Brazilian Natural Medicines. IV.[1] New Noroleanane-Type Triterpene and Ecdysterone-Type Sterol Glycosides and Melanogenesis Inhibitors from the Roots of *Pfaffia glomerata*

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The ethyl acetate and 1-butanol soluble fractions of the roots of *Pfaffia glomerata* were found to show inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. From the ethyl acetate and 1-butanol soluble fractions, we isolated a new noroleanane-type triterpene, pfaffianol A, its glycosides, pfaffiaglycosides A and B, and ecdysterone-type sterol glycosides, pfaffiaglycosides C, D, and E, together with eight known constituents. The structures of new constituents were determined on the basis of physicochemical and chemical evidence. Among them, pfaffianol A (IC_{50}=44 μM) and pfaffoside C (IC_{50}=92 μM) substantially inhibited melanogenesis without cytotoxic effects. The inhibitory effects were stronger than that of reference compound, arbutin (IC_{50}=174 μM).

Key words *Pfaffia glomerata*; Brazil ginseng; pfaffiaglycoside; noroleanane-type triterpene; ecdysterone-type sterol glycoside; melanogenesis inhibitor

The Amaranthaceae plant, *Pfaffia* (*P.*) *glomerata*, which is so-called as Brazil ginseng in Japan, is widely cultivated in South American countries such as Brazil, Ecuador, and Panama. The roots of this plant are used as a Brazilian folk medicine for a tonic and treatment of diabetes. The extract from the roots of *P. glomerata* has been reported to possess gastroprotective,[2] anti-oxidant,[3] and anti-inflammatory effects.[4] On the other hand, ecdysterone has been characterized as the principal constituent from the roots.[5,6] However, chemical and pharmacological studies on the roots of *P. glomerata* have not been investigated sufficiently yet. During the course of characterization studies on the bioactive constituents of Brazilian natural medicines,[1,7—10] the ethyl acetate (EtOAc) and 1-butanol (1-BuOH) soluble fractions of the roots of *P. glomerata* were found to show inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. From the EtOAc and 1-BuOH soluble fractions, we have isolated a new noroleanane-type triterpene, pfaffianol A (**1**), two new noroleanane-type triterpene glycosides, pfaffiaglycosides A (**2**) and B (**3**), and three ecdysterone-type sterol glycosides, pfaffiaglycosides C (**4**), D (**5**), and E (**6**), with eight known constituents. Furthermore, we examined the inhibitory effects of the fractions and principal constituents on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. In this paper, we describe the isolation and structure elucidation of the new constituents

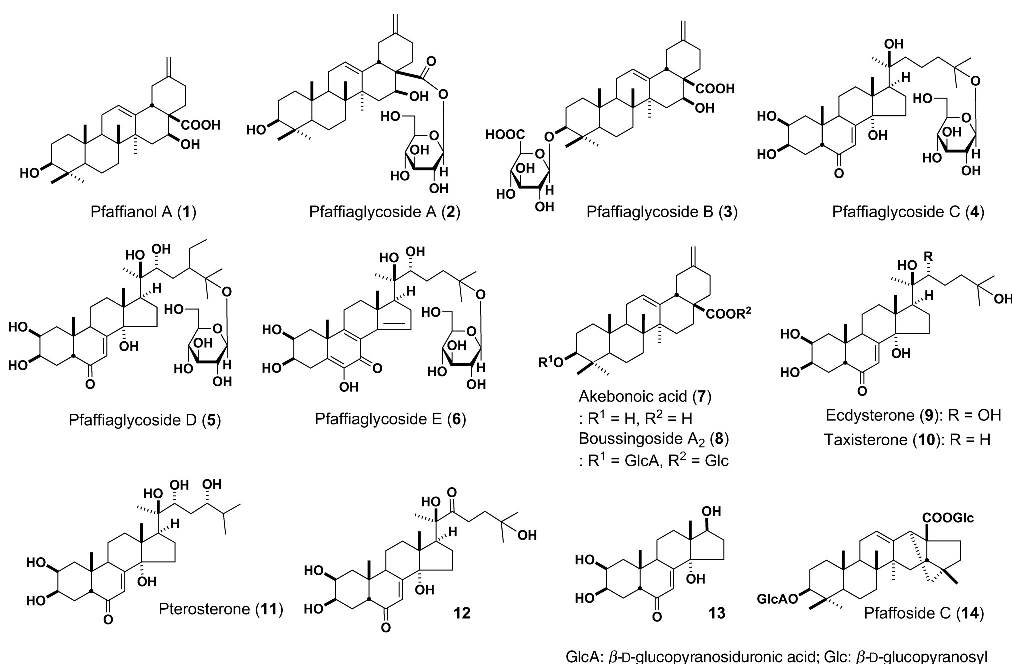

Chart 1

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(**1**—**6**) and the effects on melanogenesis of the principal constituents from the roots of *P. glomerata*.

