Six Immunosuppressive Features from an Ascomycete, *Zopfiella longicaudata*, Found in a Screening Study Monitored by Immunomodulatory Activity

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In a screening study on immunomodulatory fungal metabolites, three known anthraquinones, carviolin (roseo-purpurin) (1), 1-*O*-methylemodin (2), ω-hydroxyemodin (citreorosein) (4), and a new anthraquinone, ω-acetylcarviolin (3), together with a known steroid, ergosta-4,6,8(14),22-tetraen-3-one (5) and a new steroid, 25-hydroxyergosta-4,6,8(14),22-tetraen-3-one (6) were isolated from an Ascomycete, *Zopfiella longicaudata*, and found to have moderate immunosuppressive activities. The structure–activity relationships of these metabolites are discussed.

Key words immunosuppressive fungal metabolite; *Zopfiella longicaudata*; Ascomycete; anthraquinone; ergostane; structure–activity relationship

In our screening program on immunomodulatory constituents from fungi, many metabolites with various structures from four *Gelasinospora* fungi,[1—3] a *Diplogelasinospora* fungus,[4] a *Microascus* fungus,[5] an *Emericella* fungus,[6] an *Eupenicillium* fungus,[7] and a *Chaetomium* fungus,[8] have been isolated as immunosuppressive compounds. The EtOAc extract of the Ascomycete *Zopfiella longicaudata* (CAIN) VON ARX showed an appreciable suppressive effect on the proliferation (blastogenesis) of mouse splenic lymphocytes stimulated with mitogens, concanavalin A (Con A), and lipopolysaccharide (LPS). Solvent partitions followed by repeated chromatographic fractionations of the extract, monitored by immunomodulatory activity, afforded six compounds tentatively designated ZL-1 (**1**)—6 (**6**) as the immunosuppressive constituents of this fungus. This paper deals with the structures and immunosuppressive activities of these six constituents.

The EtOAc extract of *Z. longicaudata* IFM4630[9] cultivated on sterilized moistened-rice medium suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 82.7% at 50 μg/ml. The EtOAc extract was partitioned between *n*-hexane and water into an *n*-hexane layer and an aqueous suspension. The aqueous suspension was further partitioned between EtOAc and water into an EtOAc layer and an aqueous layer [yields (%) of the *n*-hexane, EtOAc, and aqueous layers after evaporation of the solvents from the EtOAc extract: 86.6, 8.8, and 3.2, respectively]. The *n*-hexane, EtOAc, and aqueous layers suppressed the Con A-induced proliferation by 10.3, 45.5, and 16.1% at 25 μg/ml, respectively. Repeated chromatographic fractionation monitored by the immunomodulatory activity of the EtOAc layer afforded four components, ZL-1 (**1**)—4 (**4**), and that of the *n*-hexane layer afforded ZL-5 (**5**) and -6 (**6**) [yields (%) of **1**, **2**, **3**, **4**, **5**, and **6** from the EtOAc extract: 0.68, 0.006, 0.004, 0.003, 0.14, and 0.058, respectively].

ZL-1 (**1**), obtained as yellow needles that were positive in the $FeCl_3$ test, had $C_{16}H_{12}O_6$ as the molecular formula. The ^{1}H- and ^{13}C-NMR spectral data of **1** including the two-dimensional ^{1}H–^{1}H shift correlation (COSY), ^{1}H-detected heteronuclear correlation through multiple quantum coherence (HMQC), and ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) NMR data (see Table 1) showed that ZL-1 might be 6,8-dihydroxy-3-(hydroxymethyl)-1-methoxyanthraquinone (**1**), which was isolated as carviolin from a Fungi Imperfecti, *Penicillium carmino-violaceum*[10,11] or as roseo-purpurin from *Penicillium roseo-purpureum*[12] in 1940. In comparison of the melting point of ZL-1 with that of carviolin (roseo-purpurin) reported in the literature,[10—12] ZL-1 was finally identified as carviolin (roseo-purpurin) (**1**), as shown in Fig. 1.

