Hirseins A and B, Daphnane Diterpenoids from *Thymelaea hirsuta* That Inhibit Melanogenesis in B16 Melanoma Cells

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Received January 7, 2009

Two new daphnane diterpenoids, hirseins A (**1**) and B (**2**), were isolated from the aerial parts of *Thymelaea hirsuta*, and their structures were elucidated on the basis of spectroscopic data interpretation. Hirsein B (**2**) is an unusual daphnane in possessing a coumaroyl moiety. NOESY correlations of **2** implied that isomerization of the coumaroyl group in **2** was caused by equilibrium between the *E* (**2e**) and *Z* (**2z**) forms. Compounds **1** and **2** were found to inhibit melanogenesis in B16 murine melanoma cells.

During research on bioactive compounds extracted from Tunisian medicinal plants, we have focused on *Thymelaea hirsuta,* which belongs to the family Thymelaeaceae and is native to North Africa. In Tunisia, this plant has been used traditionally as an antiseptic and anti-inflammatory agent and for the treatment of hypertension by external application. It was previously reported that five daphnane diterpenoids were isolated from *T. hirsuta*,[1] and extracts of this species from Algeria showed antioxidant activity.[2] However, the chemical constituents of this plant and their bioactivity have not been investigated in recent years. It was found that an extract of *T. hirsuta* decreased the synthesized melanin content in B16 murine melanoma cells without cytotoxicity.[3] The bioassay-guided fractionation of this plant implied that the major bioactive components are daphnane diterpenoids. Daphnane diterpenoids have been found in plants of the families Thymelaeaceae and Euphorbiaceae.[4] These compounds are known to have various bioactivities, such as antileukemic and neurotrophic effects.[4−6] In this paper, we report on the structures of two new daphnane diterpenoids, hirseins A (**1**) and B (**2**), from *T. hirsuta*, and their antimelanogenesis activities.

The aerial parts of *T. hirsuta* (500 g) were extracted with MeOH. The MeOH extracts were partitioned between EtOAc and H_2O. The EtOAc-soluble portion (9.9 g) was subjected to silica gel column chromatography, separation through a C_{18} Sep-Pak cartridge, and reversed-phase HPLC to yield two new daphnane diterpenoids, hirseins A (**1**, 0.00044%) and B (**2**, 0.00032%).

Hirsein A (**1**), $[\alpha]^{25}_{D}$ +21 (*c* 1.0, MeOH), showed a pseudomolecular ion peak at *m/z* 671 $(M + Na)^+$ in the ESIMS. The molecular formula of **1** was deduced as $C_{37}H_{44}O_{10}$ from HRESIMS [*m/z* 671.2810 $(M + Na)^+$, Δ −2.2 mmu]. The IR spectrum indicated the presence of hydroxyl (3421 cm^{-1}), ester carbonyl (1700 cm^{-1}), and unsaturated carbonyl (1654 and 1637 cm^{-1}) groups, whereas the UV absorption at 236 nm implied that **1** possesses an α,β-unsaturated ketone. The gross structure of **1** was deduced by detailed analysis of the ^1H and ^{13}C NMR data (Table 1) aided by 2D NMR experiments ($^1H-^1H$ COSY, HMQC, and HMBC). The ^{13}C NMR data indicated that the molecule possesses one unsaturated carbonyl carbon, one ester carbonyl carbon, eight disubstituted olefin carbons, six aromatic carbons, one orthoester carbon, four oxymethines, one oxymethylene, three methines, four methylenes, four methyl groups, and four oxygenated quaternary carbons (Table 1). The HMBC correlations of H-19 to C-1, C-2, and C-3 revealed that a methyl group is attached at C-2. The $^1H-^1H$ COSY connectivities of H-1 and H-10 and HMBC correlations of H-1 to C-3 and C-4, and H-19 to C-1, C-2, and C-3, indicated the presence of a cyclopentenone moiety (ring A). The $^1H-^1H$ COSY connectivities of H-7 and H-8 and the HMBC correlations of H-5 to C-4, C-6, and C-7, H-7 to