The roots of Brazilian *P. glomerata* were extracted with methanol (MeOH). The MeOH extract (20.3% from the roots) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (2.0%) and an aqueous phase. The aqueous phase was further extracted with 1-BuOH to give 1-BuOH- (4.1%) and H_2O- (14.1%) soluble fractions. The EtOAc-soluble fraction [inhibition (%): 70.4±1.5 ($p<0.01$) at 100 μg/ml] and 1-BuOH-soluble fraction [inhibition (%): 25.0±3.3 ($p<0.01$) at 100 μg/ml] were found to show the inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. The EtOAc-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and finally HPLC to afford a pfaffiaglycoside A (**2**, 0.00036%) together with a known compound, 22-oxo-20-hydroxyecdysone (**12**, 0.0008%).[11] The 1-BuOH-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and finally HPLC to afford pfaffianol A (**1**, 0.0014%), pfaffiaglycosides B (**3**, 0.00027%), C (**4**, 0.00009 %), D (**5**, 0.00005%), and E (**6**, 0.00005%), together with seven known compounds, akebonoic acid (**7**, 0.00008%),[12] boussingoside A_2 (**8**, 0.0011%),[13] ecdysterone (**9**, 0.46%),[14] taxisterone (**10**, 0.0023%),[15] pterosterone (**11**, 0.0018%),[14] 2β,3β,14α,17β-tetrahydroxy-5β-androst-7-en-6-one (**13**, 0.00053%),[16] and pfaffoside C (**14**, 0.0037%).[17]

Structures of Pfaffianol A (1), Pfaffiaglycosides A—E (2—6) Pfaffianol A (**1**) was obtained as a white powder with a positive optical rotation ($[\alpha]_D^{26}$ +33.5° in MeOH). The IR spectrum of **1** showed absorption bands at 3420, 1710 and 1655 cm^{-1} ascribable to hydroxy, carboxy and olefin functions. In the electron ionization-mass spectra (EI-MS) of **1**, a molecular ion peak was observed at *m/z* 456 (M^+), and high-resolution EI-MS (HR-EI-MS) analysis revealed the molecular formula of **1** to be $C_{29}H_{44}O_4$. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[18] showed signals assignable to five methyls [δ 0.87, 1.00, 1.01, 1.24, 1.36 (3H each, all s, H_3-25, 24, 26, 23, 27)], two methines bearing an oxygen function [δ 3.45 (1H, dd, J=4.0, 11.5 Hz, H-3), 4.66 (1H, dd, J=4.6, 11.5 Hz, H-16)], three olefinic protons [δ 4.77, 4.81 (1H each, both s like, H_2-29), 5.52 (1H, dd like, H-12)], and a carboxy carbon (δ_C 180.0, C-28). The proton and carbon signals of **1** in the ^{1}H- and ^{13}C-NMR spectra resembled those of akebonoic acid,[19] except for the signals around the 16-position. The double quantum filter correlation spectroscopy (DQF COSY) experiment on **1** indicated the presence of partial structures written in bold lines (Fig. 1), and in the heteronuclear multiple bond connectivity spectroscopy (HMBC) experiment, long-range correlations were observed between the following protons and carbons: H-12 and C-9, 14, 18; H-16 and C-28; H-18 and C-16, 19, 20, 22, 28; H-23 and C-3, 4, 5, 24; H-24 and C-3, 4, 5, 23; H-25 and C-1, 5, 9, 10; H-26 and C-7, 8, 9, 14; H-27 and C-8, 15; H-29 and C-20. These results supported that the structure of **1** was noroleana-