ZL-2 (**2**), obtained as yellow needles that were positive in the $FeCl_3$ test, had $C_{16}H_{12}O_5$ as the molecular formula. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1** showed that ZL-2 might be 6,8-dihydroxy-1-methoxy-3-methylanthraquinone (1-*O*-methylemodin) (**2**), which was obtained synthetically in 1977,[13] and isolated from an anamorphic fungus, *Phialophora alba* in 1994.[14] In comparison of the ^{1}H-NMR spectrum and the melting point of ZL-2 with those of 1-*O*-methylemodin reported in the literature,[13,14] ZL-2 was finally identified as 1-*O*-methylemodin (**2**), as shown in Fig. 1.

ZL-3 (**3**) was obtained as orange powder being positive to the $FeCl_3$ test (mp 249—252 °C). The molecular formula of **3** was determined to be $C_{18}H_{14}O_7$ by high-resolution electron-impact (HR-EI)-MS. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **3** with those of **1** indicated that the $-CH_2OH$ group at position 3 in **1** was replaced with a $-CH_2OAc$ group in **3** in reference to the acetylation shift rule,[15,16] because the signals of $\underline{CH_3}CO-$ newly appeared at δ_H 2.14 (3H, s), and δ_C 20.6 (q) and 170.2 (s), and the signal of $-\underline{CH_2}O-$ at position 3 was shifted to δ_H 5.22 (+0.58) (2H, s) and δ_C 64.5 (+2.3) (t), and that of C-3 was shifted to δ_C 144.5 (−6.9) (s) in the spectra of **3** (see Table 1). This was also supported by the fact that absorption of ester C=O newly appeared at 1740 cm^{-1} in the IR spectrum of **3**. Accordingly, the structure of ZL-3 was deduced to be 6,8-dihydroxy-3-(acetoxymethyl)-1-methoxyanthraquinone (ω-acetylcarviolin) (**3**), as shown in Fig. 1. To our knowledge, this is the first time that ω-acetylcarviolin (**3**) has been isolated from a natural source.

ZL-4 (**4**), obtained as yellow powder that was positive in the $FeCl_3$ test, had $C_{15}H_{10}O_6$ as the molecular formula. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **4** (see Table 1)

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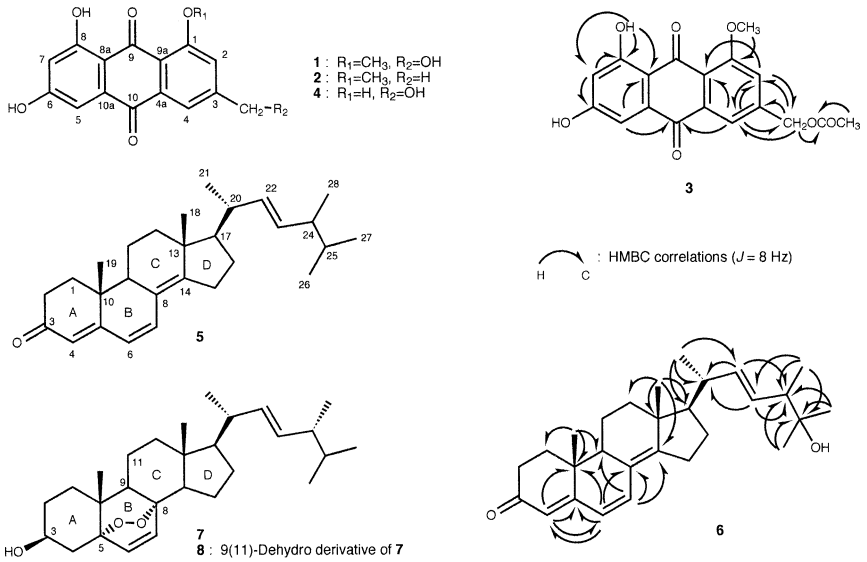

Fig. 1

Table 1. NMR Data for ZL-1, -2, -3, and -4, δ (ppm) from TMS as an Internal Standard

