Blazeispirane and protoblazeispirane derivatives from the cultured mycelia of the fungus *Agaricus blazei*

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Abstract

Two blazeispirane derivatives including blazeispirols G and I were isolated from the cultured mycelia of the fungus *Agaricus blazei* Murill and were established to be (20S, 22S, 23R, 24S)-14β,22: 22,25-diepoxy-5-methoxy-des-A-ergosta-5,7,9-triene-11α,23-diol and (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-5-methoxy-des-A-ergosta-5,7,9,11-tetraene-23,28-diol by comparison of extensive 1D and 2D NMR spectral data with that of blazeispirol A. Furthermore, four blazeispirol derivatives blazeispirols, U, V, V$_1$ and Z$_1$ were isolated form the same source described above. Their structures were determined to be (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-23-hydroxyergosta-4,6,8,11-tetraen-3-one, (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-6α,7α,23-trihydroxy-ergosta-4,8,11-trien-3-one, (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-6β,7α,23-trihydroxyergosta-4,8,11-trien-3-one and (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-23-hydroxy-4,5-seco-ergosta-6,8-diene-3,5-dione by extensive 1 D and 2D NMR spectral data.
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Keywords: Agaricus blazei; Agaricaceae; Basidiomycetes; Higher fungi; des-A-Steroid; Blazeispirane; Protoblazeispirane; Blazeispirols G, I, U, V, V$_1$ and Z$_1$

1. Introduction

Agaricus blazei is an important fungus for producing bioactive compounds. There are many reports of poly-saccharides (Kawagishi et al., 1989; Mizuno et al., 1990, 1998; Ito et al., 1997), cytotoxic ergosterol derivatives (Kawagishi et al., 1988) and antimutagenic and bactericidal substances (Osaki et al., 1994) from the fruiting bodies of *A. blazei*. However, the chemical examination of the secondary metabolites of the cultured mycelia of this species has not been reported (Ito et al., 1997). We recently reported the isolation and structure determination of a number of unprecedented skeletal compounds, blazeispirols A (**1**), B, C (**2**), D, E, F, X, Y and Z (**3**) from the cultured mycelia of *A.blazei* (Hirotani et al., 1999, 2000a, 2001, 2002) and the biosynthesis of blazeispirol A (Hirotani et al., 2000b). Blazeispirols A–F are the first demonstration of steroids without ring A in a living organism, for which we have proposed the col-

lective name "blazeispirane skeleton". In this paper, we report the isolation and structural elucidation of two additional new des-A-ergostane-type compounds named blazeispirols G (**4**) and I (**5**), and also new blazeispirol derivatives named blazeispirols U (**6**), V (**7**), V$_1$ (**8**) and Z$_1$ (**9**) from the cultured mycelia of *A. blazei* (Fig. 1).

2. Results and discussion

The cultured mycelia of *A. blazei* Murill were extracted sequentially with methanol at room temperature. The concentrated methanolic extract was diluted with water and partitioned with chloroform and ethyl acetate. Each fraction was dried in vacuo. The chloroform extract (18.86 g) was subjected to silica gel chromatography, with fractions A–G being subsequently obtained. Further purification by ODS column chromatography and normal- and reversed-phase HPLC afforded compounds **4–9** from fractions D, E and F.

Compound **4**, blazeispirol G, had a molecular formula of $C_{25}H_{36}O_5$ (*m/z* 416.2578), with a 16 mass unit

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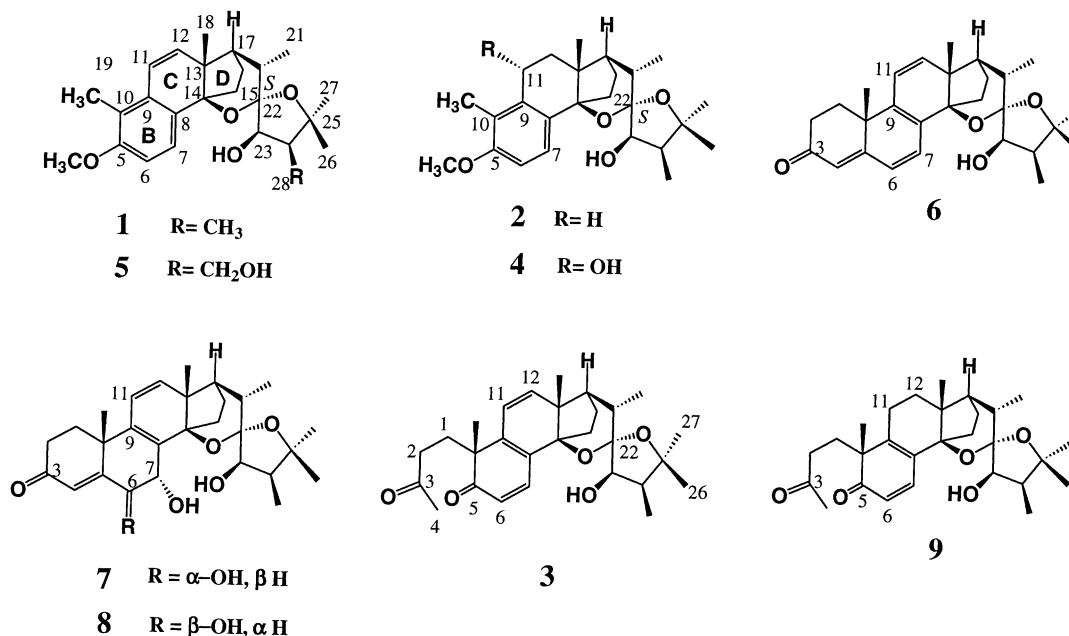*E-mail address:* hirotanim@pharm.kitasato-u.ac.jp (M. Hirotani).