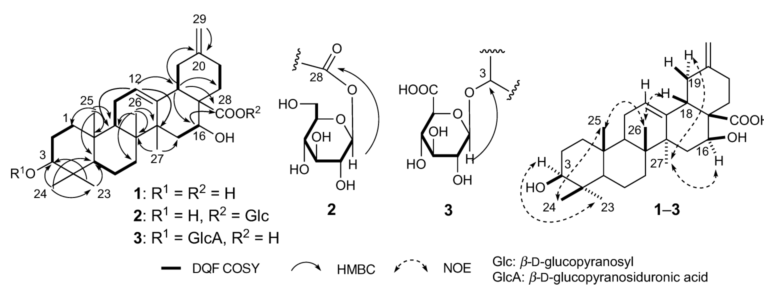

Fig. 1. Selected DQF, HMBC and NOE Correlations

Table 1. ^{13}C-NMR (125 MHz) Data for **1**—**6**[a]

Carbon	1	2	3	4	5	6	Carbon	1	2	3	4	5	6
1	38.2	38.1	38.1	37.7	38.0	42.5	19	41.5	41.8	41.5	24.5	24.7	27.4
2	28.0	28.0	26.5	68.1	68.1	69.8	20	148.3	148.2	148.3	74.3	76.9	76.1
3	78.0	78.0	89.1	68.0	68.0	73.4	21	29.9	29.9	29.9	27.0	21.5	20.7
4	39.1	39.0	39.4	32.4	32.8	28.1	22	32.9	31.8	32.9	45.2	76.0	77.6
5	55.7	55.8	55.7	51.2	51.4	132.8	23	28.7	28.7	28.1	18.8	31.9	26.4
6	18.7	18.7	18.3	203.7	203.7	144.4	24	16.5	16.5	16.8	42.8	47.6	39.7
7	33.1	33.2	33.0	121.5	121.6	180.8	25	15.5	15.7	15.5	77.4	80.9	77.2
8	39.8	39.9	39.7	166.4	166.1	127.4	26	17.2	17.4	17.2	27.1[b]	22.0[b]	27.5[b]
9	47.2	47.2	47.0	34.2	34.4	163.6	27	26.9	26.8	26.9	27.2[b]	26.7[b]	27.7[b]
10	37.2	37.2	36.9	38.7	38.6	41.6	28	180.0	174.8	180.0		47.6	
11	23.7	23.8	23.7	20.9	21.5	24.4	29	107.6	107.4	107.6		14.2	
12	123.5	122.9	123.5	31.8	31.8	36.8							
13	144.6	142.4	142.7	47.4	48.1	47.2	1′		96.1	107.1	98.7	98.7	98.7
14	44.3	44.5	44.3	84.5	84.1	142.2	2′		73.9	75.4	75.4	75.4	75.4
15	38.9	39.4	38.5	31.5	31.9	124.2	3′		78.3	77.8	78.7	78.8	78.8
16	65.0	65.2	65.0	21.9	21.5	32.3	4′		71.1	73.3	71.8	72.0	72.0
17	50.7	51.6	50.7	53.9	49.9	55.7	5′		79.3	78.0	78.2	78.6	78.6
18	49.6	49.7	49.6	17.9	17.8	18.5	6′		62.3	172.4	62.9	63.4	63.4

a) Measured in pyridine-d_5; *b*) reversible.

dienoic acid with two hydroxy groups at the 3- and 16-positions. Next, the stereostructure of **1** was characterized by nuclear Overhauser enhancement spectroscopy (NOESY) experiment, which showed NOE correlations between the following proton pairs: H-3α and H_3-23; H-16α and H_3-27; H-18 and H_3-26; H-19α and H_3-27; H_3-24 and H_3-25; H_3-25 and H_3-26 (Fig. 1). On the basis of this evidence, the structure of pfaffianol A (**1**) was characterized as shown.

