 3-O-β-glucopyranoside (**6**)*

Amorphous solid; $[\alpha]_D^{24}$ −60.6 (*c* = 0.090, EtOH); IR ν_{max}^{KBr} cm^{-1}: 3436, 2956, 2869, 1631, 1460, 1384, 1158, 1084; FABMS *m*/*z*: 629 $[M+Na]^+$; HRFABMS *m*/*z*: 629.4031 $[M+Na]^+$ (calcd for 629.4030: $C_{35}H_{58}O_8Na$).

*2.4. X-ray crystallographic analysis of **2***

$C_{28}H_{44}O_4 \cdot H_2O$, *Mr*. 462.65, monoclinic, space group: *C*2, *a* = 13.041 (6) Å, *b* = 9.687 (5) Å, *c* = 21.584 (10) Å, *β* = 106.669 (8)°, *V* = 2612 (2) $Å^3$, *Z* = 4, *F*(0 0 0) = 1016, μ(Mo Cα) = 0.079 mm^{-1}, measured independent reflections = 5501, number of reflections used for refinement = 3264 ($I > 2\sigma(I)$), parameters used for refinement = 293, final *R* = 0.0576 (for $I > 2\sigma(I)$) and w*R* = 0.1421 (for all data), (δ/σ) max = 0.000, $\Delta\rho$max = 0.273 e $Å^{-3}$, and $\Delta\rho$min = −0.333 e $Å^{-3}$. To determine the position of the

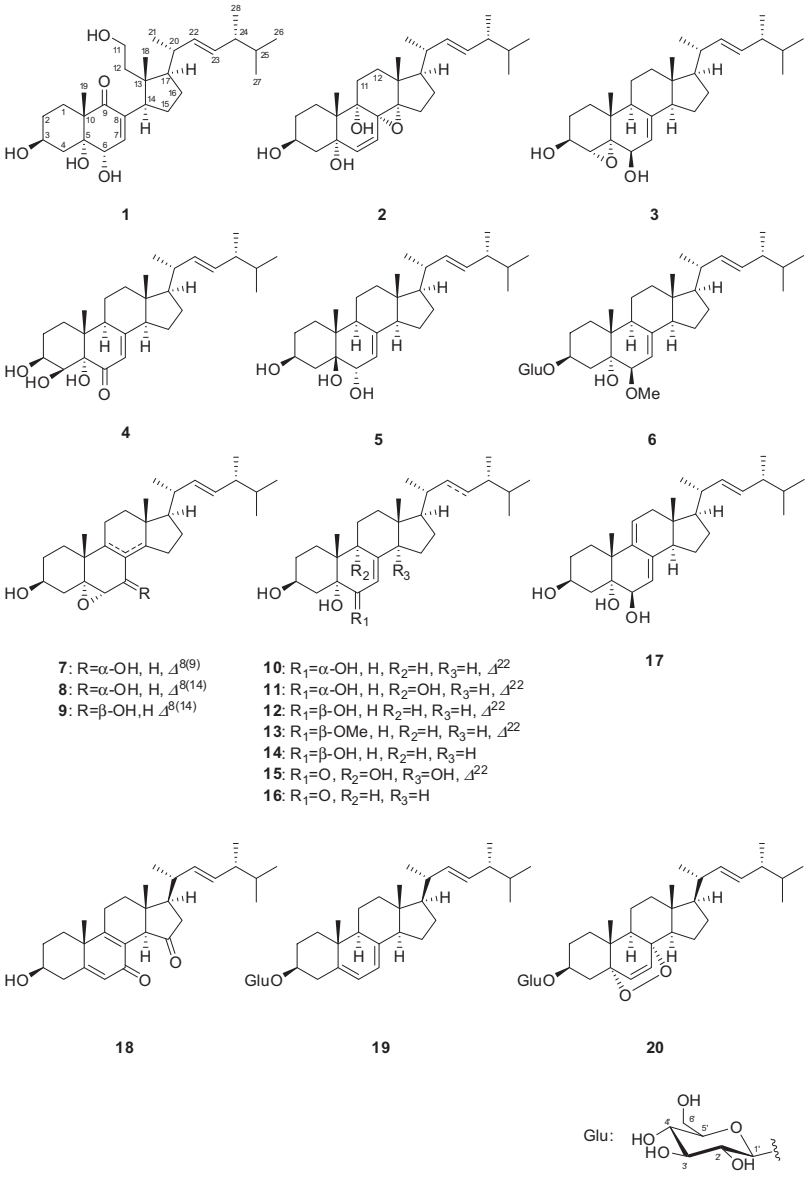

Fig. 1. Structures of compounds.