Fig. 1. Structures of blazeispirol A (**1**), C (**2**), Z (**3**), G (**4**), I (**5**), U (**6**), V (**7**), V₁ (**8**) and Z₁ (**9**).

excess relative to that of blazeispirol C (**2**) ($C_{25}H_{36}O_4$) reported previously (Hirotani et al., 2002). The IR spectrum showed the presence of an alcohol (3420 cm^{-1}). Its ^{13}C NMR spectrum showed 25 carbons, and the DEPT spectrum suggested the presence of seven methyls, three methylenes, seven methines, and eight quaternary carbons. Five oxygenated carbon signals were seen at δ 66.1, 82.9, 84.1, 84.9 and 107.7, three of which were quaternary carbons. The fourth and fifth were hydroxyl methine carbons which were supported by the presence of two proton signals at δ 5.14 and 3.85, respectively. One of the quaternary carbons appeared very much deshielded at δ 107.7, reminiscent of an acetal system in the side chain like that of blazeispirol C (**2**). The ^{13}C NMR spectrum of **4** was closely similar to that of **2** except for the observation of the downfield shift of carbon 11 and related carbons 9 and 12 (Table 1).

In the NOESY spectrum of **4**, H-11 was observed to have a cross-peak with CH₃-18, suggesting that H-11 and CH₃-18 were 1,3-diaxial to each other. Therefore, the orientation of the hydroxyl group of C-11 also the α-configuration. Thus, the structure of **4** was determined to be (20*S*, 22*S*, 23*R*, 24*S*)-14β,22: 22,25-diepoxy-5-methoxy-des-*A*-ergosta-5,7,9-triene-11α,23-diol.

Compound **5**, blazeispirol I, $C_{25}H_{34}O_5$ showed UV absorptions at 224, 258, 266, 275 nm and hydroxyl absorption (3430 cm^{-1}) in the IR spectrum. The ^{13}C NMR spectrum of **5** was very similar to that of **1**, except for the downfield shift of the C-28 and C-24 carbon signals (δ 59.2 and 51.9) and upfield shift of the C-23

Table 1
^{13}C NMR (100 MHz) spectroscopic data for blazeispirols A (**1**), C, (**2**) Z, (**3**), G (**4**), I (**5**), U (**6**), V (**7**), V₁ (**8**) and Z₁ (**9**)

	1[a]	2[a]	3[a]	4	5	6	7	8	9
1	–	–	32.6	–	–	32.6	34.2	35.3	33.6
2	–	–	38.9	–	–	33.8	33.5	34.2	38.7
3	–	–	207.7	–	–	198.0	197.8	198.9	207.9
4	–	–	29.9	–	–	124.1	122.4	127.7	29.9
5	156.4	155.9	204.9	156.8	156.4	165.9	166.4	163.6	206.1
6	108.6	108.3	123.9	110.1	108.6	125.1	69.1	76.0	123.6
7	121.4	123.5	140.9	123.7	121.4	129.8	66.1	67.5	140.9
8	132.0	132.8	130.7	132.8	131.8	131.0	132.9	132.0	131.3
9	130.3	133.4	142.7	135.1	130.3	138.2	134.1	133.4	146.9
10	122.5	124.2	50.6	126.0	122.5	38.7	39.0	36.9	52.5
11	122.4	23.8	121.4	66.1	122.4	120.5	120.0	120.3	22.3
12	139.1	29.4	143.7	41.1	139.0	142.4	142.8	141.9	28.6
13	47.0	42.9	48.2	46.4	47.0	48.3	48.1	48.1	43.2
14	84.0	83.5	82.8	82.9	84.1	82.6	82.7	82.7	82.2
15	37.1	38.8	36.8	38.4	37.1	36.8	37.9	38.1	37.5
16	25.0	21.0	25.6	21.4	24.9	25.4	25.7	25.7	20.7
17	50.7	50.5	50.6	50.1	50.5	50.7	50.5	50.5	49.8
18	15.7	14.7	15.3	16.1	15.6	15.0	14.7	14.5	14.7
19	10.8	11.1	25.5	12.5	10.8	27.8	23.3	24.4	25.5
20	33.5	34.0	33.6	33.4	33.5	33.5	33.7	33.8	34.0
21	16.4	16.7	16.3	16.5	16.3	16.3	16.3	16.4	16.6
22	107.4	107.8	107.7	107.7	107.6	107.5	107.5	107.6	108.1
23	85.0	85.1	84.7	84.9	82.9	84.7	84.8	84.8	84.7
24	44.1	44.1	44.2	44.0	51.9	44.1	44.0	44.0	44.0
25	84.1	84.0	84.6	84.1	82.7	84.3	84.6	84.6	84.6
26	25.7	25.7	25.8	25.6	25.7	25.6	25.6	25.5	25.0
27	30.7	30.8	30.7	30.7	31.7	30.5	30.2	30.3	30.7
28	8.7	8.6	8.6	8.6	59.2	8.6	8.5	8.5	8.5
OMe	55.6	55.6	–	55.5	55.6	–	–	–	–