Position	ZL-1 (**1**) in DMSO-d_6		ZL-2 (**2**) in acetone-d_6		ZL-3 (**3**) in DMSO-d_6		ZL-4 (**4**) in acetone-d_6	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		160.6 (s)		162.0 (s)		160.6 (s)		162.6 (s)
1-OH							12.14 (s)	
1-O$\underline{CH_3}$	3.93 (3H, s)	56.4 (q)	4.00 (3H, s)	56.8 (q)	3.96 (3H, s)	56.6 (q)		
2	7.49 (br s)	116.2 (d)	7.42 (br s)	120.3 (d)	7.57 (br s)	118.0 (d)	7.32 (br s)	120.9 (d)
3		151.4 (s)		147.9 (s)		144.5 (s)		153.1 (s)
3-$\underline{CH_3}$			2.50 (3H, s)	22.0 (q)				
3-$\underline{CH_2}$OH	4.64 (2H, s)	62.2 (t)					4.79 (2H, s)	62.9 (t)
3-$\underline{CH_2}$OCOCH$_3$					5.22 (2H, s)	64.5 (t)		
3-CH$_2$O$\underline{C}$OCH$_3$						170.2 (s)		
3-CH$_2$OCO$\underline{CH_3}$					2.14 (3H, s)	20.6 (q)		
4	7.75 (br s)	116.8 (d)	7.67 (br s)	121.0 (d)	7.74 (br s)	117.8 (d)	7.76 (br s)	117.3 (d)
4a		134.7 (s)[a]		136.1 (s)[b]		135.0 (s)[c]		131.2 (s)
5	7.04 (d, 2.4)	107.0 (d)	7.17 (d, 2.5)	107.5 (d)	7.06 (d, 2.0)	107.1 (d)	7.28 (d, 2.4)	109.0 (d)
6		164.1 (s)		164.7 (s)		164.2 (s)		165.5 (s)[d]
7	6.56 (d, 2.4)	108.3 (d)	6.63 (d, 2.5)	109.2 (d)	6.58 (d, 2.0)	108.3 (d)	6.68 (d, 2.4)	108.1 (d)
8		164.5 (s)		166.2 (s)		164.5 (s)		165.7 (s)[d]
8-OH	13.25 (s)		13.40 (s)		13.16 (s)		12.20 (s)	
8a		110.2 (s)		111.8 (s)		110.3 (s)		109.7 (s)
9		186.3 (s)		187.8 (s)		186.2 (s)		191.0 (s)
9a		118.3 (s)		119.1 (s)		119.3 (s)		114.4 (s)
10		182.3 (s)		183.2 (s)		182.1 (s)		181.4 (s)
10a		134.1 (s)[a]		135.5 (s)[b]		134.0 (s)[c]		133.6 (s)

Multiplicities and coupling constants (in Hz) in parentheses. *a*—*d*) Assignmens with the same superscript in the same column may be interchangeable.

with those of **2** indicated that ZL-4 might be 1,6,8-trihydroxy-3-(hydroxymethyl)anthraquinone (**4**), which was isolated as ω-hydroxyemodin from *Penicillium cyclopium*[17] or as citreorosein from *Penicillium citreo-roseum*[18] in 1940 and synthesized in 1982.[19] In comparison of the melting point of ZL-4 with that of ω-hydroxyemodin (citreorosein) reported in the literature,[17,19] ZL-4 was finally identified as ω-hydroxyemodin (citreorosein) (**4**), as shown in Fig. 1.

ZL-5 (**5**), obtained as pale yellow optically active plates, had $C_{28}H_{40}O$ as the molecular formula. In comparison of the ^{1}H- and ^{13}C-NMR spectral data, the melting point, and the specific rotation of ZL-5 with those of ergosta-4,6,8(14),22-tetraen-3-one, which was isolated from some Basidiomycetes, *Fomes officinalis*,[20] *Scleroderma polyrhizum*,[21] *Astraeus hygrometricus*,[22] *Ganoderma applanatum*, and *G. neo-japonicum*,[23] ZL-5 was finally identified with ergosta-4,6,8(14),22-tetraen-3-one (**5**), as shown in Fig. 1.

ZL-6 (**6**) was obtained as pale yellow optically active powder (mp 128—130 °C, $[\alpha]_D^{24}$ +568°($CHCl_3$)). The molecular formula was determined to be $C_{28}H_{40}O_2$ by HR-FAB-MS. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **6**, assigned with the aid of the HMQC and HMBC NMR spectra, with those of **5** showed that the signals of C$\underline{H_3}$-26, C$\underline{H_3}$-27, and C-25 were shifted to δ_H 1.15 (+0.32) (3H, s), δ_H 1.18 (+0.33) (3H, s), and δ_C 72.4 (+39.3) (s), in **6**, respectively (see Table 2), indicating that ZL-6 is a 25-hydroxylated derivative of

Table 2. NMR Data for ZL-5 and -6 in $CDCl_3$, δ (ppm) from TMS as an Internal Standard

