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Agariblazeispirols A and B, an unprecedented skeleton from the cultured mycelia of the fungus, *Agaricus blazei*

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Abstract—Agariblazeispirols A and B, which have a unique steroidal skeleton, have been isolated from the cultured mycelia of *Agaricus blazei* (Agaricaceae). The absolute structure of Agariblazeispirol A was established to be (20*S*,22*R*,23*R*,24*S*)-13β,22:22,25-diepoxy-5-methoxy-14β-methyl-18-nor-des-*A*-ergosta-5,7,9,11-tetraen-23-ol by extensive 1D and 2D NMR spectral data, and X-ray analysis. The structure of Agariblazeispirol B was elucidated to be a stereoisomer of agariblazeispirol A at its carbon 22, (20*S*,22*S*,23*R*,24*S*)-13β,22:22,25-diepoxy-5-methoxy-14β-methyl-18-nor-des-*A*-ergosta-5,7,9,11-tetraen-23-ol by comparison of extensive 1D and 2D NMR spectral data with those of agariblazeispirol A. Both compounds showed a moderate circumvention of drug resistance on mouse leukemia P388/VCR cells.

Agaricus blazei is known as a precious fungus which produces many bioactive compounds in its fruiting bodies. The polysaccharides obtained from it have been revealed to have immuno-stimulating activities.[1–4] Ergosterol derivatives showed cytotoxity,[5] and linoleic acid derivatives were suggested to have antimutagenic and bactericidal effects.[6] In our previous papers we reported the isolation and structure elucidation of many blazeispirol derivatives, which have an unprecedented skeleton, from the cultured mycelia of *Agaricus blazei*.[7–11] In further investigation of the same source, we found two new unique skeletal compounds, Agariblazeispirols A (**1a**) and B (**2**), named by their fungus name and structure. Their structures were established by spectral data and X-ray analysis. As described in the previous paper,[11] the methanol extract of the mycelia of *A. blazei* cultured in a medium of 65.8 L was partitioned between chloroform and water. The $CHCl_3$ extract (18.86 g) was chromatographed on silica gel using a gradient solvent of toluene–AcOEt (0–100%). In a previous paper,[11] we reported the isolation and structure determination of blazeispirane and protoblazeispirane derivatives.

In this paper we describe the structure elucidation of two new additional skeletal compounds, agariblazeispirols A (**1a**) and B (**2**).

Agariblazeispirol A (**1a**),[12] colorless needles, mp 122.5–126°C (73.1 mg: 0.39% yield from the $CHCl_3$ extract), $[\alpha]_D^{30}$ +139.4° (*c* 0.33, $CHCl_3$), had the molecular formula $C_{25}H_{34}O_4$ as shown by the high-resolution EI mass spectrum. The presence of a hydroxyl group was indicated from its IR absorption at 3500 cm^{-1}. Its ^{13}C NMR spectrum showed 25 carbons, and the DEPT spectrum suggested the presence of seven methyls, two methylenes, eight methines, and eight quaternary carbons similarly to blazeispirol A.[7,8] Four oxygenated carbon signals were seen at δ 73.9, 82.1, 95.2 and 115.8; three of them were quaternary carbons. The remaining one was proved to be a hydroxyl methine carbon by the correlation with the proton signal at δ 4.08. Two carbon signals (δ 73.9 and 82.1) were identical with those of C-23 and C-25 (δ 75.1 and δ 82.1) of blazeispirol B which is a stereoisomer of blazeispirol A at C-22. The remaining two carbon signals (δ 95.2 and 115.8) were suggested to be C-14 and C-22 and were observed shifted downfield at 8 to 11 ppm relative to those of C-14 and C-22 in blazeispirols A and B.[10] These data indicate that **1a** should have a diepoxy moiety similar to that of blazeispirols A and B but not the same. In the NOESY spectra of **1a**, NOEs were observed between methyl proton H-19 (δ 2.20) and