Pfaffiaglycoside A (**2**) was obtained as a white powder with a positive optical rotation ($[\alpha]_D^{23}$ +27.9° in MeOH). The IR spectrum of **2** showed strong absorption bands at 3430 and 1072 cm^{-1} suggestive of the glycosidic structure together with absorption bands at 1718 and 1655 cm^{-1} due to carboxy and olefin functions. In the positive-ion fast atom bombardment (FAB)-MS of **2**, a quasimolecular ion peak was observed at *m/z* 641 $(M+Na)^+$. The HR-FAB-MS analysis revealed the molecular formula of **2** to be $C_{35}H_{54}O_9$. Acid hydrolysis of **2** liberated the aglycone, pfaffianol A (**1**), together with D-glucose, which was identified by HPLC analysis using an optical rotation detector.[20] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[18] of **2** showed signals assignable to an aglycon moiety [δ 0.91, 0.98, 1.06, 1.19, 1.28 (3H each, all s, H_3-25, 24, 26, 23, 27), 3.40 (1H, dd, J=4.1, 11.5 Hz, H-3), 4.60 (1H, dd, J=4.0, 11.5 Hz, H-16), 4.73, 4.77 (1H each, both s like, H_2-29), 5.43 (1H, dd like, H-12)], together with a β-D-glucopyranosyl moiety [δ 6.10 (1H, d, J=8.0 Hz, H-1′)]. The position of the glycoside linkage was determined by a HMBC experiment, which showed long-range correlations between H-1′ and C-28. Furthermore, the DQF COSY and HMBC experiments on **2** showed correlations as shown in Fig. 1. This evidence led us to formulate the structure of pfaffiaglycoside A (**2**) as shown.

Pfaffiaglycoside B (**3**), obtained as a white powder with a positive optical rotation ($[\alpha]_D^{23}$ +35.3° in MeOH), showed strong absorption bands at 3430 and 1070 cm^{-1} suggestive of the glycosidic structure together with absorption bands at 1710 and 1655 cm^{-1} due to carboxy and olefin functions in the IR spectrum. The molecular formula $C_{35}H_{52}O_{10}$ was determined from the positive-ion FAB-MS at *m/z* 655 $(M+Na)^+$ and by HR-FAB-MS measurement. Acid hydrolysis of **3** liberated the aglycone, pfaffianol A (**1**), together with D-glucuronic acid, which was identified by GLC analysis of the trimethylsilyl thiazolidine derivative.[21—23] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[18] of **3** showed signals assignable to an aglycon moiety [δ 0.87, 1.00, 1.01, 1.24, 1.36 (3H each, all s, H_3-25, 24, 26, 23, 27), 3.45 (1H, dd, J=4.0, 11.5 Hz, H-3), 4.66 (1H, dd, J=4.6, 11.5 Hz, H-16), 4.78, 4.82 (1H each, both s like, H_2-29), 5.52 (1H, dd like, H-12)], together with a β-D-glucopyranosiduronic acid moiety [δ 4.98 (1H, d, J=8.0 Hz, H-1′)]. The position of the glycoside linkage was determined by a HMBC experiment, which showed long-range correlations between H-1′ and C-3. Furthermore, the DQF COSY and HMBC experiments on **3** showed correlations as shown in Fig. 1. This evidence led us to formulate the structure of pfaffiaglycoside B (**3**) as shown.

Pfaffiaglycoside C (**4**) was obtained as a white powder with negative optical rotation ($[\alpha]_D^{23}$ −16.1° in MeOH). The IR spectrum of **4** showed absorption bands at 3430, 1670, and 1074 cm^{-1} assignable to hydroxy, unsaturated carbonyl, and ether functions. In the positive-ion FAB-MS of **4**, a quasimolecular ion peak was observed at *m/z* 649 $(M+Na)^+$. The HR-FAB-MS analysis revealed the molecular formula of **4** to be $C_{33}H_{54}O_{11}$. The acid hydrolysis of **4** liberated D-glucose, which was identified by HPLC analysis.[20] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[18] of **4** showed signals due to five methyls [δ 1.05, 1.12, 1.34, 1.37, 1.50 (3H each, all s, H_3-19, 18, 26, 27, 21)], two methines bearing an oxygen function [δ 4.15 (1H, m, H-2), 4.21 (1H, m, H-3)], an olefinic proton [δ 6.19 (1H, d, J=2.0 Hz, H-7)], a carbonyl carbon (δ_C 203.7, C-6), and a β-D-glucopyranosyl moiety [δ 4.96 (1H, d, J=8.0 Hz, H-1′)]. The proton and carbon signals of the aglycon part in the ^{1}H- and ^{13}C-NMR spectra of **4** were superimposable on those of taxisterone (**10**),[15] except for the signals due to the terminal side chain part (C-25—C-27), while the proton and carbon signals due to the glycoside moiety including terminal side chain part (C-25—C-27) were very similar to those of ecdysterone 25-*O*-β-D-glucopyranoside.[24] As shown in Fig. 2, the DQF COSY experiment on **4** indicated the presence of partial structures written in bold lines, and in the HMBC experiment, long-range correlations were observed between the following protons and carbons: H-2 and C-4; H-3 and C-1; H-5 and C-3, 4, 6, 10; H-7 and C-5, 6, 8, 9; H-17 and C-13, 15, 16, 20; H-18 and C-12, 13, 14, 17; H-19 and C-1, 5, 9, 10; H-21 and C-20, 22; H-26, 27 and C-24, 25; H-1′ and C-25. On the basis of this evidence, the structure of pfaffiaglycoside C (**4**) was characterized as shown.