Position	ZL-5 (**5**)		ZL-6 (**6**)	
	δ_H	δ_C	δ_H	δ_C
1		34.1 (t)		34.1 (t)
2		34.1 (t)		34.1 (t)
3		199.5 (s)		199.5 (s)
4	5.74 (s)	123.0 (d)	5.74 (s)	123.1 (d)
5		124.5 (s)		124.6 (s)
6	6.03 (d, 9.6)	124.4 (d)	6.04 (d, 9.6)	124.5 (d)
7	6.61 (d, 9.6)	134.0 (d)	6.60 (d, 9.6)	133.9 (d)
8		164.3 (s)		164.3 (s)
9		44.3 (d)		44.3 (d)
10		36.7 (s)		36.8 (s)
11		18.9 (t)		18.9 (t)
12		35.6 (t)		35.6 (t)
13		44.0 (s)		44.0 (s)
14		156.1 (s)		155.6 (s)
15		25.4 (t)		25.3 (t)
16		27.7 (t)		27.7 (t)
17		55.7 (d)		55.5 (d)
18	0.96 (3H, s)	19.0 (q)	0.97 (3H, s)	19.0 (q)
19	1.00 (3H, s)	16.6 (q)	1.00 (3H, s)	16.7 (q)
20		39.3 (d)		39.3 (d)
21	1.06 (3H, d, 6.7)	21.2 (q)	1.08 (3H, d, 6.7)	21.1 (q)
22	5.23 (m)	135.0 (d)	5.37 (m)	138.3 (d)
23	5.23 (m)	132.5 (d)	5.37 (m)	129.8 (d)
24		42.9 (d)		48.2 (d)
25		33.1 (d)		72.4 (s)
26	0.83 (3H, d, 6.7)	20.0 (q)	1.15 (3H, s)	27.0 (q)
27	0.85 (3H, d, 6.7)	19.6 (q)	1.18 (3H, s)	26.5 (q)
28	0.93 (3H, d, 7.0)	17.6 (q)	1.02 (3H, d, 6.7)	15.7 (q)

Multiplicities and coupling constants (in Hz) in parentheses.

5, *i.e.*, 25-hydroxyergosta-4,6,8(14),22-tetraen-3-one (**6**), as shown in Fig. 1. 25-Hydroxyergosta-4,6,8(14),22-tetraen-3-one (**6**) has been isolated for the first time from a natural source.

The immunosuppressive activities (IC_{50} values) of ZL-1 (**1**)—6 (**6**) were calculated against Con A-induced (T cell) and LPS-induced (B cell) proliferation of mouse splenic lymphocytes, as shown in Table 3. In a comparison of the IC_{50} values of **1**—**6** with those of other compounds shown in Table 3, **1**—**6** exhibited moderate immunosuppressive activities. As immunosuppressive anthraquinones, emodin (1,6,8-trihydroxy-3-methylanthraquinone),[24] questin (1,6-dihydroxy-8-methoxy-3-methylanthraquinone), and rubrocristin (1,4,6-trihydroxy-8-methoxy-3-methylanthraquinone)[5] were already known. Huang *et al*. estimated that the immunosuppressive activity of emodin might be partly mediated through H_2O_2 generated from its semiquinone form, and the free OH group at the β-position of the anthraquinone nucleus played an important role in its immunosuppressive effect.[24] The fact that the four immunosuppressive anthraquinones ZL-1 (**1**)—4 (**4**), have their free OH groups at the β-position of their anthraquinone nucleus supports the estimation by Huang *et al*.[24] Meanwhile, in the ergosterol homologues, it is known that although ergosterol itself is not immunosuppressive, both ergosterol peroxide (5α,8α-epidioxyergosta-6,22-dien-3β-ol) (**7**) and 9(11)-dehydroergosterol peroxide (**8**) are immunosuppressive[25,26] (see Fig. 1). This shows that the immunosuppressive activities of **7** and **8** mainly result from their characteristic moiety over the ring A—C portion, namely, the 5α,8α-epidioxyergosta-6-en-3β-ol system. Also in the case of ZL-5 (**5**) and -6 (**6**), their characteristic moiety over rings A—C, *i.e.*, the ergosta-4,6,8(14)-trien-3-one system may be important to exert their immunosuppressive activities.