Table 1. ^1H and ^{13}C NMR Data (500 MHz, $CDCl_3$) of Hirsein A (**1**)

position	δ_C	δ_H, mult. (*J* in Hz)	HMBC[a]
1	160.4, CH	7.59, d (2.3)	C-3, 4, 10
2	136.9, C		
3	209.5, C		
4	72.2, C		
5	72.1, CH	4.26, s	C-4, 6, 7
6	60.5, C		
7	64.4, CH	3.63, s	C-6, 8
8	35.5, CH	3.66, brs	C-7, 9, 14
9	78.3, C		
10	47.5, CH	3.92, d (2.7)	
11	44.2, CH	2.50, q (7.3)	C-12, 13, 18
12	78.6, CH	5.10, s	C-9, 13, 14, 18, 1″
13	84.2, C		
14	80.8, CH	4.91, d (2.6)	C-9, 1′
15	142.9, C		
16	113.7, CH_2	5.02, s	C-13, 17
17	18.8, CH_3	1.87, s	C-13, 15, 16
18	18.2, CH_3	1.38, d (7.3)	C-9, 12
19	9.84, CH_3	1.78, d (1.4)	C-1, 2, 3
20a	65.1, CH_2	3.96, d (12.4)	C-7
20b	65.1, CH_2	3.80, d (12.4)	C-6
1′	117.8, C		
2′	128.1, CH	6.16, m	C-1′
3′	145.9, CH	6.16, m	C-2′, 4′
4′	33.0, CH	2.15, m	C-2′, 3′
5′	31.3, CH	1.43, m	C-4′
6′	28.3, CH_2	1.29, m	C-7′
7′	22.4, CH_2	1.29, m	C-6′
8′	13.9, CH_2	0.89, m	C-6′, 7′
1″	166.0, C		
2″	118.1, CH	5.74, d (15.2)	C-1″, 3″, 5″, 9″
3″	146.3, CH	7.20, m[b]	
4″	135.3, C		
5″	128.0, CH	7.71, m	C-7″
6″	128.6, CH	7.38, m	C-4″
7″	129.6, CH	7.38, m	
8″	126.0, CH	7.38, m	C-4″
9″	128.0, CH	7.71, m	C-7″

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] The signal of H-3″ was overlapped with that of $CHCl_3$.

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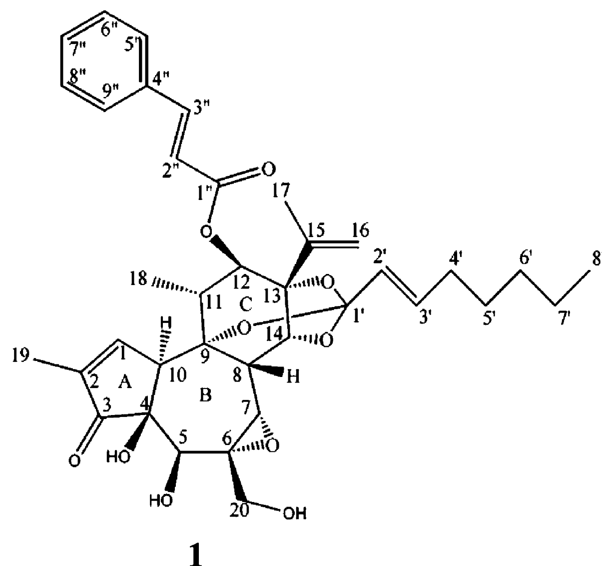
10.1021/np800808h CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/13/2009