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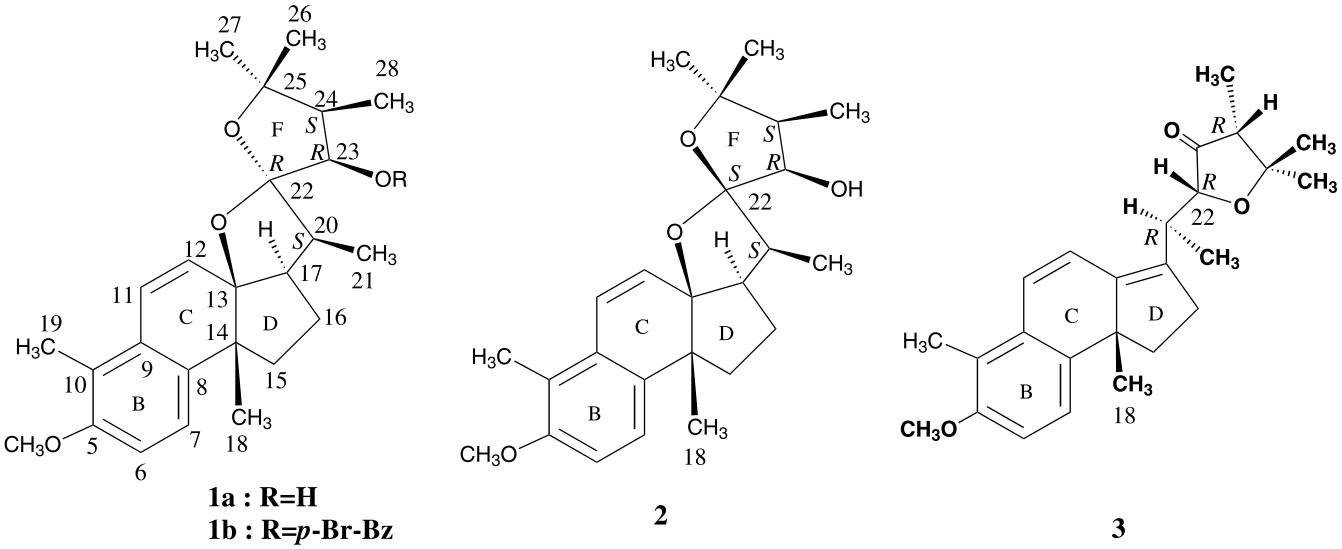

Figure 1. Structures of agariblazeispirols A (**1a**), A *p*-bromobenzoate (**1b**), B (**2**) and **3**.

H-11 (δ 6.60), and H-11 and H-12 (δ 6.34), methoxy methyl proton (δ 3.80) and H-6 (δ 6.70), H-6 and H-7 (δ 7.16), respectively. These results lead to the conclusion that the rings B and C in **1a** have a 5,7,9,11-tetraene structure the same as blazeispirol A. In the HMBC spectrum, the proton signal H-11 showed correlations with oxygenated carbon (δ 95.2: ^3J) that was deduced to be C-13. Proton signals at both H-7 and H-12 had correlation to the quaternary carbon (δ 51.2: ^3J) that revealed it was C-14. Depending on the evidence of HMBC spectral data that the methyl proton signal (δ 1.16) was correlated with three carbons (δ 51.2, 95.2 and 134.0) supposed to be C-14, C-13 and C-8, its position was considered to be shifted from C-13 to C-14 and assigned to be an 18-methyl group. The very deshielded oxygenated carbon (δ 115.8) showed correlation with H-17 (δ 2.39) and H-21 (δ 1.01) in the HMBC spectrum.

From these NMR data we are led to the conclusion that **1a** had an 18-nor-des-*A*-ergostane-type structure possessing 13,22:22,25-diepoxy. And the relative structure of **1a** was determined as 13,22:22,25-diepoxy-5-methoxy-14-methyl-18-nor-des-*A*-ergosta-5,7,9,11-tetraen-23-ol.

In order to determine the absolute structure of **1a**, X-ray analysis[13] was performed using *p*-bromobenzoate[14] of it (**1b**) as shown in Figure 2.