Table 3. Immunosuppressive Effects of ZL-1 -ZL-6, and Emodin, Questin, Azathioprine, Cyclosporin A, and Tacrolimus on the Con A-Induced and LPS-Induced Proliferation of Mouse Splenic Lymphocytes.

Compound	IC_{50} (μg/ml)	
	Con A-induced	LPS-induced
ZL-1 (**1**)	4.5	4.0
ZL-2 (**2**)	3.0	4.0
ZL-3 (**3**)[a]	14.4	10.0
ZL-4 (**4**)	9.0	9.0
ZL-5 (**5**)	7.6	5.5
ZL-6 (**6**)[a]	1.7	3.0
Emodin	0.2	0.2
Questin	0.3	0.3
Azathioprine	2.7	2.7
Cyclosporin A	0.04	0.07
Tacrolimus	1.5×10^{-5}	1.6×10^{-3}

The IC_{50} value of each sample was calcutated from the correlation curve between the sample concentration (horizontal axis) and the cell proliferation (vertical axis). The curve of each sample was drawn with 7 points, each of which represented the mean of three experiments on each correlation between 7 different concentrations and cell proliferations. *a*) New compounds.

Experimental

The general procedures for chemical experiments and other experimental conditions, including those for the evaluation of suppressive activity (IC_{50} values) of samples against the proliferation of mouse splenic lymphocytes stimulated with Con A and LPS, were the same as those described in our previous reports [this method is based on the formation ratio of MTT-formazan from exogenous 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) in lymphocytes].[7,8] Chemical shifts are expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard.

Isolation of ZL-1—6 from *Z. longicaudata* *Z. longicaudata* IFM4630[9] was cultivated on sterilized moistened rice in Roux flasks (200 g/flask×100) at 25 °C for 21 d. The moldy rice was extracted with EtOAc (35 l) with shaking at room temperature for 6 h twice to give an EtOAc solution (*ca*. 70 l), which gave, after evaporation *in vacuo*, an EtOAc extract (100.3 g). A concentrated solution of the EtOAc extract (50.0 g) in MeOH (50 ml) was suspended in H_2O (1.0 l). The suspension was partitioned with *n*-hexane (1.0 l) twice into an *n*-hexane layer (after evaporation *in vacuo*, 43.3 g) and an aqueous suspension. The aqueous suspension was further partitioned with EtOAc (1.0 l) twice into an EtOAc layer (4.4 g) and an aqueous layer (1.6 g). The *n*-hexane, EtOAc, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 10.3%, 45.5%, and 16.1% at 25 μg/ml, respectively. The EtOAc layer was subjected to chromatography on a silica gel [PSQ100B (Fuji silysia)] column with $CHCl_3$–MeOH (50 : 1, v/v), (10 : 1), (10 : 1), (5 : 1), (5 : 1), MeOH, and MeOH to give the seven fractions 1a—1g (1760, 69, 482, 945, 178, 213, and 283 mg), which suppressed 50% of the Con A-induced proliferation of the lymphocytes at >25, <10, 10—25, 10—25, 10—25, >25, and 10—25 μg/ml, respectively. Fractions 1d, 1e, and 1g were treated with MeOH to afford **1** (204 mg), **1** (30 mg), and **1** (107 mg) as precipitates, respectively. The acetone-soluble portion (54 mg) of fraction 1b (64 mg) was chromatographed on a silica gel [C-60 (Nacalai tesque)] column with *n*-hexane–acetone (2 : 1), (2 : 1), (2 : 1), (2 : 1), and MeOH to give five fractions 2a—2e (21, 5, 2, 4, and 10 mg), respectively. Fractions 2b and 2d were recrystallized from MeOH to afford **2** (3 mg) and **3** (2 mg), respectively. Fraction 1c (480 mg) was divided with acetone into an acetone-soluble portion (400 mg) and an acetone-insoluble portion (73 mg). The acetone-soluble portion (113 mg) was chromatographed on a silica gel (C-60) column with *n*-hexane–acetone (2 : 1)—(1 : 1) to give six fractions 3a—3f (3, 31, 37, 3, 10, and 2 mg, respectively). The MeOH-soluble portion (32 mg) of fraction 3c (37 mg) was chromatographed on a Sephadex LH-20 (Pharmacia) column with MeOH to afford **4** (1.5 mg). The *n*-hexane layer (6.0 g) was subjected to chromatography on a silica gel