[a] The data for compunds **1**, **2** and **3** are cited from Hirotani et al., 2001, 2002.

and C-25 carbon signals (δ 82.9 and 82.7) relative to those of **1** (δ 8.7 and 44.1, and 85.0 and 84.1), respectively (Table 1). The downfield shift (from δ 8.7 to 59.2) indicated that the C-28 methyl carbon had been changed into a hydroxymethyl carbon. This supported the downfield and upfield shifts seen for the C-28, C-24, and C-23, C-25 carbons being due to a substitution effect. In addition, this was supported by the disappearance of the methyl proton signal (δ 1.04) seen in **1** and appearance of the methylene proton signals (δ 3.83 and 3.98) in the ^{1}H NMR spectrum of **5** (Table 2). These data led to the conclusion that the structure of **5** was 28-hydroxymethylblazeispirol A, (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-des-A-ergosta-5,7,9,11-tetraene-23,28-diol.

The molecular formula of compound **6**, blazeispirol U, was also determined to be $C_{28}H_{36}O_4$ (m/z 436.2631) by HREI-Ms. The IR absorptions at 3440 and 1640 cm^{-1} suggested the presence of a hydroxyl and an α,β-unsaturated carbonyl group. The ^{13}C and ^{1}H NMR spectra of **6** showed 28 carbon atoms and six methyl signals, which suggested an ergostane skeleton with a spiroacetal structure moiety as a side chain like that of blazeispirol A. This was supported by the fact that the ^{13}C and ^{1}H NMR spectroscopic data from the C-20–C-28 carbons and protons were almost identical with that of the spiroacetal structure of **1** (Tables 1 and 2). In addition, an NOE correlation was observed between the proton signals of H-20 (δ 2.47) and H-18 (δ 0.90), and between H-17 (δ 1.95) and H-12 (δ 5.94), respectively, indicating that rings C and D in **6** have the same structures as in **1**. In the rings A, B and C of **6**, the ^{1}H NMR signals exhibited five olefinic proton signals at δ 5.80 (1H, s), 5.94 (1H, d, J = 10 Hz) and 6.70 (1H, d, J = 10 Hz), and 6.24 (1H, d, J = 9.5 Hz) and 6.68 (1H, d, J = 9.5 Hz). Additionally, the ^{13}C spectrum showed eight olefinic carbon signals (δ 120.5, 124.1, 125.1, 129.8, 142.4, each methine carbons, and 131.0, 138.2, 165.9, each quaternary carbons). In the HMBC spectrum, cross-peaks were also observed between two proton signals, H-6 (δ 6.24) and H-11 (δ 6.70), to the quaternary carbon C-8 (δ 131.0) and H$_3$-19 (δ 1.30) showed correlations with two quaternary carbons (δ 138.2 and 165.9). At the same time, H-2 methylene proton (δ 2.60), which exhibited an NOE correlation to the H$_3$-19 methyl proton, was correlated to the C-3 carbonyl carbon signal (δ 198.0) in the HMBC spectrum. Accordingly, compound **6** has the 3-oxo-ergosta-4,6,8,11-tetraene structure in rings A, B and C. Blazeispirol U (**6**) is, therefore, (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-23-hydroxyergosta-4,6,8,11-tetraen-3-one.

Compound **7**, blazeispirol V, $C_{28}H_{38}O_6$, mp 276–277.5 °C had UV absorptions at 237 nm and hydroxyl (3470 and 3330 cm^{-1}) absorptions in the IR spectrum. The ^{13}C and ^{1}H NMR spectra of **7** showed 28 carbon atoms and six methyl signals, which suggested an ergostane skeleton with a spiroacetal structure moiety as a side chain like that of **6**. The ^{13}C NMR spectrum of

7 was closely similar to that of **6** except for upfield shifts of two hydroxyl methine carbons C-6 (δ 69.1) and C-7 (δ 66.1), and the change of the chemical shifts of neighboring carbons (C-4, C-8 and C-9) of C-6 and C-7. The configuration of the hydroxyl groups was determined by NOESY spectral data and the ^{1}H–^{1}H coupling constant. The NOEs between H-19 (δ 1.34) and H-6 (δ 4.46), and H-4 (δ 6.28) and 6-OH (δ 2.66) established the α-hydroxyl group in the C-6 position. This was supported by the fact that the coupling constant of H-4 and H-6 is 2 Hz (Collins et al., 1963). The configuration of another hydroxyl group (7-OH) was decided to be the α-configuration by analysis of the coupling constant value (4.5 Hz) of H-6 and H-7. These data suggested that compound **7** was (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-6α,7α,23-trihydroxyergosta-4,8,11-trien-3-one.