The result of X-ray analysis of **1b** confirmed our data analysis and established its absolute structure as (20*S*,22*R*,23*R*,24*S*)-13β,22:22,25-diepoxy-5-methoxy-14β-methyl-18-nor-des-*A*-ergosta-5,7,9,11-tetraen-23-ol.

We were extremely interested to find the structure of the E ring of **1a** comprised of not a 14β,22-epoxy moiety (pyran ring) as observed in other blazeispirol derivatives but a 13β, 22-epoxy moiety (furan ring) hardly found in other widespread sterol derivatives.

Agariblazeispirol B (**2**),[15] colorless powder (83.7 mg: 0.44% yield from the $CHCl_3$ extract), $[\alpha]_D^{22}$ +33.8° (*c* 0.13, $CHCl_3$), had also the molecular formula $C_{25}H_{34}O_4$ as shown by the high-resolution EI mass spectrum. The presence of a hydroxyl group was indicated from its IR absorption at 3440 cm^{-1}. Its ^{13}C NMR spectrum showed 25 carbons, and the DEPT spectrum suggested the presence of seven methyls, two methylenes, eight methines, and eight quaternary carbons like that of agariblazeispirol A. Four oxygenated carbon signals were seen at δ 83.6, 84.8, 94.0 and 114.8, three of which were quaternary carbons. The fourth was a hydroxyl methine carbon which was supported by the presence of a proton signal at δ 3.98. One of the quaternary carbons appeared very much deshielded at δ 114.8, reminiscent of an acetal system. As shown in Table 1, ^{13}C NMR data from C-5 to C-17 and C-19 carbons of **2** were almost identical with that of **1a** except for the carbon signal data of the spiroketal moiety. These data suggested that **2** was a stereoisomer of agariblazeispirol A (**1a**) derived from the spiroketal structural moiety. To clarify the stereochemistry, the NOESY data for compound (**2**) were compared in detail to that of agariblazeispirol A (**1a**) of which the orientation of C-22 has been determined to be *R*-configuration by X-ray analysis (Fig. 2). In the NOE experiment, both **1a** and **2** were observed on the same NOEs of the protons connected to the rings B, C, D and E as shown in Figure 3. On the contrary, the NOEs of the protons connected to the ring F were observed to have quite different cross-peaks. These results indicate that the orientation of C-22 of agariblazeispirol A and B is different. In the ring F of agariblazeispirol A, cross-peaks were observed for the following signals: OH-23/CH_3-18, H-23/CH_3-21 and H-15β. On the contrary in agriblazeispirol B, cross-peaks were observed only for the following signals: H-23/H-12 and H-20 instead of the above NOEs in agariblazeispirol A. From these results, the C-22 orientation of agariblazeispirol B has been determined to be *S*-configuration. Thus, the struc-