Table 1
^{1}H and ^{13}C NMR data for **1**, **2** and **3** (δ in ppm).[a]

	1[b]			**1**[c]			**2**[c]			**3**[c]			**3**[d]		
	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C
1α	2.73	dt (3.9, 13.8)	28.5	2.01	m	27.3	2.30	dt (4.1, 13.5)	24.9	1.29	m	29.4	1.07	m	29.3
1β	1.89	m		1.65	m		1.47	m		1.35	m		1.23	m	
2α	1.86	m	31.5	1.26	m	31.9	1.94	m	29.6	1.93	m	27.1	1.68	m	26.4
2β	2.25	m		1.48	m		1.55	m		1.36	m		1.24	m	
3	4.60	tt (5.4, 11.0)	66.4	3.96	m	66.6	4.28	tt (5.6, 11.1)	66.7	4.03	brt (8.3)	65.9	3.68	m	64.5
4	α 3.00	dd (5.4, 13.1)	40.2	A 1.57	m	38.4	α 1.91	m	42.0	3.17	d (1.2)	62.5	2.98	m	62.3
	β 2.11	dd (11.0, 13.1)		B 2.21	m		β 1.69	m							
5			80.3			79.6			72.9			65.8			65.6
6	4.87	d (1.8)	71.3	4.43	brs	70.7	5.96	d (10.0)	140.7	3.45	brs	72.0	3.17	m	70.1
7	6.69	d (1.8)	143.6	6.26	brs	141.9	5.18	d (10.0)	124.2	5.40	dt (2.3, 5.0)	118.0	5.22	dd (2.6, 5.3)	119.6
8			136.5			137.4			67.4			144.5			140.8
9			204.3			204.6			75.6	2.04	m	45.0	1.82	m	44.5
10			50.2			49.6			42.3			33.5			33.0
11	A 4.10	dd (5.5, 9.9)	58.3	A 3.65	m	59.2	1.88	m (2H)	28.3	1.57	m (2H)	21.4	1.52	m (2H)	20.83
	B 4.32	dd (5.4, 9.9)		B 3.84	m										
12	A 1.65	m	42.4	A 1.09	m	40.7	A 1.51	m	28.2	α 1.33	m	38.9	α 1.31	m	38.3
	B 1.95	m		B 1.60	m		B 1.59	m		β 2.06	m		β 1.98	m	
13			46.4			46.0			42.2			43.5			42.8
14	3.72	dd (8.4, 11.4)	43.0	3.37	t (9.7)	43.2			84.1	1.94	m	54.7	1.86	m	53.9
15α	1.49	m	26.9	1.59	m (2H)	26.1	1.49	m	25.6	1.47	m	22.9	1.51	m	22.4
15β	1.56	m					2.01	m		1.59	m		1.39	m	
16α	1.56	m	26.0	1.73	m	25.9	1.95	m	26.6	1.75	m	27.9	1.68	m	27.4
16β	1.40	m		1.44	m		1.46	m		1.30	m		1.27	m	
17	1.85	m	50.5	1.73	m	49.7	1.60	m	52.0	1.30	m	55.9	1.30	m	55.1
18	0.83	s	17.9	0.65	s	17.8	0.93	s	15.5	0.63	s	12.3	0.58	s	11.9
19	1.40	s	19.9	1.23	s	19.7	1.12	s	21.5	1.28	s	18.9	1.14	s	18.1
20	2.25	m	39.1	2.14	m	38.9	2.11	m	39.4	2.04	m	40.4	2.02	m	39.9
21	1.10	d (6.8)	21.7	1.03	d (6.8)	21.4	1.02	d (6.7)	21.0	1.03	d (6.7)	21.1	1.00	d (6.5)	20.80
22	5.29	dd (8.0, 15.2)	135.3	5.23	m	134.5	5.19	dd (7.5, 15.2)	134.5	5.16	dd (8.2, 15.2)	135.4	5.18	dd (8.0, 15.3)	135.1
23	5.21	dd (7.5, 15.2)	132.6	5.22	m	132.9	5.26	dd (7.6, 15.2)	132.8	5.23	dd (7.3, 15.2)	132.1	5.24	dd (7.4, 15.3)	131.3
24	1.85	m	43.2	1.86	m	43.0	1.87	m	42.8	1.85	m	42.8	1.87	m	41.8
25	1.40	m	33.3	1.49	m	33.1	1.48	m	33.0	1.48	m	33.1	1.47	m	32.3
26	0.82	d (6.9)	19.8	0.82	d (6.8)	19.7	0.82	d (6.7)	19.6	0.82	d (6.8)	19.6	0.80	d (6.8)	19.3
27	0.84	d (6.9)	20.1	0.84	d (6.8)	20.0	0.84	d (6.7)	19.9	0.84	d (6.8)	19.9	0.82	d (6.8)	19.6
28	0.93	d (6.9)	17.7	0.92	d (7.1)	17.6	0.92	d (6.7)	17.5	0.92	d (6.7)	17.6	0.89	d (6.8)	17.1
3-O<u>H</u>													5.11	d (5.0)	
5-O<u>H</u>							5.78	d							
6-O<u>H</u>													4.91	d (4.9)	
9-O<u>H</u>							3.37	d							

[a] Assignments were based on 2D NMR including HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY.
[b] Measured in C_5D_5N (^{1}H: 400 MHz; ^{13}C: 100 MHz).
[c] Measured in $CDCl_3$ (^{1}H: 600 MHz; ^{13}C: 150 MHz).
[d] Measured in DMSO-d_6 (^{1}H: 600 MHz; ^{13}C: 150 MHz).