Pfaffiaglycoside D (**5**), obtained as a white powder with positive optical rotation ($[\alpha]_D^{23}$ +23.8° in MeOH), showed absorption bands at 3430, 1670, and 1072 cm^{-1} assignable to hydroxy, unsaturated carbonyl, and ether functions in the IR spectrum. The positive-ion FAB-MS of **5** exhibited a quasimolecular ion peak at *m/z* 693 $(M+Na)^+$. The molecular formula $C_{35}H_{58}O_{12}$ of **5** was determined from the quasimolecular ion peak and by HR-FAB-MS measurement. The acid hydrolysis of **5** liberated D-glucose, which was identified by HPLC analysis.[20] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[18] of **5** showed signals assignable to an aglycon part [δ 1.03 (3H, t, J=7.4 Hz, H_3-29), 1.05, 1.21, 1.31,

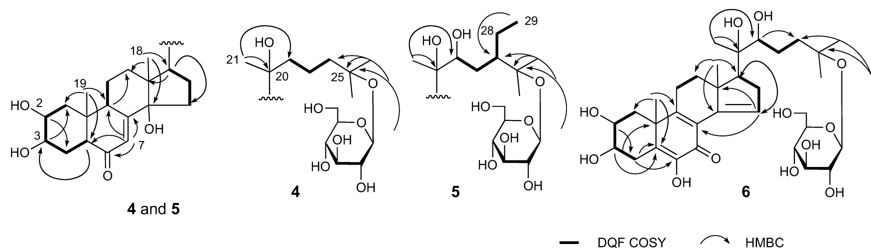

Fig. 2. Selected DQF and HMBC Correlations

Table 2. Inhibitory Effects of Constituents on Melanogenesis in B16 Melanoma 4A5 Cells

Compound No.	Inhibition (%) for melanogenesis					
Conc. (μM)	0	1	3	10	30	100
Pfaffianol A (**1**)	0.0±3.1	−1.0±3.4	−6.6±2.2	7.9±5.0	34.7±3.7**	80.0±2.5**
Pfaffiaglycoside B (**3**)	0.0±2.0	−4.0±2.3	−2.5±2.3	−1.2±1.6	−17.0±4.2	−12.6±4.3
Boussingoside A_2 (**8**)	0.0±3.4	−3.3±4.6	2.2±4.9	4.2±2.9	2.5±8.6	22.1±2.4*
Ecdysterone (**9**)	0.0±2.9			−6.6±3.1	−27.2±18.3	−6.1±7.6
Taxisterone (**10**)	0.0±2.2	0.9±3.4	−4.2±3.1	−1.1±6.6	1.9±3.5	−10.1±6.4
Pterosterone (**11**)	0.0±3.3	−4.4±1.8	−8.9±5.1	−3.5±2.7	−9.1±3.2	−3.3±3.0
12	0.0±2.9	0.6±2.5	−5.4±3.5	0.6±3.2	7.4±2.2	−0.5±2.9
13	0.0±6.4	1.8±3.0	−2.6±4.0	−1.3±5.2	1.3±5.8	2.3±4.2
Pfaffoside C (**14**)	0.0±1.4	4.5±2.3	2.2±2.1	10.8±1.9**	28.3±2.2**	51.4±1.2**
	Inhibition (%) for melanogenesis					
Conc. (μM)	0	10	30	100	300	1000
Arbutin	0.0±1.4	10.6±0.6**	20.4±0.5**	38.1±0.9**	61.5±0.6**	83.7±0.5**

Each value represents the mean±S.E.M. (n=4). Significantly different from the control * $p<0.05$, ** $p<0.01$. The cell viabilities of compounds, **1**, **3**, **8**—**14**, and arbutin at 100 μM are more than 86%.