Finally, the structure of **7** was confirmed by X-ray structural analysis, whose crystal were grown in a methanol–acetonitrile mixed solution as colorless plates. A view of the solid-state confirmation is provided in Fig. 2. This result is in agreement with the above conclusions based on the physicochemical evidence.

Compound **8**, blazeispirol V$_1$, had the same molecular formula of $C_{28}H_{38}O_6$ (M$^+$ 470, m/z 470.2657 HRMS) as **7**. The ^{13}C and ^{1}H NMR spectra of **8** showed 28 carbon atoms and six methyl signals, which suggested an ergostane skeleton with a spiroacetal structure moiety as a side chain like that of **7**. The ^{13}C NMR spectrum of **8** was very similar to that of **7**, except for the downfield shift of the C-4, C-6 carbon signals (δ 127.7, 76.0), and upfield shift of the C-5 carbon signal (δ 163.6) relative to that of **7** (δ 122.4, 69.1, and 166.4), respectively (Table 1). These data suggested that **8** was a stereoisomer of **7** derived from the C-6 hydroxyl group. In the NOESY spectrum of **8**, an NOE was detected between H6 (δ 4.36) and H-4 (δ 5.98), but not between H-6 and H-19, confirming the β-orientation of the C(6)-OH group. This was supported by the observation of the downfield shift of the H-19 methyl proton signal (δ 1.47) relative to that of **7**. On the basis of this spectroscopic evidence, compound **8** was identified as (20S, 22S, 23R, 24S)-14β,22:22,25-diepoxy-6β,7α,23-trihydroxyergosta-4,8,11-trien-3-one.

Compound **9**, blazeispirol Z$_1$, had a molecular formula of $C_{28}H_{40}O_5$ (M$^+$ 456, m/z 456.2860 HRMS), a two mass excess more than that of **3**. It showed in the UV spectrum an absorption at 326 (log ϵ 3.43) characteristic of a steroid dienone (Scott, 1964). The IR spectrum of **9** showed the presence of an acetyl carbonyl (1720 cm^{-1}) and an α,β-unsaturated carbonyl (1660 cm^{-1}) group. The ^{13}C and ^{1}H NMR spectrum of **9** showed 28 carbon atoms and seven methyl signals, which suggested an ergostane skeleton with a spiroacetal structure moiety as a side chain like that of blazeispirol Z (**3**). The ^{1}H NMR spectrum of **9** was very similar to that of **3** except for the chemical shift and the

Table 2
^{1}H NMR (400 MHz) spectroscopic data (δ in ppm, J in Hz) for blazeispirols A (**1**), C, (**2**) Z, (**3**) G (**4**), I (**5**), U (**6**), V (**7**), V$_1$ (**8**) and Z$_1$ (**9**)