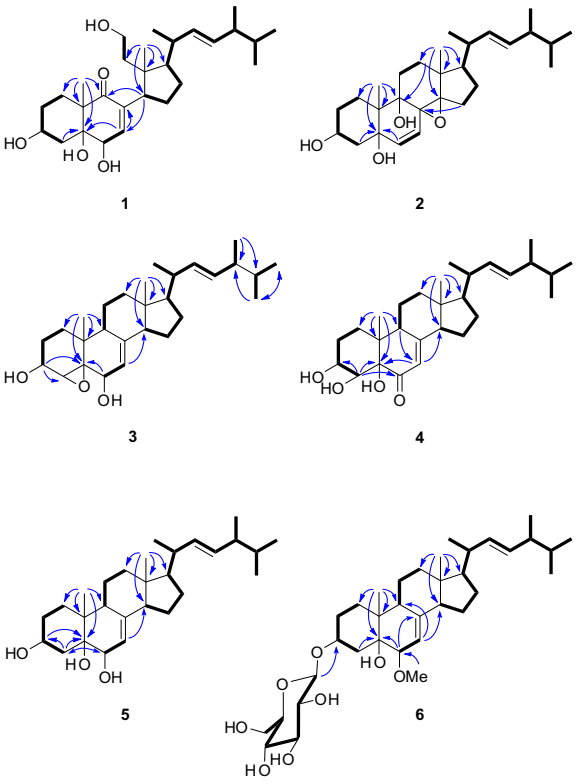

Fig. 2. Key HMBC and ^{1}H–^{1}H COSY correlations of compounds.

hydroxy and epoxy groups, a single crystal of **2** obtained from acetone was mounted on an X-ray diffractometer equipped with graphite monochromated Mo Kα radiation (λ = 0.71073 Å) at 220 K. X-ray diffraction data were collected using a *Bruker AXS SMART APEX CCD* camera. Crystal structures were solved by a direct method using the SHELXS-97 program. Atomic scattering factors were taken from International Tables for X-ray Crystallography. Positional parameters of non-H-atoms were refined by a full-matrix least-squares method with anisotropic thermal parameters using the SHELXL-97 program. CCDC 1476620 (for **2**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at the Cambridge Crystallographic Date Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.5. Acid hydrolysis and determination of sugar configurations

The determination of absolute sugar configurations was carried out according to a method reported previously with slight modifications [12]. A solution of **6** (0.92 mg) in 2 M CF_3COOH_{aq} (1 mL) was heated at 90 °C for 3.5 h. The mixture was extracted with AcOEt (3 times). The H_2O layer was concentrated to dryness. The residue was then dissolved in pyridine (0.1 mL) and stirring with L-cysteine methyl ester hydrochloride (0.5 mg) at 60 °C for 1 h. The reaction mixture was treated with *o*-tolylisothiocyanate, and heated at 60 °C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC [column: *Cosmosil 5C_{18}-PAQ column* (Nacalai Tesque, Inc., Kyoto, Japan), 250 × 4.6 mm i.d. (5 μm); mobile phase: MeCN–H_2O (2:8, v/v) in 1% AcOH; detection: refractive index; flow rate: 1.0 mL/min; column temperature: 35 °C] to identify the derivatives of D-glucose by comparison of their retention times with those of authentic samples (t_R: D-glucose 26.7 min, L-glucose 23.8 min).

2.6. Cell cultures

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

2.7. Determination of RAW264.7 cell proliferation

RAW264.7 cell proliferation was examined in accordance with a method reported previously [13]. Briefly RAW264.7 cells (5×10^4 cells in 100 μL) were seeded onto a 96-well microplate, and incubated for 24 h. DMEM containing test samples (100 μL total volume, final concentration of 30, 10, 3, or 1 μM) dissolved in dimethyl sulfoxide (DMSO, final concentration 0.2%) was added.

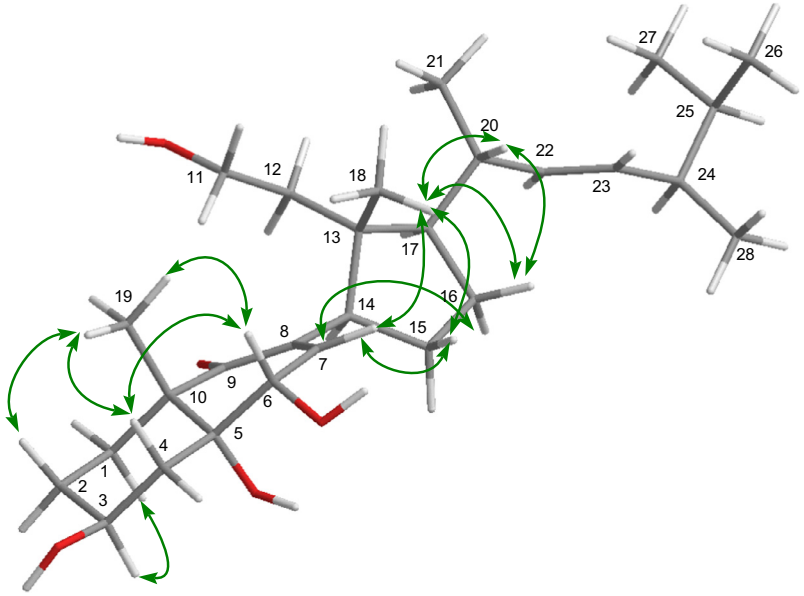

Fig. 3. Key NOE correlations of compound **1**.