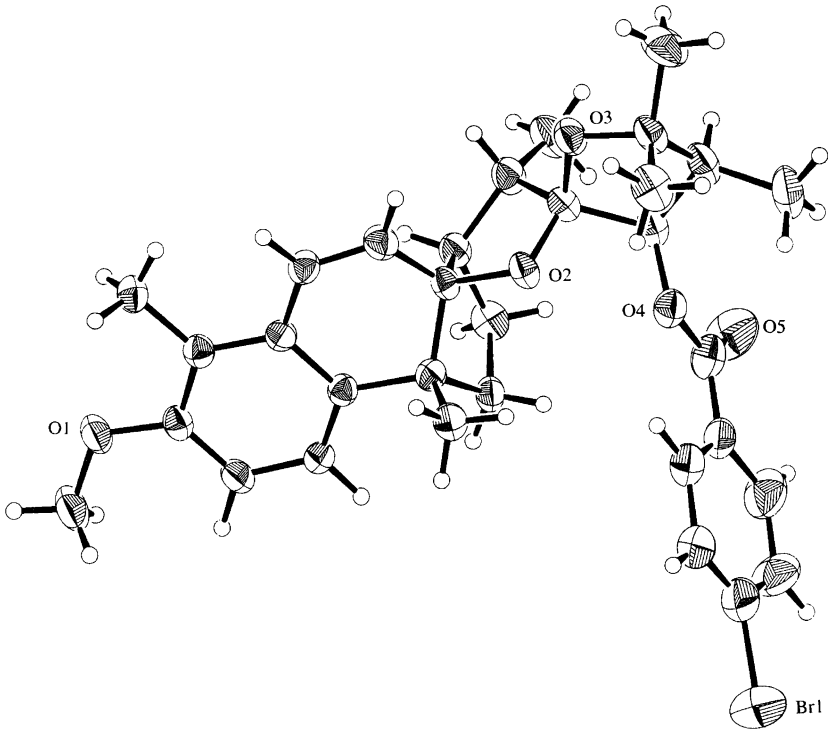

Figure 2. ORTEP drawing of **1b**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of **1a** and **2**, and HMBC spectral data of **1a**

Position	**1a**			**2**	
	δ_H[a]	δ_C[a]	^{1}H–^{13}C long-range correlations[b]	δ_H[a]	δ_C[a]
5	–	156.2		–	155.9
6	6.70 d (8.5)	109.2	C-5, C-8, C-10,	6.70 d (8.5)	109.2
7	7.16 d (8.5)	122.5	C-5, C-9, C-14	7.19 d (8.5)	122.6
8	–	134.0		–	134.6
9	–	131.4		–	131.4
10	–	122.5		–	122.3
11	6.60 d (10)	122.4	C-9, C-10, C-12, C-13	6.56 d (10)	122.6
12	6.34 d (10)	133.9	C-9, C-14	5.90 d (10)	134.7
13	–	95.2		–	94.0
14	–	51.2		–	51.3
15	2.42 dd (13.5, 8)	36.9	C-13, C-14, C-16, C-17	2.21 dd (12, 8)	36.1
	1.94 ddd (13.5, 10.5, 9.5)		C-8, C-14, C-16	2.29 dd (12, 8)	
16	1.38 dddd (13.5, 11, 10.5, 8)	21.8	C-13, C-15, C-17,	1.28 m	23.0
	1.62 ddd (13.5, 9.5, 4.5)		C-13, C-14, C-15, C-17, C-20	1.79 ddd (13, 8, 3)	
17	2.39 ddd (11, 7, 4.5)	55.2	C-15, C-20, C-21, C-22	2.34 ddd (11, 7, 3)	56.6
18	1.16 s	23.1	C-12, C-13, C-14, C-15	1.11 s	22.8
19	2.20 s	11.1	C-5, C-9, C-10	2.20 s	11.1
20	2.72 qd (7, 7)	45.4	C-16, C-17, C-21, C-22, C-23	2.63 dq (7, 7)	40.8
21	1.01 d (7)	10.8	C-17, C-20, C-22	1.08 d (7)	12.2
22	–	115.8		–	114.8
23	4.08 dd (6.5, 5.5)	73.9	C-20, C-22, C-25, C-28	3.98 dd (4, 4)	84.8
24	1.89 qd (7, 6.5)	46.8	C-22, C-23, C-25, C-26, C-27	2.39 dq (7, 4)	44.4
25	–	82.1		–	83.6
26	1.26 s	24.6	C-24, C-27	1.16 s	25.8
27	1.23 s	29.7	C-24, C-25, C-26	1.36 s	30.5
28	1.07 d (7)	9.3	C-23, C-24, C-25	1.02 d (7)	8.4
OCH_3	3.80 s	55.5	C-5	3.80 s	55.5
OH	3.00 d (5.5)	–	C-22, C-23, C-24	1.42 d (4)	–

[a] Spectra obtained in $CDCl_3$ referenced to $CHCl_3$ at δ 7.26 (^{1}H) and 77.0 (^{13}C).
[b] ^{1}H–^{13}C long-range correlations from HMBC.