H	1[a]	2[a]	3[a]	4	5	6	7	8	9
1	–	–	2.20 *dd* (12, 4.5) 1.97 *m*	–	–	2.30 *ddd* (13, 5.5, 2) 1.99 *ddd* (13, 13, 7.5)	2.19 *ddd* (13, 5, 2.5) 1.79 *ddd* (14, 14, 5)	2.15 *ddd* (13, 5, 2.5) 1.76 *ddd* (14, 13, 5)	2.10 *m* 1.94 *m*
2	–	–	1.85 *ddd* (9.5,9.5,4.5) 2.16 *m*	–	–	2.60 *dd* (13.5, 5.5) 2.50 *m*	2.54 *dd* (17, 14.5, 5) 2.50 *m*	2.66 *ddd* (17, 14, 5) 2.49 *m*	1.95 *ddd* (13,13,4), 2.20 *m*
4	–	–	2.03 *s*	–	5.80 *s*	6.28 *dd* (2)		5.98 *bs*	2.06 *s*
6	6.73 *d* (8.5)	6.76 *d* (7.5)	6.07 *d* (10)	6.84 *d* (8.5)	6.72 *d* (8.5)	6.24 *d* (10)	4.46 *dd* (9.5, 4.5, 2)	4.36 *dd* (4.5, 3)	6.03 *d* (10)
7	7.24 *d* (8.5)	7.30 *dd* (8, 0.5)	7.41 *dd* (10,0.5)	7.30 *d* (8.5)	7.24 *d* (8.5)	6.68 *d* (10)	4.63 *dd* (8, 4.5)	4.59 *dd* (6.5, 3)	7.31 *d* (10)
11	6.54 *d* (10)	2.70 *m*	5.96 *bd* (9.5)	5.14 *dd* (8,8)	6.54 *dd* (10, 1)	6.07 *d* (10)	5.82 *d* (10)	5.85 *d* (10)	2.20 *m*2.40 *m*
	–	–	–	–			–		
12	5.89 *d* (10)	1.91 *ddd* (13.5,11,8) 1.53 *ddd* (13.5,7,1.5)	6.05 *d* (9.5)	2.15 *dd* (13.5,8)1.80 *dd* (13.5,8)	5.89 *d* (10)	5.94 *d* (10)	5.94 *d* (10)	5.93 *d* (10)	1.77 *m*1.42 *m*
15	2.44 *ddd* (13.5, 9.5, 5.5) 1.80 *ddd* (13.5, 12.5, 3.5)	2.52 *dd* (9,9) 1.74 *m*	2.59 *ddd* (13,9.5, 5.5) 1.66 *ddd* (13,12.5,3.5)	1.92 *ddd* (13,10,4) 2.59 *ddd* (13,10,4)	2.42 *ddd* (14, 9.5, 5.5) 1.80 *ddd* (14, 12.5, 3.5)	2.54 *dd* (13.5, 5.5) 1.67 *ddd* (13.5, 12.5, 2.5)	2.60 *ddd* (13, 9, 5.5) 1.66 *ddd* (13, 12.5, 3.5)	2.60 *ddd* (13, 9.5, 5.5) 1.69 *ddd* (13, 13, 3.5)	2.66 *ddd* (13.5, 9.5, 4.5) 1.58 *ddd* (13.5, 11.5, 4.5)
16	2.05 *ddd* (13, 9.5, 3.5) 1.46 *m*	2.03 *dd* (9,9) 1.74 *m*	1.39 *m* 2.06 *m*	2.15 *ddd* (13,10,4) 1.82 *dddd* (13,10,6.5,4)	2.04 *ddd* (13, 9.5, 3.5) 1.45 *m*	2.03 *dd* (12.5, 4) 1.35 *ddd* (12.5, 12.5, 6)	2.03 *ddd* (13, 9.5, 3.5) 1.34 *ddd* (12.5, 5.5)	2.04 *ddd* (13, 9.5, 3.5) 1.37 *ddd* (13, 13, 5.5)	1.76 *m* 2.04 *m*
17	1.94 *dd* (6, 3.5)	1.72 *dd* (6,3)	1.98 *dd* (6, 3.5)	1.71*dd* (6.5,3.0)	1.94 *dd* (6, 3.5)	1.95 *dd* (6, 3.5)	1.95 *dd* (6, 3.5)	1.95 *dd* (6, 3.5)	1.69 *dd* (6.5, 3)
18	0.90 *s*	0.92 *s*	0.93 *s*	0.86 *s*	0.89 *s*	0.90 *s*	0.90 *s*	0.95 *s*	0.89 *s*
19	2.20 *s*	2.20 *s*	1.20 *s*	2.31*s*	2.19 *s*	1.30 *s*	1.34 *s*	1.47 *s*	1.23 *s*
20	2.54 *qdd* (7, 3.5, 1)	2.63 *qd* (7, 3)	2.49 *qd* (7, 4.5)	2.56 *qd* (6.5,3.0)	2.53 *qdd* (7, 3.5, 1)	2.47 *qdd* (7, 3, 1)	2.48 *qdd* (7.5, 3.5, 1)	2.49 *qd* (7,3.5)	2.60 *qd* (7, 4.5)
21	1.14 *d* (7)	1.14 *d* (7)	1.12 *d* (7)	1.13 *d* (6.5)	1.15 *d* (7)	1.10 *d* (7)	1.10 *d* (7)	1.11 *d* (7)	1.11 *d* (7)
23	3.95 *dd* (4.5, 4.5)	3.90 *dd* (5.5,4)	3.90 *d* (4.5)	3.85 *d* (4.5)	4.23 *d* (4)	3.89 *bd* (4)	3.88 *dd* (5, 5)	3.91 *dd* (4.5, 4.5)	3.90 *d* (4.5)
24	2.64 *qd* (7, 4.5)	2.59 *qd* (7, 4)	2.48 *qd* (7, 4.5)	2.55 *dq* (4.5, 7.5)	2.73 *ddd* (9, 5.5, 4)	2.46 *qd* (7, 5)	2.44 *qd* (7, 5)	2.45 *qd* (7, 4.5)	2.42 *qd* (7, 4.5)
26	1.16 *s*	1.18 *s*	1.16 *s*	1.17 *s*	1.21 *s*	1.13 *s*	1.12 *s*	1.13 *s*	1.16 *s*
27	1.42 *s*	1.48*s*	1.39 *s*	1.47 *s*	1.48 *s*	1.34 *s*	1.36 *s*	1.36 *s*	1.40 *s*
28	1.04 *d* (7)	1.01 *d* (7)	1.02 *d* (7)	1.00 *d* (7.5)	3.83 *dd* (10.5, 5.5) 3.98 *dd* (10.5, 9)	1.00 *d* (7)	1.00 *d* (7)	0.99 *d* (7)	1.00 *d* (7)
OCH$_3$	3.80 *s*	3.80 *s*	–	3.81*s*	3.80 *s*	–	–	–	–
23-OH	1.41 *d* (4.5)	1.44 *d* (5.5)	1.44 *d* (5.5)	–	–	1.60	1.42 *d* (5)	1.48 d (4.5)	–
6-OH							2.66 (9.5)	1.79 *d* (4.5)	
7-OH							1.25 *d* (8)	1.50 *d* (6.5)	