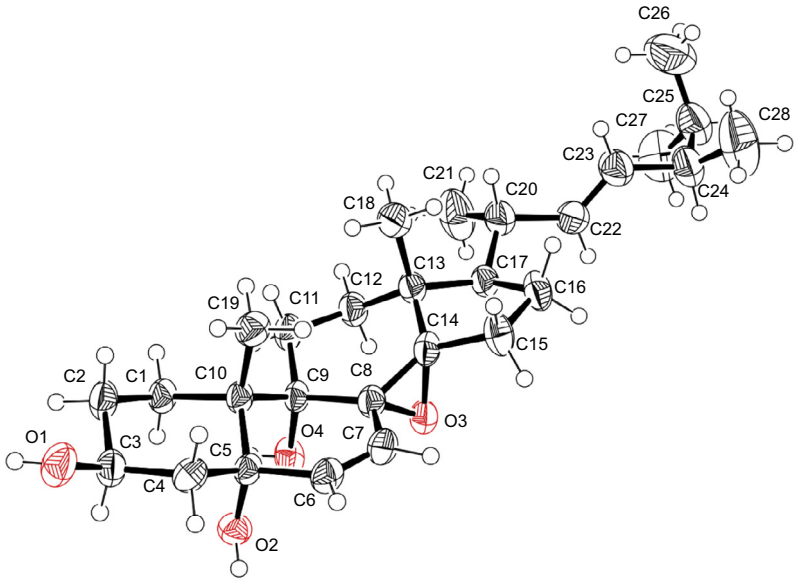

Fig. 4. ORTEP drawing of compound **2**.

After treatment for 24 h, MTT solution was added. After a 3-h incubation, 20% sodium dodecyl sulfate in 0.1 M HCl was added to dissolve the formazan produced in the cells. The absorbance of each well was read at 570 nm using a microplate reader. The optical density of vehicle control cells was assumed to be 100%.

2.8. Inhibitory assay of NO production

An inhibitory assay of NO production was examined in accordance with a method reported previously [14] with a minor modifications. Briefly RAW264.7 cells (5×10^4 cells in 100 μL) were seeded into a 96-well microplate, and incubated for 24 h. DMEM containing test samples (100 μL total volume, final concentration of 30, 10, 3, or 1 μM) dissolved in DMSO (final concentration 0.2%), and LPS (final concentration of 5 μg/mL), was added. After treatment for 24 h, the supernatant of culture medium was transferred to another 96-well microplate, and then 50 μL of 0.15% *N*-(1-naphtyl)ethylenediamine in H_2O and 1.5% sulfanilamide in 7.5% phosphoric acid were added. After incubation for 30 min, the absorbance of each well was read at 570 nm using a microplate reader. The optical density of vehicle control cells was assumed to be 100%.

3. Results and discussion

Compounds **2**, **3**, **5**, **7** [15], **8** [15], **12** [15], **13** [16], **14** [17], **15** [18], **18** [19] were isolated from sample 1, and Compounds **1**, **4**, **6**, **9** [20], **10** [21], **11** [18], **16** [9], **17** [20], **19** [22], **20** [22] were isolated from sample 2 (Fig. 1). Among them, **1**–**6** were new compounds.

Compound **1** was obtained as colorless crystals, with a molecular formula of $C_{28}H_{46}O_5$ by HRFABMS. The IR spectrum showed absorptions indicating hydroxy groups (ν_{max} 3340 cm^{-1}), and the UV spectrum showed the presence of a conjugated enone (λ_{max} 238.0 nm). The ^{13}C NMR spectrum of **1** suggested that 3 out of 6 degrees of unsaturation came from two carbon-carbon double bonds and a carbonyl, thus **1** was shown to be tricyclic. ^1H and ^{13}C NMR spectra (δ_H and δ_C in ppm) in C_5D_5N displayed signals for two annular methyls [δ_H 0.83 (s), 1.40 (s)], four secondary methyls [δ_H 0.82 (d), 0.84 (d), 0.93 (d), 1.10 (d)], a hydroxy methylene [δ_H 4.10 (dd), 4.32 (dd); δ_C 58.3 (t)], seven sp^3 methines including two oxymethines [δ_H 4.60 (tt), 4.87 (d); δ_C 66.4 (d), 71.3 (d)], three sp^3 quaternary carbons including an oxycarbon [δ_C 80.3 (s)] a trisubstituted olefin [δ_H 6.69 (d); δ_C 136.5 (s), 143.6 (d)], a disubstituted olefin [δ_H 5.21 (dd, J = 7.5, 15.2 Hz), 5.29 (dd, J = 8.0, 15.2 Hz); δ_C 132.6 (d), 135.3 (d)], and a ketone [δ_C 204.3 (s)] (Table 1). In the HMBC experiment (Fig. 2), it was suggested that **1** was a 9,11-*seco*-ergostane-type steroid, because correlations from Me-19 to the ketone at C-9 [δ_C 204.3 (s)], Me-18 to C-12, and H_2-12 to C-11 [δ_C 58.3 (t)] were observed. Its structure was similar to 3β,5α,6α-trihydroxy-9,11-*seco*cholest-7,22-dien-9-one [23] except for the presence of an additional secondary methyl group. The HMBC and ^1H–^1H COSY experiments revealed that the additional secondary methyl group was at C-24, and established plane structure as shown Fig. 2. The hydroxy group at C-3 was in an usual β-equatorial configuration [δ_H 4.60 (tt); δ_C 66.4 (d)]. The order of the side chain, C-20–C-28 was seen to be the same as common ergost-22-ene by ^1H–^1H COSY and HMBC spectra. The configuration of hydroxy group at C-5 was α-orientation because the pyridine-induced deshielding effects (δ: δC_5D_5N–$\delta CDCl_3$) [20,24] were observed at H-1α ($\Delta\delta$ 0.72) and H-3α ($\Delta\delta$ 0.64) in the ^1H NMR spectrum, and the NOE correlation between Me-19 and H-6 in the NOESY experiment suggested that the configuration of the hydroxy group at C-6 was established as the α-orientation (Fig. 3). The stereochemistry of C-24 was established as *R* by comparison of ^{13}C NMR chemical shifts in $CDCl_3$ at 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 [25,26]. Therefore, **1** was determined as (22*E*)-3β,5α,6α,11-tetrahydroxy-9(11)-*seco*-ergosta-7,22-dien-9-one (Fig. 1). Although some 9,11-*seco*-cholestane-type steroids have been reported [27–30], this is first report of a 9,11-*seco*-ergostane-type steroid.