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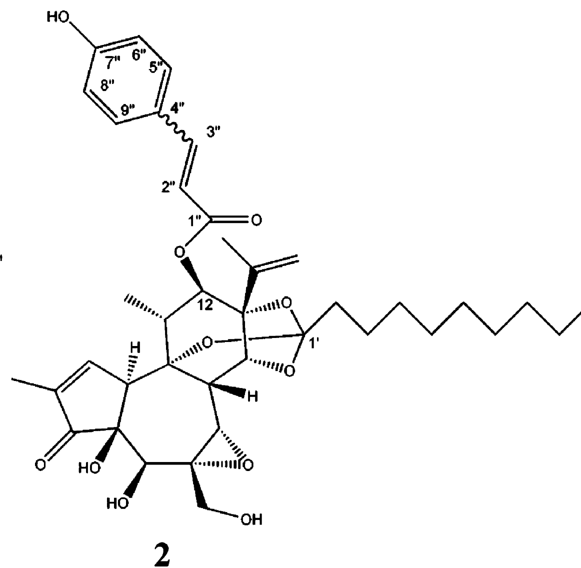

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C-6 and C-8, and H-8 to C-7 and C-9 suggested the presence of a cycloheptane moiety (ring B). The HMBC correlations of H-20 to C-6 and C-7 suggested that an oxymethylene group is attached to C-6. The $^1H-^1H$ COSY connectivities between H-14 and H-8 and the HMBC correlations of H-8 to C-9, H-11 to C-12 and C-13, H-12 to C-9, C-13, and C-14 indicated the presence of a cyclohexane moiety (ring C). The $^1H-^1H$ COSY connectivities between H-11 and H-18 revealed a methyl carbon connected to C-11. Attachment of an isoprenyl moiety to C-13 was indicated by HMBC correlations of H-16 to C-13 and C-17 and H-17 to C-13, C-15, and C-16. The $^1H-^1H$ COSY connectivities between H-8 and H-14 and an HMBC correlation of H-11 to C-9 indicated that ring B is connected to ring C, and the $^1H-^1H$ COSY connectivity between H-1 and H-10 and the HMBC correlations of H-5 to C-3 and C-4 suggested ring B is attached to ring A. The HMBC correlation of H-12 to C-1″ indicated that the ester carbonyl (C-1″) is connected to C-12. From the chemical shifts of the ^{13}C NMR and DEPT spectra of **1**, it was revealed that C-14 is an oxymethine and C-9 and C-13 are oxygenated quaternary carbons. The $^1H-^1H$ COSY connectivities of H-2′ to H-3′, H-4′ to H-8′ and an HMBC correlation of H-4′ to C-3′ revealed the presence of a 2-octene moiety. The *E*-geometry of the disubstituted double bond at C-2′-C-3′ was deduced from the carbon chemical shifts of allylic carbons (C-4′, δ_C 33.0).[7] The $^1H-^1H$ COSY connectivities of H-2″ to H-3″ and H-5″ to H-9″ and the HMBC correlations of H-2″ to C-5″ and C-9″ suggested the occurrence of a cinnamate moiety. The *E*-geometry of the disubstituted double bond at C-2″−C-3″ was deduced from the $^1H-^1H$ coupling constants ($J_{2'',3''}$ 15.2 Hz). When compared with NMR data of known compounds, it was suggested that C-1′ is an orthoester carbon.[8] The HMBC correlations of H-14 and H-2′ to the orthoester carbon (C-1′) showed that the orthoester was formed by a 2-octenoic acid unit and three hydroxyl groups at C-9, C-13, and C-14. The HMBC correlations of H-2″ and H-12 to the ester carbonyl carbon C-1″ (δ_C 166.0) indicated that the cinnamate is connected to C-12. Therefore, hirsein A (**1**) was assigned as 5β-hydroxyresiniferonol-6α,7α-epoxy-12β-cinnamoyloxy-9,13,14-*ortho*-2*E*-octanoate.