1.47, 1.59 (3H each, all s, H_3-19, 18, 26, 27, 21), 3.98 (1H, m, H-22), 4.15 (1H, m, H-2), 4.20 (1H, m, H-3), 6.29 (1H, d, J=2.0 Hz, H-7)] and a β-D-glucopyranosyl moiety [δ 5.09 (1H, d, J=8.0 Hz, H-1′). The proton and carbon signals of the aglycon part in the ^{1}H-NMR spectra of **5** were similar to those of makisterone C,[25] except for the signals due to the terminal side chain part (C-25—C-27), while the proton and carbon signals due to the glycoside moiety were superimposable on those of ecdysterone 25-O-β-D-glucopyranoside.[24] In the HMBC experiment, long-range correlations were observed between the following protons and carbons: H-2 and C-4; H-3 and C-1; H-5 and C-3, 4, 6, 10; H-7 and C-5, 6, 8, 9; H-17 and C-13, 15, 16, 20; H-18 and C-12, 13, 14, 17; H-19 and C-1, 5, 9, 10; H-21 and C-17, 20, 22; H-26, 27 and C-24, 25; H-29 and C-24; H-1′ and C-25. On the basis of this evidence, the structure of pfaffiaglycoside D (**5**) was characterized as shown.

Pfaffiaglycoside E (**6**), obtained as a white powder with positive optical rotation ($[\alpha]_D^{23}$ +24.6° in MeOH), showed absorption bands due to hydroxy, unsaturated carbonyl, and ether functions in the IR spectrum. The molecular formula $C_{33}H_{50}O_{12}$ of **6** was determined from the positive-ion FAB-MS [m/z 661 $(M+Na)^+$] and by HR-FAB-MS measurement. The acid hydrolysis of **6** liberated D-glucose, which was identified by HPLC analysis.[20] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[18] of **6** showed signals assignable to an aglycon part [δ 1.42, 1.43, 1.47, 1.58, 1.74 (3H each, all s, H_3-18, 26, 27, 21, 19), 3.84 (1H, m, H-22), 4.08 (1H, m, H-3), 4.46 (1H, m, H-2), 7.47 (1H, dd like, H-15)] and a β-D-glucopyranosyl moiety [δ 5.06 (1H, d, J=8.0 Hz, H-1′)]. The proton and carbon signals of the aglycon part in the ^{1}H- and ^{13}C-NMR spectra of **6** were similar to those of podecdysone B 25-O-β-D-glucopyranoside,[24] except for the signals around the B ring part (C-5—C-10). As shown in Fig. 2, the DQF COSY experiment on **6** indicated the presence of partial structures written in bold lines, and in the HMBC experiment, long-range correlations were observed between the following protons and carbons: H-2 and C-1, 3, 4; H-3 and C-2, 4, 5; H-4 and C-3, 5, 6, 10; H-15 and C-8, 13, 16, 17; H-18 and C-12, 13, 14, 17; H-19 and C-1, 5, 9, 10; H-21 and C-17, 20, 22; H-22 and C-23, 24; H-26, 27 and C-24, 25; H-1′ and C-25. On the basis of this evidence, pfaffiaglycoside E (**6**) was characterized as shown.

Inhibitory Effects of Constituents on Melanogenesis in B16 Melanoma 4A5 Cells Melanin production is principally responsible for skin color, and melanin pigmentation is a major defense mechanism against ultraviolet rays from the sun. On the other hand, the excess melanin formation after sunlight exposure for long periods of time causes some dermatological disorders such as melasma, freckles, post-inflammatory melanoderma, and solar lentigines. To develop melanogenesis inhibitors, we have reported the inhibitory effects of several diarylheptanoids and flavonoids in theophylline-stimulated B16 melanoma 4A5 cells recently.[26,27] As a continuing of these studies, the inhibitory effects of the constituents from the roots of *P. glomerata* on melanogenesis were examined. Among the principal constituents, pfaffianol A (**1**, IC_{50}=44 μM) and pfaffoside C (**14**, IC_{50}=92 μM) substantially inhibited melanogenesis without cytotoxic effects. The effects of **1** and **14** were stronger than that of reference compound, arbutin (IC_{50}=174 μM). On the other hand, ecdysterone (**9**) and its derivatives **10**—**13** did not show such effects (Table 2).