Compound **2** was obtained as colorless crystals, with a molecular formula of $C_{28}H_{44}O_4$ and 7 degrees of unsaturation. The IR spectrum indicated the presence of a hydroxy group (3336 cm^{-1}). The ^1H, ^{13}C NMR and HSQC spectra suggested the presence of two tertiary methyls [δ_H 0.93 (s), 1.12 (s)], four secondary methyls [δ_H 0.82 (d), 0.84(d), 0.92 (d), 1.02 (d)], an oxymethine [δ_H 4.28 (tt); δ_C 66.7 (d)], six quaternary carbons including four oxycarbons [δ_C 67.4 (s), 72.9 (s), 75.6 (s), 84.1 (s)], two disubstituted olefins [δ_H 5.19 (dd, J = 7.5, 15.2), 5.26 (dd, J = 7.6, 15.2), δ_C 132.8 (d), 134.5 (d); δ_H

Table 2
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data for **4**, **5** and **6** (δ in ppm).[a]

	4[b]			**5**[b]			**5**[c]			**6**[d]		
	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C	^{1}H	(mult, *J* in Hz)	^{13}C
1α	1.70	m	30.0	1.34	m	24.7	1.17	m	25.2	2.01	m	33.3
1β	1.56	m		1.86	m		1.72	m		1.57	m	
2α	1.75	m	25.7	1.88	m	28.4	1.72	m	28.1	2.30	m	30.2
2β	1.69	m		1.64	m		1.49	m		1.88	m	
3	3.93	m	67.8	4.26	t (2.7)	67.7	4.01	brs	66.1	4.86	tt (5.0, 11.4)	75.9
4α	4.15	d (3.5)	72.3	1.53	m	30.0	1.24	m	30.1	2.49	m	38.04
4β				1.97	dt (2.7, 15.0)		1.79	m		2.64	dd (11.4, 13.2)	
5			77.0			76.9			76.3			75.51
6			200.6	4.27	m	72.1	3.94	brs	70.6	3.59	d (1.8)	83.8
7	5.65	t (2.1)	119.7	5.09	brd (4.7)	119.0	4.87	d (1.7)	121.9	5.68	dt (1.8, 7.3)	115.9
8			167.9			140.4			138.6			143.5
9	2.55	td (2.6, 10.5)	45.0	2.12	m	41.1	2.11	m	40.9	2.49	m	44.1
10			40.4			39.9			40.5			38.00
11	1.67	m (2H)	20.9	1.55	m (2H)	22.6	1.50	m (2H)	22.4	1.59	m (2H)	23.4
12α	2.12	m	38.6	1.26	m	39.4	1.27	m	39.2	2.02	m	39.8
12β	1.44	m		2.04	m		1.95	m		1.27	m	
13			44.9			43.8			43.7			43.9
14	2.14	m	56.00	1.83	m	55.0	1.83	m	54.6	1.88	m	55.4
15	α 1.49	m	22.4	α 1.53	m	22.5	α 1.43	m	22.7	1.61	m (2H)	22.3
	β 1.61	m		β 1.43	m		β 1.35	m				
16	α 1.36	m	27.7	α 1.73	m	28.1	1.67	m (2H)	28.3	1.74	m	28.5
	β 1.79	m		β 1.28	m					1.34	m	
17	1.36	m	56.01	1.27	m	55.8	1.25	m	55.6	1.22	m	56.2
18	0.61	s	12.7	0.56	s	12.1	0.52	s	12.4	0.66	s	12.5
19	1.19	s	15.1	0.93	s	18.1	0.81	s	18.4	1.21	s	18.5
20	2.04	m	40.2	2.03	m	40.3	2.00	m	40.5	2.02	m	40.9
21	1.03	d (6.4)	21.0	1.01	d (6.8)	21.0	0.99	d (6.4)	21.4	1.06	d (6.5)	21.5
22	5.15	dd (7.6, 15.2)	134.9	5.15	dd (7.3, 15.2)	135.4	5.17	dd (8.2, 15.2)	135.8	5.20	dd (8.5, 15.3)	136.2
23	5.23	dd (8.5, 15.2)	132.5	5.22	dd (7.9, 15.2)	132.0	5.23	dd (7.3, 15.2)	131.9	5.26	dd (7.7, 15.3)	132.2
24	1.85	m	42.7	1.85	m	42.8	1.85	m	42.4	1.88	m	43.1
25	1.48	m	33.0	1.47	m	33.0	1.46	m	32.9	1.48	m	33.4
26	0.82	d (6.8)	19.6	0.82	d (6.8)	19.6	0.80	d (6.7)	19.9	0.87	d (6.7)	19.9
27	0.83	d (6.8)	19.9	0.83	d (6.8)	19.9	0.81	d (6.7)	20.2	0.88	d (6.7)	20.2
28	0.91	d (6.8)	17.5	0.91	d (6.8)	17.5	0.89	d (7.0)	17.7	0.97	d (7.0)	17.9
1′										5.02	d (8.3)	103.2
2′										4.08	t (8.3)	75.53
3′										4.23	t (8.3)	78.7
4′										4.30	t (8.3)	71.7
5′										3.82	ddd (2.4, 5.0, 8.3)	78.4
6′A										4.41	dd (5.0, 11.8)	62.9
6′B										4.51	dd (2.4, 11.8)	
3-OH							5.33	d (3.2)				
5-OH							4.98	s				
6-OH							4.34	d (5.0)				
6-OMe										3.37	s	57.8