Hirsein B (**2**) was obtained as a mixture of isomers. Because it was difficult to separate each compound, the structure of **2** was analyzed as a mixture. Hirsein B (**2**), $[\alpha]^{25}_D$ +42 (*c* 1.0, MeOH), showed a pseudomolecular ion peak at *m/z* 695 $(M + H)^+$ in the ESIMS. The molecular formula of hirsein B (**2**) was deduced to be $C_{39}H_{50}O_{11}$ by HRESIMS [*m/z* 695.3395 $(M + H)^+$, Δ −3.6 mmu]. The IR spectrum indicated the presence of hydroxyl (3629 cm^{-1}), ester carbonyl (1700 cm^{-1}), and unsaturated carbonyl (1627 and 1604 cm^{-1}) groups, whereas the UV absorption at 229 nm implied that **2** possesses an α,β-unsaturated ketone. The gross structure of hirsein B (**2**) was deduced by a detailed analysis of the ^1H and ^{13}C NMR data (Table 2) aided by 2D NMR experiments ($^1H-^1H$ COSY, HMQC, and HMBC). The ^1H and ^{13}C NMR spectra were similar to those of other compounds based on a daphnane skeleton.[5] A $^1H-^1H$ coupling constant ($J_{5'',6''}$ 8.5 Hz) showed that **2** possesses a disubstituted benzene ring. The HMBC correlations of H-3″ to C-1″, H-5″ to C-3″, H-12 to C-1″ and the molecular formula of **2** revealed a coumaroyl group connected to C-12. From the $^1H-^1H$ coupling constants ($J_{e2',e3'}$ 15.9 Hz and $J_{z2',z3'}$ 8.5 Hz), hirsein B (**2**) was concluded to be a mixture of isomers containing *E*- and *Z*-coumaroyl moieties. The HMBC correlations of H-14 and H-2′ to C-1′ indicated that the orthoester was formed by a decanoate and three hydroxyl groups at C-9, C-13, and C-14. The relative stereochemistry of **2** was deduced from the NOESY spectrum (Figure 1). The NOESY correlations of H-8 to H-11 and H-14, indicated that the six-membered ring (ring C) of compound **2** was fixed in a boat conformation by a 1,4,5-triaxially connected orthoester group at C-9, C-13, and C-14. The H-12 signal appeared as a broad singlet because of a 90° dihedral angle with two vicinal protons (H-12 and H-11), indicating that the ester group connected to C-12 was β-oriented. The NOESY correlations of H-5 to H-10 indicated that these protons should be α-oriented, and that the hydroxyl groups of C-4 and C-5 are in a β-orientation. In the NOESY spectrum of **2**, H-7 exhibited a correlation with H-20b, indicating that the oxymethylene group at C-6 and H-7 are β-oriented, whereas the epoxy group at C-6 and C-7 is in the α-position. In hirsein B-z (**2z**), the NOESY correlations of H-5″ to H-8, H-6″ to H-7, and H-2″ to H-16 implied that the coumaroyl group is folded above ring B (Figure 1). This suggested that the *Z*-coumaroyl group above ring B has an anisotropic effect on ring B; thus, the chemical shifts of protons on ring B shifted to a higher field. The estimated relative stereochemistry indicated the presence of a hydrogen bond between a hydroxyl group of the coumaroyl and a hydroxyl group at C-5 (Figure 1). On the other hand, in hirsein B-e (**2e**), no NOESY correlations between the *E*-coumaroyl group and ring B were observed, indicating that the *E*-coumaroyl group of hirsein B-e (**2e**) was that outside of the main skeleton (Figure 1). This implied that the isomerization in the coumaroyl group of hirsein B (**2**) was caused by equilibrium between the thermodynamic stabilization in hirsein B-e (**2e**) and the electrostatic stabilization of the hydrogen bond between the hydroxyl groups of the coumaroyl and C-5 in hirsein B-z (**2z**). Consequently, hirsein B (**2**) was assigned as 5β-hydroxyresiniferonol-6α,7α-epoxy-12β-coumaroyloxy-9,13,14-*ortho*-decanoate.