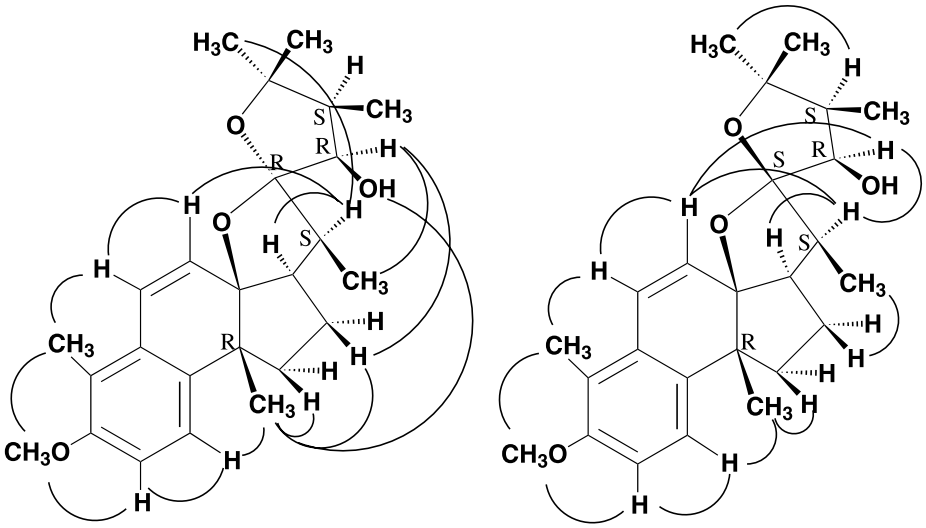

Figure 3. NOESY interaction in agariblazeispirols A (**1a**) and B (**2**).

ture of **2** was determined to be (20*S*,22*S*,23*R*,24*S*)-13β,22:22,25-diepoxy-5-methoxy-14β-methyl-18-nor-des-*A*-ergosta-5,7,9,11-tetraen-23-ol.

These results suggest that agariblazeispirols A and B were biosynthesized derived from blazeispirol A along with cleavage of the diepoxyl structure and rearrangement of methyl from C(13) to C(14) with formation of a furan ring. In order to clarify this suggestion, we carried out reaction of blazeispirol A with $BF_3{\cdot}Et_2O$.[16,17] After working up, many kinds of compounds were detected on TLC. After purification by HPLC, compound (**3**) was isolated. Compound (**3**),[18] colorless powder (7.0 mg: 7.3% yield), $[\alpha]_D^{28}$ +269.6° (*c*, 0.11, $CHCl_3$); had the molecular formula $C_{25}H_{32}O_3Na$ as shown by the high-resolution FAB mass spectrum. The structure was determined to be (20*R*,22*R*,24*R*)-22,25-epoxy-5-methoxy-14β-methyl-18-nor-des-*A*-ergosta-5,7,9,11,13(17)-pentaen-23-one by 1D and 2D NMR spectral data as shown in Figure 1. The result supports that agariblazeispirols A and B should be biosynthesized derived from blazeispirol A. Plausible synthetic and biosynthetic mechanisms are shown in Figure 4.

Agariblazeispirols A (**1a**) and B (**2**) showed no cytotoxicity on P388/S cells and P388/VCR cells at a concentration of 6.3 μg/ml. However, these compounds showed growth inhibitory activity on P388/VCR at concentrations of 2.4 and 4.7 μg/ml for **1a** and **2**, respectively, in the presence of 13 ng/ml of vincristine, which did not possess the growth inhibitory activity on P388/VCR cells. Therefore, it is considered that these compounds can overcome multidrug resistance of mouse leukemia P388/VCR cells.[19]