[a] Assignments were based on 2D NMR including HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY.
[b] Measured in $CDCl_3$.
[c] Measured in DMSO-d_6.
[d] Measured in C_5D_5N.

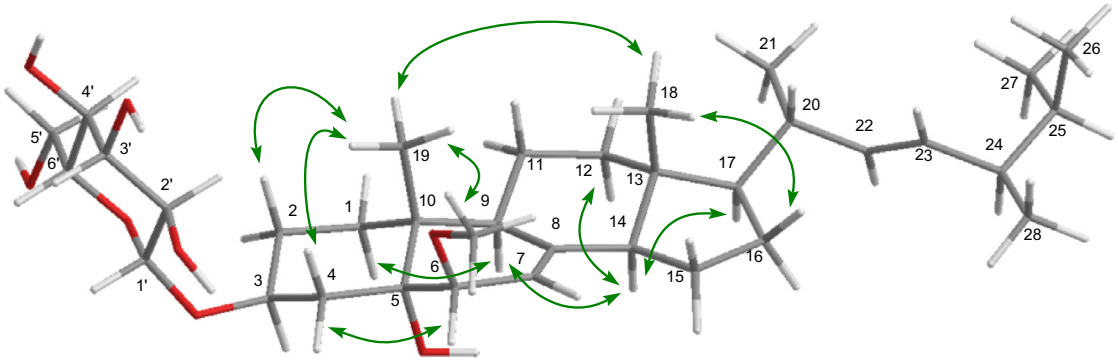

Fig. 5. Key NOE correlations of compound **6**.

Table 3
Inhibitory effects of NO production by ergostane-type steroids from fruiting bodies of *Pleurotus eringii*.