In a previous paper, we reported that *T. hirsuta* extracts decreased the synthesized melanin content in B16 murine melanoma cells.[3] To determine the inhibitory effect of hirseins A (**1**) and B (**2**) on

Table 2. ^{1}H and ^{13}C NMR Data (500 MHz, $CDCl_3$) of Hirsein B (**2e** and **2z**)

	hirsein B-e (**2e**)		hirsein B-z (**2z**)	
position	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
1	161.5	7.55, brs	160.6	7.55, brs
2	136.7		136.4	
3	209.7		209.6	
4	72.1		72.1	
5	72.0	4.28, s	72.0	4.23, s
6	60.3		58.4	
7	68.3	3.55, s	66.4	3.22, s
8	35.4	3.49, brs	35.4	2.84, d (2.3)
9	78.3		78.3	
10	47.7	3.75, d (2.8)	47.5	3.67, d (2.7)
11	44.3	2.44, q (7.3)	44.0	2.29, m
12	79.6	5.05, s	79.6	4.87, s
13	83.6		83.6	
14	80.3	4.74, d (2.3)	80.3	4.17, d (2.4)
15	143.1		142.7	
16a	113.5	4.99, s	113.4	4.92, s
16b	113.5	4.97, s	113.4	4.71, s
17	18.7	1.83, s	18.7	1.68, s
18	18.3	1.31, d (7.3)	18.1	1.31, d (7.3)
19	9.86	1.68, m	9.81	1.68, m
20a	65.5	4.45, d (12.3)	64.6	3.96, m
20b	65.5	3.75, m	64.6	3.39, m
1′	119.9		119.4	
2′	34.8	1.91, m	34.6	1.80, m
3′	23.2	1.25, m	22.6	1.25, m
4′	23.2	1.25, m	22.6	1.25, m
5′	23.2	1.25, m	22.6	1.25, m
6′	23.2	1.25, m	22.6	1.25, m
7′	23.2	1.25, m	22.6	1.25, m
8′	23.2	1.25, m	22.6	1.25, m
9′	23.2	1.25, m	22.6	1.25, m
10′	14.1	0.87, m	14.1	0.87, m
1″	166.1		165.9	
2″	115.8	6.21, d (15.9)	114.7	5.78, d (12.2)
3″	145.4	7.55, d (15.9)	144.6	7.06, d (12.2)
4″	127.6		126.6	
5″	130.6	7.42, d (8.5)	130.1	7.15, d (8.4)
6″	115.8	6.90, d (8.5)	114.9	6.83, d (8.4)
7″	157.1		157.1	
8″	115.8	6.90, d (8.5)	114.9	6.83, d (8.4)
9″	130.6	7.42, d (8.5)	130.1	7.15, d (8.4)

the melanogenesis and cell viability of B16 cells, the melanin content and cell viability were determined. B16 cells treated with **1** or **2** had a significantly lower melanin content than the control (Figure 2). Hirsein A (**1**) at 0.1 μM reduced the melanin content by 37%, whereas the positive control, arbutin at 100 μM, reduced the melanin content by 33%. On the other hand, hirsein B (**2**) reduced the melanin content by 26% compared with untreated cells. Guava PCA results comparing the viability of control cells with treated cells were 99.3% (for **1**) and 97.5% (for **2**). These results indicate that hirseins A (**1**) and B (**2**) inhibited melanogenesis without any significant reduction in cell viability. The viability results suggest that the reduction in melanin content is most likely due to mechanisms that inhibit the synthesis or activity of melanogenesis enzymes rather than by cell death or cytotoxicity.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco DIP-370. UV spectra were recorded on a HITACHI U-2000A spectrometer. IR spectra were recorded on a JASCO FT/IR-300 spectrometer. ^{1}H and ^{13}C NMR spectra were measured and recorded on a Bruker Avance 500 MHz spectrometer in $CDCl_3$. The resonances of $CDCl_3$ at δ_H 7.26 and δ_C 77.0 were used as internal references for NMR spectra. ESIMS were recorded on a JEOL JMS-T100LC mass spectrometer.