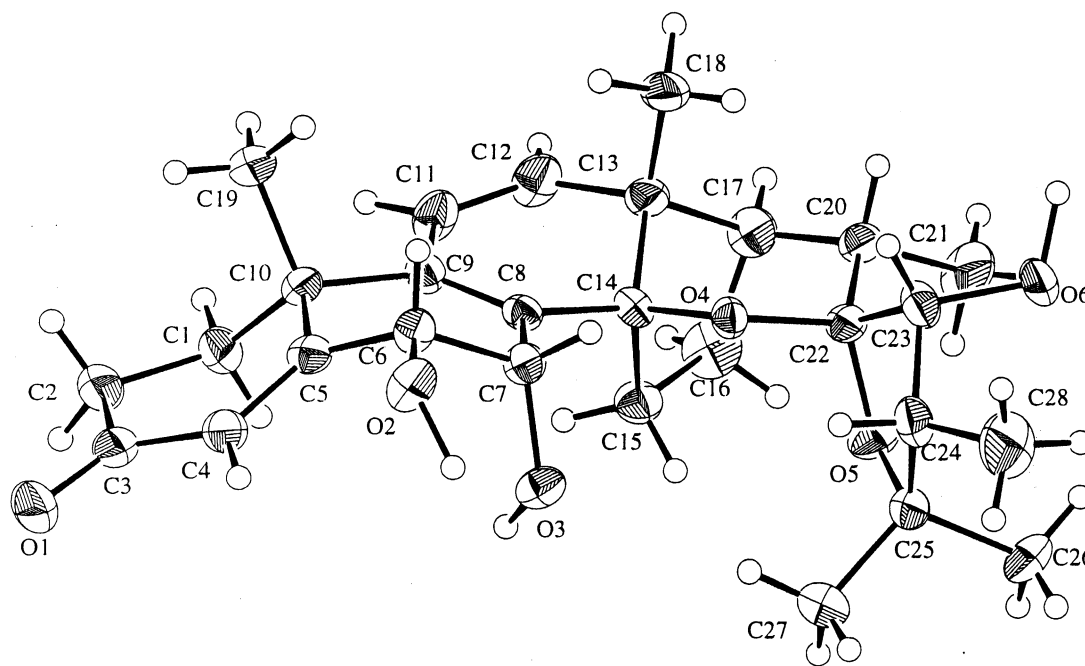[a] The data for compunds **1**, **2** and **3** are cited from Hirotani et al., 2001, 2002.

Fig. 2. ORTEP drawing for blazeispirol V (**7**).

coupling constants observed for the pair of aromatic protons (δ 5.96 and 6.05) to the two methylene protons (δ 2.20 *m* and 2.40 *m*; 1.77 *m* and 1.42 *m*). The ^{13}C NMR spectrum of **9** was also closely similar to that of **3** except for the appearance of two methylene carbon signals (δ 22.3 and 28.6) instead of the olefinic methine carbon signals (δ 121.4 and 143.7) seen in the spectrum of **3**. On the basis of these observations, compound **9**, blazeispirol Z_1 was identified to be (20*S*, 22*S*,23*R*,24*S*)-14β,22:22,25-diepoxy-23-hydroxy-4,5-seco-ergosta-6,8-diene-3,5-dione. It seems likely that blazeispirol Z_1 is a direct intermediate of blazeispirol C (**2**).

In this paper, we have described two blazeispirane derivatives, in which the A-ring had been lost, including blazeispirol G and I. Additionally, we have also described four blazeispirol derivatives which have the ergostane skeleton. These compounds are a group of naturally occuring steroids built on an ergostane skeleton in which C-14, C-22 and C-25 are appropriately oxidized to form a 14, 22: 22, 25-diepoxy structure. For convenience, this basic structure was designated as the "pro-toblazeispirane" skeleton as shown in Fig. 3 and some of them seem to be precursors of blazeispirane derivatives.

3. Experimental

3.1. General

Optical rotation:JASCO DIP-370 (CHCl$_3$), UV: Hitachi 340 (alcohol), IR: JASCO FT/IR-200 (KBr discs and NaCl film), EIMS: JEOL JMS-AX505 H, FABMS: JEOL JMS-AX505 HA, ^{1}H and ^{13}C NMR: Varian Unity 400 (400 and 100 MHz respectively in CDCl$_3$). HPLC was performed by Senshu Pak ODS (10 × 300 mm and 20 × 300 mm) coupled with a UV detector and a differential refractometer.

3.2. Culture conditions for Agaricus blazei mycelium

The pure mycelia were subcultured for 2 weeks and grown in a 500-ml Erlenmeyer flask with 125 ml of medium containing 10 g sucrose, 30 g malt extract, 5 g yeast extract in 1 l of dist. H$_2$O according to the previous paper (Hirotani et al., 2002). Usually, each flask was seeded with 5 of the 10 mm plugs cut from the potato dextrose agar culture.