	Inhibitory ratio of NO % (Cell viability %)[a]				
	1 μM	3 μM	10 μM	30 μM	IC_{50} (μM)
1	91.0 ± 1.6 (98.5 ± 1.2)	80.2 ± 3.0** (92.7 ± 1.7)	52.5 ± 2.9** (102.6 ± 0.6)	−5.8 ± 0.7** (98.9 ± 1.1)	10.3
2	103.0 ± 2.0 (99.1 ± 1.5)	102.4 ± 0.7 (98.5 ± 4.0)	107.8 ± 2.1 (97.7 ± 3.3)	82.1 ± 3.9** (98.8 ± 1.5)	>30
3	101.8 ± 7.4 (90.5 ± 0.6)	96.4 ± 8.4 (87.7 ± 0.2)	89.9 ± 6.0 (79.8 ± 0.7)	84.5 ± 5.4 (81.6 ± 0.3)	>30
4	97.2 ± 4.2 (99.4 ± 0.4)	100.0 ± 7.7 (95.6 ± 0.2)	70.0 ± 5.1** (84.9 ± 0.6)	33.1 ± 2.5** (82.8 ± 0.6)	18.1
5	99.5 ± 4.0 (95.2 ± 0.7)	98.5 ± 5.4 (94.5 ± 0.9)	102.0 ± 5.0 (90.9 ± 1.0)	109.1 ± 10.5 (92.6 ± 0.5)	>30
6	104.3 ± 6.4 (100.4 ± 0.3)	98.1 ± 2.2 (98.8 ± 0.2)	98.7 ± 0.6 (91.4 ± 0.2)	92.6 ± 2.3 (79.4 ± 0.9)	>30
7	90.1 ± 0.8 (94.2 ± 1.0)	73.9 ± 3.9** (90.4 ± 0.8)	59.2 ± 1.8** (78.5 ± 0.4)	16.0 ± 1.0** (37.6 ± 2.0)	12.4
8	96.9 ± 2.6 (100.6 ± 1.1)	95.5 ± 2.0 (93.9 ± 1.7)	91.5 ± 3.3 (82.0 ± 0.3)	53.0 ± 1.9* (51.3 ± 1.6)	>30
9	92.8 ± 0.4 (98.6 ± 1.6)	86.1 ± 3.6** (96.5 ± 1.3)	64.3 ± 1.2** (94.1 ± 1.7)	12.8 ± 1.5** (98.7 ± 3.6)	14.3
10	105.0 ± 2.3 (103.3 ± 0.4)	102.4 ± 2.0 (101.1 ± 0.6)	105.8 ± 1.4 (101.2 ± 1.2)	94.7 ± 3.3 (98.6 ± 0.4)	>30
11	107.1 ± 3.9 (99.0 ± 0.6)	106.8 ± 1.2 (99.2 ± 0.5)	98.2 ± 3.3 (92.3 ± 0.7)	61.6 ± 2.0** (97.1 ± 0.9)	>30
12	96.5 ± 6.1 (97.2 ± 0.7)	102.9 ± 1.7 (95.3 ± 0.9)	94.9 ± 8.5 (95.0 ± 0.8)	77.1 ± 4.7* (75.9 ± 0.6)	>30
13	97.6 ± 1.5 (95.9 ± 0.9)	100.3 ± 4.0 (98.3 ± 1.3)	94.5 ± 1.4 (77.1 ± 0.4)	17.9 ± 1.6** (32.0 ± 3.4)	20.4
14	100.7 ± 2.2 (83.3 ± 0.5)	92.7 ± 2.0 (66.8 ± 0.7)	97.9 ± 6.3 (56.2 ± 0.8)	44.9 ± 4.9** (57.7 ± 0.5)	28.4
15	91.1 ± 5.7 (84.6 ± 0.5)	99.0 ± 3.0 (79.8 ± 0.3)	82.7 ± 3.0* (73.4 ± 0.6)	79.6 ± 2.4** (42.7 ± 0.6)	>30
16	104.3 ± 3.0 (95.4 ± 2.5)	104.4 ± 4.6 (85.2 ± 0.7)	109.0 ± 1.2 (51.2 ± 0.4)	116.7 ± 3.5* (33.7 ± 0.3)	>30
17	97.4 ± 0.6 (93.1 ± 0.7)	98.6 ± 1.6 (94.4 ± 0.1)	104.8 ± 1.7 (87.0 ± 0.9)	47.3 ± 1.4** (84.4 ± 1.4)	29.8
18	94.7 ± 0.9 (93.7 ± 1.3)	89.8 ± 3.4* (98.5 ± 0.4)	64.1 ± 1.4** (91.9 ± 0.4)	9.6 ± 0.6** (47.7 ± 0.5)	12.4
19	94.6 ± 4.6 (96.2 ± 1.7)	101.4 ± 9.3 (91.9 ± 0.3)	111.0 ± 9.0 (74.2 ± 1.3)	113.6 ± 8.3 (67.3 ± 1.2)	>30
20	93.8 ± 3.5 (95.3 ± 0.7)	85.7 ± 2.2* (93.1 ± 0.8)	77.0 ± 4.3** (84.2 ± 0.5)	62.8 ± 3.0** (60.9 ± 1.3)	>30
L-NMMA[b]	43.1 ± 1.1** (101.5 ± 0.9)	93.3 ± 2.2 23.9 (101.9 ± 0.4)	91.4 ± 0.8 (98.5 ± 0.9)	68.9 ± 4.5** (109.4 ± 0.5)	

Significant differences from the vehicle control group shown as $^{*}p<0.05$ and $^{**}p<0.01$.

[a] Produced NO (%) and cell viability (%) were determined based on the absorbance at 570 nm, respectively, by comparison with values for DMSO (100%). Each value represents the mean ± standard error (S.E.) of four determinations. The concentration of DMSO in the sample solution was 2 mL/mL.

[b] Positive control.

5.18 (d, J = 10.0), 5.96 (d, J = 10.0); δ_C 124.2 (d), 140.7 (d)] (Table 1). Because of the correlations at Me-18/C-14 [δ_C 84.1 (s)], Me-19/C-5 [δ_C 72.9 (s)] and C-9 [δ_C 75.6 (s)], and H-15/C-8 [δ_C 67.4 (s)] in the HMBC spectrum (Fig. 2), the four quaternary oxycarbons were at C-5, C-8, C-9, and C-14. Considering 7 of unsaturation, 4 out of 7 unsaturation came from steroid frame, and 2 from double bonds. Therefore, the remaining 1 of unsaturation predicted due to an epoxy ring, which was assumed to situate at the C-8, 9 or C-8, 14 position. An X-ray diffraction analysis conducted to confirm the position of the hydroxy and epoxy groups, and it also established the relative stereostructure of **2** (Fig. 4). As a result, hydroxy groups attached at C-5α and C-9α, and the epoxy group attached at C-8,14 position, and the relative structure was determined successively. Therefore the structure of **2** was determined to be (22*E*)-8,14-epoxyergosta-6,22-diene-3β,5α,9α-triol (Fig. 1).

Compound **3** was obtained as a white solid, with a molecular ion at m/z 428.3295 $[M]^+$ ($C_{28}H_{44}O_3$) in the HREIMS. The ^1H and ^{13}C NMR spectra suggested that compound **3** would be structural isomer of gargalol A [4β,5β-epoxy-(22*E*)-ergosta-7,22-diene-3β,6α-diol] [31] except for the different stereochemistry at C-4, C-5, and C-6. The stereochemistry of epoxy group at C-4 and C-5 was α-orientation because the NOE correlation between H-4β and Me-19 was observed although it was not strong. The configuration of the hydroxy groups at the 6 position was β-orientation, because a NOE correlation between 6β-O<u>H</u> and Me-19 was observed by ROESY experiment in DMSO-d_6. Therefore the structure of **3** was determined to be (22*E*)-4α,5α-epoxyergosta-7,22-diene-3β,6β-diol (Fig. 1).