Plant Collection. The aerial part of the plant was collected from the Kasserine area of Tunisia in March 2006. The voucher specimen is maintained at Alliance for Research on North Africa (ARENA), Graduate School of Life and Environmental Sciences, University of Tsukuba. The leaves were dried in the shade at 25 °C.

Extraction and Isolation. Leaves (500 g) of *T. hirsuta* were extracted with MeOH (8 L) and evaporated to dryness in vacuo at 30 °C. The MeOH extract (28.5 g) was partitioned between EtOAc (500 mL × 3) and H_2O (500 mL). The EtOAc-soluble portion (9.9 g) was divided into 13 fractions using silica gel column chromatography (ϕ4.6 × 35 cm, $CHCl_3$/MeOH, 98:2 → 0:100). A fraction (3.91 g) eluted with $CHCl_3$/MeOH (95:5) was subjected to silica gel column chromatography (ϕ2.2 × 35 cm, $CHCl_3$/MeOH, 98:2 → 0:100). A fraction (704 mg) eluted with $CHCl_3$/MeOH (98:2) was applied to a C_{18} Sep-Pak cartridge (Waters, MeOH/H_2O, 1:1 → $CHCl_3$/MeOH, 1:6) and the fraction (138 mg) containing daphnane diterpenoids was further separated by reversed-phase HPLC (Inertsil ODS3, GL Sciences, ϕ1.0 × 25 cm, flow rate 2.0 mL/min; CH_3CN/H_2O, 13:7) to give hirsein A (**1**, 2.2 mg, t_R 37.5 min). A fraction (563 mg) that eluted with $CHCl_3$/MeOH (95:5) was then separated using a C_{18} Sep-Pak cartridge (Waters; MeOH/H_2O, 1:1 → $CHCl_3$/MeOH, 1:6) and the fraction (90 mg) containing daphnane diterpenoids was further purified by reversed-phase HPLC (Inertsil ODS3, ϕ1.0 × 25 cm, flow rate 2.0 mL/min; CH_3CN/H_2O, 13:7) to afford 17 fractions. Fraction 16 (6.0 mg) eluted with CH_3CN/H_2O (100:0) was purified by reversed-phase HPLC (Inertsil ODS3, ϕ4.6 × 250 mm, flow rate 1.0 mL/min; CH_3CN/H_2O, 3:1) to give hirsein B (**2**, 1.6 mg, t_R 27.3 min).

Hirsein A (1): colorless oil; $[\alpha]^{25}_D$ +21 (c 1.0, MeOH); IR (KBr) ν_{max} 3421, 1700, 1654, 1637, 1560, and 1081 cm^{-1}; UV (MeOH) λ_{max} 236 (ε 11200) and 260 (ε 13500) nm; ^{1}H and ^{13}C NMR and HMBC correlations (Table 1); ESIMS (positive ion) m/z 671 $(M + Na)^+$; HRESIMS (positive ion) m/z 671.2810 $(M + Na)^+$; (calcd for $C_{37}H_{44}O_{10}Na$, 671.2832).