3.3. Extraction procedure and separation of the MeOH extract of the mycelia.

After 5 weeks culture (1027 flasks), the mycelia (12.77 kg, fr. wt) were harvested with nylon cloth, homo-genized with MeOH (65.8 l) in a Waring blender and
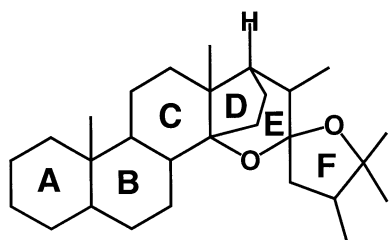

Fig. 3. Protoblazeispirane skeleton.

allowed to stand for 1 week at room temp. The homogenate was filtered and the residue was re-extracted with the same solvent (46 l), with filtrates combined and the organic solvent was removed under red. pres. The result residue was extracted with $CHCl_3$ (5 × 4 l), dried and evapd. to dryness. The $CHCl_3$ extract (18.86 g) was subjected to chromatography over silica gel (Silica gel 60, 720 g). Elution with 5.4 l toluene and 2.97 l toluene–EtOAc (19:1) (fraction A), 0.90 l (fraction B), 1.22 l and 3.67 l toluene–EtOAc (9:1) (fraction C), 2.07 l toluene–EtOAc (9:1) and 4.68 l toluene–EtOAc (8:2)(fraction D); 0.81 l toluene–EtOAc (8:2) and 3.44 l toluene–EtOAc (6:4) (fraction E); 3.44 l, 3.60 l toluene–EtOAc (4:6) and 3.60 l 100% EtOAc (fraction F), 1.80 l 100% MeOH (fraction G) yielded the crude mixture of compounds **4**, **6**, **9** (fraction D 2.0 g), compound **5** (fraction E 0.98 g), compound **7,8** (fraction F 1.91 g), Further purification was achieved by reversed-phase CC and normal- and reversed-phase HPLC.

Fr. D was subjected to reversed-phase CC (ODS-7515–12Å Senshu Scientific Co. Ltd from MeOH:H_2O = 7:3 to MeOH:H_2O = 10:0) to give 7 fractions D-1–D-7. Compounds **4**, **9** (20.1 and 12.6 mg, respectively) were isolated from fraction D-2 (323.5 mg) by HPLC (Senshu Pak Pegasil silica, ϕ 20 × 150 mm, hexane:EtOAc = 5:1, 8 ml min^{-1} flow rate, R_t, 13.8 min and 22.4 min, respectively). Compound **6** (88.1 mg) was isolated from fraction D-4 (185 mg) by HPLC (Senshu Pak ODS, ϕ 10 × 300 mm, 85% CH_3CN, 3 ml min^{-1} flow rate, Rt, 15.7 min).

Fr. E was subjected to reversed-phase CC (ODS-7515–12Å Senshu Scientific Co. Ltd from MeOH:H_2O = 5:5 to MeOH:H_2O = 10:0) to give 3 fractions E-1 - E-3. Compound **5** (3.9 mg) was isolated from fraction E-2 (20.4 mg) by HPLC (Senshu Pak Pegasil silica, ϕ 20 × 150 mm, hexane:EtOAc = 1:1, 8 ml min^{-1} flow rate, R_t, 64.6 min).

Fr. F was subjected to reversed-phase CC (ODS-7515–12Å Senshu Scientific Co. Ltd from MeOH:H_2O = 6:4 to MeOH:H_2O = 10:0) to give 4 fractions F-1–F-4. Compound **7** was isolated from fraction F-2 (253.9 mg) by HPLC (Senshu Pak Pegasil silica, ϕ 20 × 150 mm, hexane:EtOAc = 2:1, 8 ml min^{-1} flow rate, R_t, 23.9 min) and was recrystallized from MeOH:MeCN (9:1) to give colorless plates (4.6 mg). At the same time, compound **8** was isolated from a part of a different peak(Senshu Pak Pegasil silica, ϕ 20 × 150 mm, hexane: EtOAc = 2:1, 8 ml min^{-1} flow rate, R_t, 56.2 min) but still impure. Further purification was achieved by reversed phase HPLC (Senshu Pak ODS, ϕ 10 × 300 mm, 50% CH_3CN, 3 ml min^{-1} flow rate, R_t, 21.1 min) to give a colorless powder (3.8 mg).

3.4. Blazeispirol G (**4**)

Colorless oil, $[\alpha]_D^{23}$−30.9 (*c* 0.11, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 218 (4.00), 267 (3.09), 278 (3.33), 285 (3.31). IR ν_{max} (KBr) cm^{-1}: 3420, 2960, 2920, 2850,

1260, 970. HREIMS m/z: 416.2578 [M]$^+$($C_{25}H_{36}O_5$ requires 416.2563). EI-MS m/z (rel. int.):416 [M]$^+$(16), 398 [M−H_2O]$^+$(9), 380 [M−2H_2O]$^+$ (7), 346 (6), 317(14), 299 (29), 272 (10), 226 (10), 225 (27), 221 (38), 220 (100), 216 (11), 204 (29), 203 (99), 197 (20), 196 (22), 156 (13), 97 (15). ^{13}C and ^{1}H NMR, spectra see Tables 1 and 2.