Compound **4** was obtained as a white solid, with a molecular ion at m/z 444.3240 $[M]^+$ ($C_{28}H_{44}O_4$) by HREIMS. The ^1H and ^{13}C NMR spectra indicated that compound **4** had the similar structure to ergosta-7,22-diene-3β,5α,6α-triol (**10**) except for the absence of a methylene and the presence of a hydroxy methine at C-4 (Table 2). The hydroxy groups at C-3 and C-4 were established as the β-orientation (3β-OH: equatorial; 4β-OH: axial) because of NOEs between H-3α–H-1α–H-9α–H-14α, and the coupling constant of H-4 [δ_H 4.15 (d, $J_{3\alpha,4\alpha}$ = 3.5 Hz)]. Therefore, **4** was determined as (22*E*)-3β,4β,5α-trihydroxyergosta-7,22-dien-6-one (Fig. 1).

Compound **5** exhibited a $[M]^+$ ion in the HREIMS data at m/z 430.3450 compatible with the molecular formula of $C_{28}H_{46}O_3$. The ^1H and ^{13}C NMR spectra indicated that compound **5** have the same palne structure as compound **10** (Table 2). The hydroxy group at C-5 and C-6 were β and α-orientation, respectively, because NOE correlations were observed between 5-O<u>H</u> and Me-19; H-6 and Me-19 by ROESY experiment in DMSO-d_6. The configuration of the hydroxy group at C-3 was established as the β (axial)-orientation because of the coupling constants of H-3 [δ_H 4.26 (t, $J_{3\beta.2\alpha;3\beta,2\beta;3\beta.4\alpha;3\beta,4\beta}$ = 2.7 Hz)] in $CDCl_3$. Therefore, **5** was determined as (22*E*)-ergosta-7,22-diene-3β,5β,6α-triol. Compound **5** is the first case which confirmed that 5β-ergostane is present in *Pleurotus eryngii* so that some mushrooms are so.

Compound **6** was obtained as an amorphous solid, with a molecular formula of $C_{35}H_{58}O_8$. The ^1H and ^{13}C NMR spectra indicated the structure of compound **6** was glycoside of (22*E*)-6β-methoxyergosta-7,22-diene-3β,5α-diol (**13**) (Table 2). This was supported by HMBC, ^1H–^1H COSY (Fig. 2) and NOESY (Fig. 5) experiments. The glucose configuration at C-1′ was established as the β-orientation due to the coupling constant of H-1′ (J = 8.3 Hz). Acid hydrolysis of **6** was performed to determine the configuration of the glucose. Acid hydrolysis by TFA_{aq} yielded D-glucose. D-glucose was identified by derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, followed by a HPLC analysis [12]. Therefore, **6** was determined as (22*E*)-6β-methoxyergosta-7,22-diene-3β,5α-diol 3-*O*-β-D-glucopyranoside (Fig. 1).

Macrophages may be a potential therapeutic target for inflammatory diseases [32]. Activated macrophages release pro-inflammatory mediators, such as NO, reactive oxygen species, interleukin-1 beta, tumor necrosis factor-alpha, and other inflammatory mediators, which play important roles in biological defense. However, the overexpression of these mediators has been implicated in diseases such as osteoarthritis, rheumatoid arthritis, and diabetes because the increased production of pro-inflammatory mediators has been shown to induce severe or chronic inflammation [32]. Twenty ergostane-type steroids, and L-NMMA, an inducible nitric oxide synthase (iNOS) inhibitor, were evaluated for their inhibitory effects on NO production (Table 3). **1**, **4**, **7**, **9**, **13**, **14**, and **18** exhibited similar or superior NO inhibitory activities to L-NMMA. Of these, **1** and **9** exhibited no cytotoxicity at 1–30 μM. Although **7** and **18** exhibited some cytotoxicity at higher concentrations, they had superior inhibitory activities to L-NMMA at non-toxic concentrations (**7** at 3 μM; **18** at 10 and 3 μM). On the

basis of the results in the Table, the following conclusions can be drawn about the structure–activity relationship of the compounds: i) 7-en-6β-ol compounds exhibited cytotoxicity (Cell viability at 30 μM **3**: 81.6%; **12**: 75.9%; **14**: 57.7%; **17**: 84.4%) and weak inhibitory activities of NO production (IC_{50} of NO production **3**: >30 μM; **12**: >30 μM; **14**: 28.4 μM; **17**: 29.8 μM); ii) 7-en-6α-ol compounds did not exhibit cytotoxicity (Cell viability at 30 μM **1**: 98.9%; **5**: 92.6%; **10**: 98.6%; **11**: 97.1%); iii) 5α,6α-epoxy-7α-ol compounds exhibited cytotoxicity (Cell viability at 30 μM **7**: 37.6%; **8**: 51.3%), and 5α,6α-epoxy-7β-ol compounds were more active than 5α,6α-epoxy-7α-ol compounds (IC_{50} of NO production **8**: >30 μM vs **9**: 14.3 μM) with no cytotoxicity (Cell viability at 30 μM **9**: 98.7%); and iv) 3β-glycoside compounds exhibited some cytotoxicity (Cell viability at 30 μM **6**: 79.4%; **19**: 67.3%; **20**: 60.9%). These results suggested that compounds **1**, **7**, **9**, and **18** may be valuable as potential inhibitors of macrophage activation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.07.005.

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