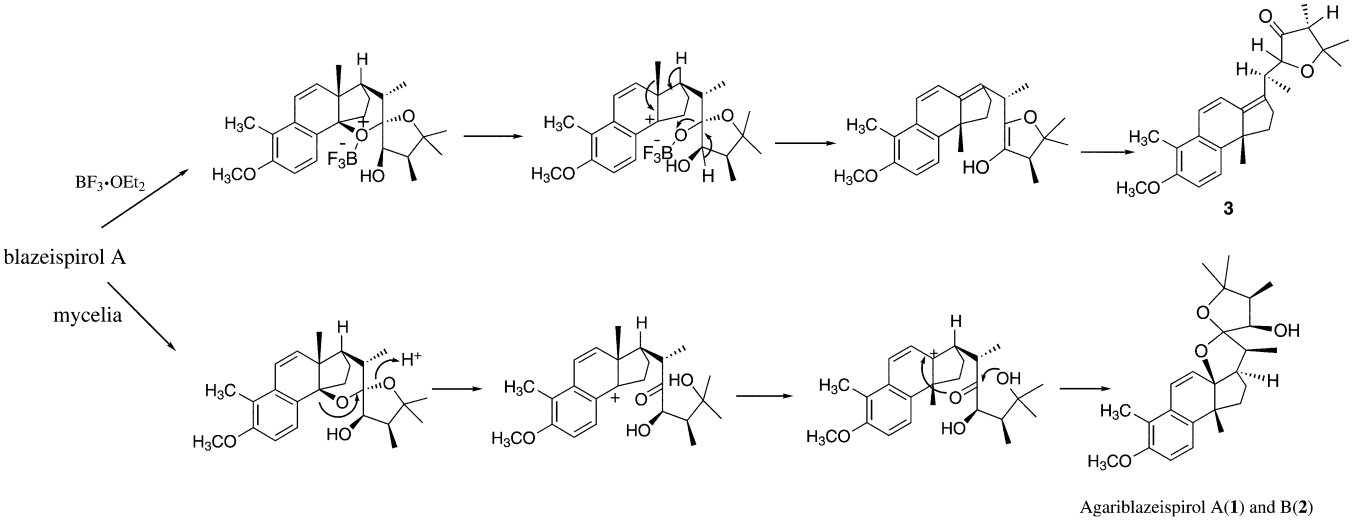

Figure 4. Plausible synthetic and biosynthetic mechanisms of compounds (**1**), (**2**) and (**3**) from blazeispirol A.