Hirsein B (2): colorless oil; $[\alpha]^{25}_D$ +42 (c 1.0, MeOH); IR (KBr) ν_{max} 3629, 1700, 1627, 1604, 1153, and 939 cm^{-1}; UV (MeOH) λ_{max} 229 (ε 16 400) and 313 (ε 15 600) nm. ^{1}H and ^{13}C NMR (Table 2). HMBC correlations ($CDCl_3$, H/C) for hirsein B-e (**2e**): 1/3, 1/4, 1/10, 5/4, 5/6, 5/7, 7/8, 7/14, 11/12, 11/18, 12/9, 12/13, 12/14, 12/15, 12/18, 12/1″, 14/ 9, 14/1′, 16a/13, 16a/17, 16b/13, 16b/17, 17/13, 17/15, 17/16, 18/9, 18/11, 18/12, 19/1, 19/2, 19/3, 20a/7, 2′/1′, 2′/3′, 10′/9′, 2″/1″, 2″/4″, 3″/1″, 3″/5″, 3″/9″, 5″/3″, 5″/6″, 5″/7″, 5″/8″, 6″/4″, 6″/7″, 8″/4″, 8″/7″, 9″/3″, 9″/6″, 9″/7″, and 9″/8″. HMBC correlations ($CDCl_3$, H/C) for hirsein B-z (**2z**): 1/3, 1/4, 1/10, 5/4, 5/6, 5/7, 7/8, 7/14, 11/12, 11/18, 12/9, 12/13, 12/14, 12/15, 12/18, 12/1″, 14/ 9, 14/1′, 16a/13, 16a/17, 16b/13, 16b/17, 17/13, 17/15, 17/16, 18/9, 18/11, 18/12, 19/1, 19/2, 19/3, 20b/6, 2′/1′, 2′/3′, 10′/9′, 2″/1″, 2″/4″, 3″/5″, 3″/9″, 5″/6″, 5″/7″, 5″/8″, 6″/4″, 6″/7″, 8″/4″, 8″/7″, 9″/6″, 9″/7″, and 9″/8″. NOESY correlations ($CDCl_3$, H/H) for hirsein B-e (**2e**): 1/5, 1/10, 5/10, 7/8, 7/14, 8/11, 8/14, 11/18, 12/18 and 20b/8. NOESY correlations ($CDCl_3$, H/H) for hirsein B-z (**2z**): 1/5, 1/10, 5/10, 7/8, 7/14, 7/6″, 7/8″, 8/11, 8/14, 8/5″, 8/6″, 8/8″, 8/9″, 11/18, 12/18, 16/2″, and 20b/7. ESIMS (positive ion) m/z 695 $(M + H)^+$; HRESIMS (positive ion) m/z 695.3395 $(M + H)^+$ (calcd for $C_{39}H_{51}O_{11}$, 695.3431).

Cells and Cell Culture. B16 murine melanoma JCRB 0202 cells were obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained as a monolayer culture in Dulbecco's modified Eagle medium (DMEM) (Sigma, St Louis, MO), supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 50 U/mL penicillin, and 50 μg/mL streptomycin (Cambrex, East Rutherford, NJ) at 37 °C with 5% CO_2.

Cell Viability Assay. The ViaCount program of Guava PCA (GE Healthcare, Ltd., Amersham, Buckinghamshire, UK) was used to determine cell viability. B16 murine melanoma cells were cultured in DMEM in 100 mm Petri dishes at a density of 5 × 10^5 cells per plate. After overnight incubation, the medium was replaced with one containing either arbutin (positive control) or compounds **1** and **2** and incubation was continued for an additional 48 h. During the cell viability assay, the medium was removed and the cells washed two times with phosphate-buffered saline (PBS) and harvested by trypsinization. The cells were then resuspended in DMEM. An aliquot of the cells, based on the cell density, was stained with ViaCount reagent (GE Healthcare, Ltd.) according to the manufacturer's instructions. The assay using ViaCount is based on the reagent differentially stained viable and nonviable cells depending on their permeability to the DNA-binding dyes in the reagent. Fluorescence of each dye is resolved operationally, allowing the quantitative assessment of viable and nonviable nucleated cells present in the suspension. The system counts the stained nucleated