(PSQ100B) column with *n*-hexane–EtOAc (20 : 1)—(1 : 1), and MeOH to give seven fractions 5a—5g (3890, 375, 163, 1040, 63, 200, and 459 mg, respectively). Fraction 5c (163 mg) was passed through a Sephadex LH-20 column with MeOH to give a fraction (135 mg), a portion of which (50 mg) was further purified by preparative TLC on a silica gel plate (Kieselgel 60, Merck) with *n*-hexane–EtOAc (3 : 1) to afford **5** (10 mg). Fraction 5e (63 mg) was chromatographed on a silica gel (PSQ100B) column with *n*-hexane–EtOAc (5 : 1)—(3 : 1) and MeOH to give the six fractions 6a—6f (2.3, 6.9, 13.3, 11.8, 15.1, and 11.6 mg, respectively). Fraction 6b (6.9 mg) was passed through a Sephadex LH-20 column with MeOH to give a fraction (6.7 mg), which was further purified by HPLC on a octadecyl silica gel (ODS) column (Develosil UG-5, Nomura) with MeOH at a flow rate of 2.0 ml/min to afford **6** (4 mg).

ZL-1 (**1**) [6,8-Dihydroxy-3-(hydroxymethyl)-1-methoxyanthraquinone, Carviolin, Roseo-purpurin]: Yellow needles from MeOH, mp 284—286 °C (lit.[10,11] 286 °C, lit.[12] 285 °C). HR-EI-MS *m*/*z*: 300.0645 (Calcd for $C_{16}H_{12}O_6$: 300.0634).

ZL-2 (**2**) [6,8-Dihydroxy-1-methoxy-3-methylanthraquinone, 1-*O*-Methylemodin]: Yellow needles from MeOH, mp 265—268 °C (lit.[13] 262—265 °C). HR-EI-MS *m*/*z*: 284.0701 (Calcd for $C_{16}H_{12}O_5$: 284.0684).

ZL-3 (**3**) [6,8-Dihydroxy-3-(acetoxymethyl)-1-methoxyanthraquinone, *ω*-Acetylcarviolin]: Orange powder from MeOH, mp 249—252 °C. EI-MS *m*/*z* (%): 342 (22, M^+), 282 (100), 253 (8), 225 (7). HR-EI-MS *m*/*z*: 342.0745 (Calcd for $C_{18}H_{14}O_7$: 342.0739). IR (KBr) cm^{-1}: 3464, 1740, 1626, 1459, 1339, 1316, 1246, 1173, 1048.

ZL-4 (**4**) [1,6,8-Trihydroxy-3-(hydroxymethyl)anthraquinone, *ω*-Hydroxy-emodin, Citreorosein]: Yellow powder from MeOH, mp 287—289 °C (lit.[17] 288 °C, lit.[19] 287—288 °C). HR-EI-MS *m*/*z*: 286.0467 (Calcd for $C_{15}H_{10}O_6$: 286.0478).

ZL-5 (**5**) [Ergosta-4,6,8(14),22-tetraen-3-one]: Pale yellow plates from MeOH, mp 115—119 °C (lit.[20] 113—114 °C). $[\alpha]_D^{24}$ +580 ° (*c*=0.50, $CHCl_3$) (lit.[20] $[\alpha]_D^{24}$ +610 ° (*c*=1.1, $CHCl_3$)). HR-FAB-MS *m*/*z*: 393.3148 (Calcd for $C_{28}H_{41}O$ $[(M+H)^+]$: 393.3158).

ZL-6 (**6**) [25-Hydroxyergosta-4,6,8(14),22-tetraen-3-one]: Pale yellow powder from MeOH, mp 128—130 °C. $[\alpha]_D^{24}$ +568 ° (*c*=0.10, $CHCl_3$). EI-MS *m*/*z* (%): 408 (1, M^+), 384 (3), 349 (8), 342 (31), 300 (24), 268 (40), 258 (78), 164 (100). HR-FAB-MS *m*/*z*: 409.3104 (Calcd for $C_{28}H_{41}O_2$ $[(M+H)^+]$:409.3107). IR (KBr) cm^{-1}: 3448, 1655, 1637, 1585, 1458, 1375, 1261, 1100, 1030, 804.

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