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12. **1a**: Colorless needles, mp 122.5–126°C, $[\alpha]_D^{30}$ +139.4° (*c* 0.33, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 229 (4.50), 260 (3.99), 268 (4.07), 278 (3.97), 305 (3.32). HREIMS *m*/*z*: 398.2472 $[M]^+$ ($C_{25}H_{34}O_4$ requires 398.2457). IR ν_{max} (KBr) cm^{-1}: 3500, 2970, 2930, 1580, 1480, 1260, 1020. EI-MS *m*/*z* (ret. int.): 398 $[M]^+$ (57), 380 $[M-H_2O]^+$ (6), 299 (50), 225 (76), 211 (38), 202 (47), 199 (36), 149 (44), 28 (100).
13. Single crystal X-ray analysis of (**1b**). Data were collected on an AFC5R Rigaku automated four-circle diffractometer, Cu Kα radiation (l=1.54178 Å), graphite monochromator. $C_{32}H_{37}O_5Br$ (581.55), crystal dimensions 0.20×0.30×0.50 mm, orthorhombic, space group $P2_12_12_1$, 296 K, a=19.094(4), b=21.172(3), c=7.436(2) Å, V=3006(1) $Å^3$, D_{calcd}=1.539g/cm^3, Z=4, $F(000)$=1422, μ=30.52 cm^{-1}. A total of 21685 reflections were collected in the 6°<2σ<135.2° range using ω–2θ scan. 10530 reflections were unique, and from these, 7193 were assumed as observed (F_o>5σ(F_o). 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).[20] 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=0.079, R_w=0.213, GOF=1.05 and a final difference map revealed no peaks greater than 0.18 e/$Å^3$. The absolute configuration was determined by the flack parameter (0.03). Additional crystallographic details, CCDC 210742 (atomic coordinates and equivalent isotopic displacement coefficients) have been deposited at the Cambridge Crystal Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK [fax:+44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].
14. Synthesis of agariblazeispirol A *p*-bromobenzoate (**1b**). A mixture of agariblazeispirol A (**1a** 30.5 mg), *p*-bromobenzoylchloride (200 mg) and pyridine(2.0 ml) was stirred at 95°C for 20 h. After being worked up, **1b** was recrystallized from *n*-hexane to afford 14.2 mg as colorless plates. **1b**: mp 147.5–148°C (found: C, 66.10; H, 6.42. $C_{32}H_{37}O_5Br$ requires C, 66.09; H, 6.41).
15. **2**: Colorless powder, $[\alpha]_D^{22}$ +33.8°(*c* 0.13, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 227 (4.12), 258 (3.46), 267 (3.60), 276 (3.48). IR ν_{max} (KBr) cm^{-1}: 3440, 2960, 2920, 1580, 1470, 1380, 1260, 1100, 970. HREIMS *m*/*z*: 398.2462 $[M]^+$ ($C_{25}H_{34}O_4$ requires 398.2457). EI-MS *m*/*z* (ret. int.): 398 $[M]^+$ (100), 380 $[M-H_2O]^+$ (22), 365 $[M-H_2O-CH_3]^+$ (6), 352 (12), 325 (8), 299 (7), 283 (12), 279 (10), 253 (10), 244 (36), 225 (83), 202 (45), 199 (31), 178 (29), 149 (27), 81 (25).
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17. Synthesis of **3**. A solution of blazeispirol A (100 mg; 0.25 mmol) in $CHCl_3$ (50 ml) was treated with $BF_3{\cdot}Et_2O$ (260 μl) and stirred for 1 h. And then the reaction was stopped by addition of Et_3N (200 μl). After workup, compound **3** was isolated using HPLC.
18. **3**: Colorless powder, $[\alpha]_D^{28}$ +269.6° (*c* 0.11, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 232 (3.91), 307 (3.94), 316 (3.93). IR ν_{max} (KBr) cm^{-1}: 2960, 2950, 1755, 1460, 1370, 1260, 1100, 970. HRFABMS *m*/*z* 403.2238 $[M+Na]^+$ ($C_{25}H_{32}O_3Na$ requires 403.2250). EIMS *m*/*z* (rel. int.) 380 $[M]^+$ (12), 365 $[M-CH_3]^+$ (80), 253 (93), 238 (100), 223 (11), 211 (10), 149 (12), 127 (6), 97 (6). ^1H NMR (400 MHz, $CDCl_3$): δ 1.06 (3H, *d*, *J*=7, H-21), 1.07 (3H, *d*, *J*=7, H-28), 1.16 (3H, *s*, H-18), 1.24 (3H, *s*, H-26), 1.37 (3H, *s*, H-27), 2.03 (1H, *ddd*, *J*=11, 10.5, 8.5, H-15β), 2.25 (3H, *s*, H-19), 2.26 (H, *dq*, *J*=7, 1, H-24), 2.30 (1H, *dd*, *J*=11, 6.5, H-15β), 2.45 (1H, *dd*, *J*=16.5, 8.5, H-16β), 2.54 (1H, *ddd*, *J*=16.5, 10.5, 6.5, H-16β), 2.99 (1H, *dq*, *J*=7, 7, H-20), 3.80 (3H, s, CH_3O), 3.97 (1H, *dd*, *J*=7, 1, H-22), 6.48 (H, *d*, *J*=10, H-12), 6.70 (1H, *d*, *J*=10, H-11), 6.72 (1H, *d*, *J*=8.5, H-6), 6.97 (1H, *d*, *J*=8.5, H-7), ^{13}C NMR (100 MHz, $CDCl_3$): δ 10.4 (C-28), 11.0 (C-19), 15.6 (C-21), 24.4 (C-26), 27.7 (C-18), 28.4 (C-27), 30.4 (C-16), 35.4 (C-20), 38.7 (C-15), 49.5 (C-14), 51.3 (C-24), 55.8 (CH_3O), 79.9 (C-25), 81.0 (C-22), 109.4 (C-6), 121.0 (C-12), 122.2 (C-10), 122.4 (C-7), 123.9 (C-11), 131.5 (C-9), 139.1 (C-8, C-13), 140.4 (C-17), 155.7 (C-5), 217.6 (C-23).
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