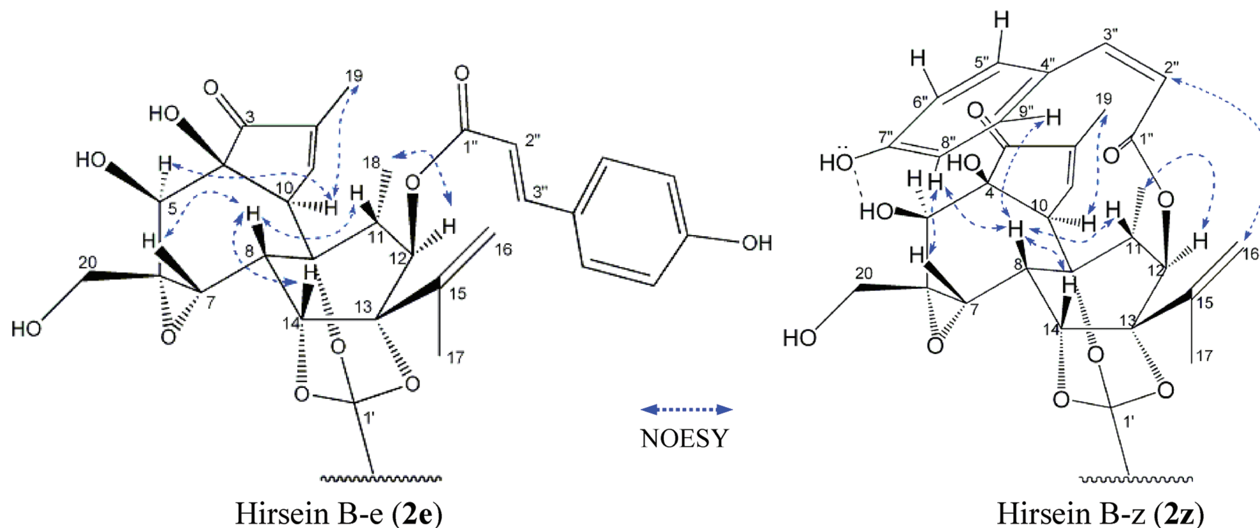

Figure 1. NOESY correlations of hirsein B (**2e** and **2z**)

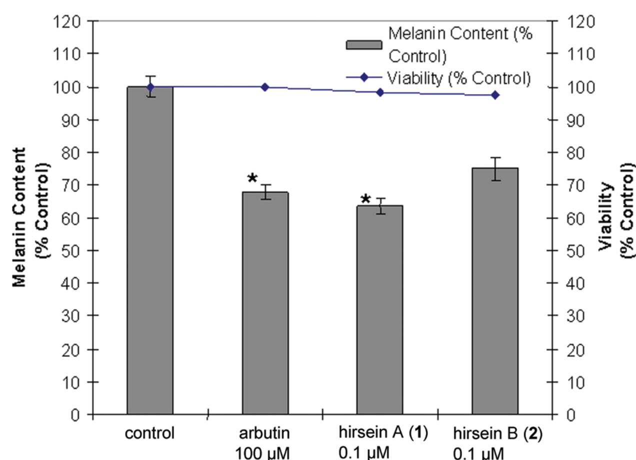

Figure 2. Effect of hirseins A (**1**) and B (**2**) and arbutin on melanogenesis in B16 murine melanoma cells. The bar graph indicates melanin content (left-hand *y*-axis). The line graph indicates cell viability (right-hand *y*-axis). Results represent means ± SD of triplicate samples. Results represent means ± SD of triplicate samples. *Statistically significant ($p < 0.005$) difference between treatments.

events and then uses the forward scatter properties to distinguish between free nuclei and cellular debris.

Measurement of Melanin Content. The melanin content was measured using the method of Hosoi et al.[9] with some modifications. B16 melanoma cells were seeded at a density of 5×10^5 cells per plate onto 100 mm Petri dishes and cultured as described above. After overnight incubation, DMEM was replaced with DMEM containing 100 μM arbutin or 0.1 μM compounds **1** and **2** and incubated further for 48 h. The medium was then removed and the cells washed two times with PBS and harvested by trypsinization (0.25% trypsin/0.02% EDTA in PBS; Sigma, St. Louis, MO). The harvested cells were pelleted and solubilized using 0.1% Triton X-100 and then purified by precipitation in 10% trichloroacetic acid. The isolated melanin was then dissolved in 8 N NaOH and incubated for 2 h at 80 °C. The amount of melanin in the solution was determined spectrophotometrically by measuring the absorbance at 410 nm. The total melanin content was estimated using the standard curve for synthetic melanin, and data were expressed as percentages of the melanin content of the control treatment.

Acknowledgment. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sports, and Culture of Japan.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP800808H