3.5. Blazeispirol I (**5**)

Colorless oil, $[\alpha]_D^{23}$−20.0(*c* 0.28, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 224 (4.39), 258 (3.72), 266 (3.81), 275 (3.73). IR ν_{max} (KBr) cm^{-1}: 3430, 2920, 2860, 1260, 1090, 970. HREIMS m/z: 414.2413 [M]$^+$ ($C_{25}H_{34}O_5$ requires 414.2406). EI-MS m/z (rel. int.): 414 [M]$^+$ (16), 396 [M−H_2O]$^+$ (31), 368 (18), 366 (35), 365 (42), 338 (25), 299 (35), 255 (15), 236 (23), 225 (33), 216 (100), 202 (21), 167 (16), 149 (38), 127 (16), 111 (19), 97 (32), 83 (31), 57 (20). ^{13}C and ^{1}H NMR, spectra see Tables 1 and 2.

3.6. Blazeispirol U (**6**)

Yellow solid, $[\alpha]_D^{23}$ + 821.7 (*c* 0.23, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 220(4.09), 272(3.92). IR ν_{max} (KBr) cm^{-1}: 3440, 2960, 2920, 1640, 1620, 1500, 1160. HREIMS m/z: 436.2631 [M]$^+$($C_{28}H_{36}O_4$ requires 436.2613). EI-MS m/z (rel. int.): 436 [M]$^+$(26), 418 [M−H_2O]$^+$ (26), 398 (5), 310 (9), 295 (18), 263 (21), 241 (10), 226 (16), 225 (14), 167 (24), 149 (38), 97 (39), 85 (89), 83 (100). ^{13}C and ^{1}H NMR, spectra see Tables 1 and 2.

3.7. Blazeispirol V (**7**)

Colorless plates mp 276.0–277.5 °C $[\alpha]_D^{23}$−15.0 (*c* 0.08, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 237 (3.94). IR ν_{max} (KBr) cm^{-1}: 3470, 3330, 2960, 2930, 1660, 1460, 1100, 980. HREIMS m/z: 470.2661 [M]$^+$ ($C_{28}H_{38}O_6$ requires 470.2668). EI-MS m/z (rel. int.): 470 [M]$^+$ (13), 452 [M−H_2O]$^+$ (85), 434 [M−2H_2O]$^+$ (29), 353 (32), 279 (53), 254 (77), 196 (37), 182 (25), 169 (22), 97 (100), 85 (49). ^{13}C and ^{1}H NMR: see Tables 1 and 2.

3.8. Blazeispirol V₁ (**8**)

Colorless powder, $[\alpha]_D^{23}$−43.3 (*c* 0.12, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 231 (4.09). IR ν_{max} (KBr) cm^{-1}: 3440, 2960, 2920, 2860, 1665, 970. HREIMS m/z: 470.2657 [M]$^+$ ($C_{28}H_{38}O_6$ requires 470.2668). EI-MS m/z (rel. int.): 470 [M]$^+$(12), 452 [M−H_2O]$^+$ (60), 434 [M−2H_2O]$^+$ (15), 353 (29), 279 (40), 257 (16), 196 (21), 182 (15), 169 (17), 97 (100), 85 (35). ^{13}C and ^{1}H NMR: see Tables 1 and 2.

3.9. Blazeispirol Z₁ (**9**)

Slight yellow solid, $[\alpha]_D^{24}$−44.9 (*c* 0.11, CHCl$_3$); UVλ_{max} (MeOH) nm (log ϵ): 223(3.53), 326 (3.43). IR ν_{max}

(KBr) cm^{-1}: 3460, 2960, 2920, 1720, 1660, 1625, 1460, 1160, 970. HREIMS m/z: 456.2860 [M]$^+$ (C$_{28}$H$_{40}$O$_5$ requires 456.2876). EI-MS m/z (rel. int.): 456 [M]$^+$ (32), 436 (12), 386 (37), 357 (11), 287 (46), 242 (15), 213 (25), 203 (24), 196 (40), 191 (87), 190 (100), 156 (13), 97 (35). ^{13}C and ^{1}H NMR: see Tables 1 and 2.

3.10. X-ray crystallographic analysis of 7

Compound **7** was crystallized from MeOH-MeCN. Data were collected on an AFC5R Rigaku automated four-circle diffractometer, Cu K_α radiation (l = 1.54178 Å), graphite monochromator. C$_{28}$H$_{38}$O$_6$ (470.60), crystal dimensions 0.40 × 0.30 × 0.30 mm, orthorhombic, space group p212121, 296 K, a = 13.826 (2), b = 24.389 (4), c = 7.476 (2), Å, V = 2521.0 (8) Å^3, Dc = 1.240g/cm^3, Z = 4, F(000) = 1016, μ = 6.94 cm^{-1}. A total of 2443 reflections were collected in the 6° < 2θ < 126.4° range using ω-2θscan. 2207 were assumed as observed (Fo > 4σ(Fo). Lorentz, polarization effects and absorption correlations were applied. Three standard reflections monitored every 97 reflections indicated no significant intensity variation. The structure was solved by direct methods (SIR92, Altomare et al., 1994). Hydrogen atoms were set in calculated positions and fixed at their positions. The structure was refined by full-matrix least-squares using anisotropic thermal parameters for all non-hydrogen atoms. The refinement converged to R = 0.047, R$_w$ = 0.072, GOF = 1.31 and a final difference map revealed no peaks greater than 0.27 e-/Å3. Complete details of the structure investigation are available upon request from the Cambridge Crystal Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.

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