Experimental

General Experimental Procedures The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and HR-EI-MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and HR-FAB-MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL EX-270 (270 MHz), JNM-LA500 (500 MHz), and JEOL ECA-600K (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL EX-270 (68 MHz) JNM-LA500 (125 MHz), and JEOL ECA-600K (150 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index detector; and HPLC column, COSMOSIL 5C18-MS-II (250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental materials were used for chromatography: normal-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, precoated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm)

(reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Plant Material The roots of *Pfaffia glomerata*, which were cultivated in Brazil, were purchased from Tamura Pharmaceutical Co., Ltd., in 2007. A voucher of the plant is on file in our laboratory (2007. Brazil-01).

Extraction and Isolation The roots of *P. glomerata* (4.75 kg) were cut and extracted four times with MeOH under reflux. Evaporation of the solvent under reduced pressure provided the MeOH extract (963 g, 20.3%), which was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (97 g, 2.0%) and aqueous layer. The aqueous layer was extracted with 1-BuOH to give 1-BuOH- (195 g, 4.1%) and H_2O- (671 g, 14.1%) soluble fractions. The EtOAc-soluble fraction (97 g) was subjected to normal-phase silica gel column chromatography [2.0 kg, $CHCl_3$–MeOH (50 : 1→20 : 1, v/v)→$CHCl_3$–MeOH–H_2O (10 : 3 : 1→7 : 3 : 1→6 : 4 : 1, v/v)→MeOH] to give 4 fractions [Fr. 1 (3.0 g), Fr. 2 (25.5 g,), Fr. 3 (30.5 g), Fr. 4 (23.0 g)]. Fraction 3 (30.5 g) was separated by reversed-phase silica gel column chromatography [750 g, MeOH–H_2O (50 : 50→60 : 40→70 : 30→80 : 20, v/v)→MeOH] to give 11 fractions [Fr. 3-1, Fr. 3-2, Fr. 3-3, Fr. 3-4 (101 mg), Fr. 3-5, Fr. 3-6, Fr. 3-7, Fr. 3-8 (3.21 g), Fr. 3-9, Fr. 3-10, Fr. 3-11]. Fraction 3-4 (101 mg) was purified by HPLC [MeOH–H_2O (80 : 20, v/v)] to give 22-oxo-20-hydroxyecdysone (**12**, 41 mg). Fraction 3-8 (3.21 g) was purified by reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (60 : 40→70 : 30→80 : 20, v/v)] and HPLC [MeOH–H_2O (80 : 20, v/v)] to give pfaffiaglycoside A (**2**, 15 mg). A part of the 1-BuOH-soluble fraction (179 g) was subjected to normal-phase silica gel column chromatography [3 kg, $CHCl_3$: MeOH : H_2O (50 : 10 : 1→40 : 10 : 1→30 : 10 : 1→7 : 3 : 1→6 : 4 : 1)→MeOH] to give 15 fractions [Fr. 1, Fr. 2, Fr. 3, Fr. 4 (3.13 g), Fr. 5, Fr. 6, Fr. 7 (4.00 g), Fr. 8, Fr-9, Fr. 10, Fr. 11, Fr. 12, Fr. 13, Fr. 14 (27.86 g), Fr. 15 (100.67 g)]. Fraction 4 (3.13 g) was separated by reversed-phase silica gel column chromatography [60 g, MeOH : H_2O (50 : 50→60 : 40→70 : 30→80 : 20→MeOH) to give 4 fractions [Fr. 4-1, Fr. 4-2 (205 mg), Fr. 4-3, Fr. 4-4]. Fraction 4-2 (205 mg) was purified by HPLC [MeOH–H_2O (70 : 30, v/v)] to give akebonoic acid (**7**, 22 mg). Fraction 7 (4.00 g) was separated by reversed-phase silica gel column chromatography [80 g, MeOH : H_2O (50 : 50→60 : 40→70 : 30→80 : 20)→MeOH] to give 14 fractions [Fr. 7-1, Fr. 7-2, Fr. 7-3, Fr. 7-4, Fr. 7-5, Fr. 7-6, Fr. 7-7, Fr. 7-8, Fr. 7-9, Fr. 7-10, Fr. 7-11, Fr. 7-12, Fr. 7-13 (300 mg), Fr. 7-14]. Fraction 7-13 (300 mg) was purified by HPLC [MeOH–H_2O (70 : 30, v/v)] to give pfaffianol A (**1**, 59 mg). Fraction 14 (27.86 g) was separated by reversed-phase silica gel column chromatography [80 g, MeOH : H_2O (20 : 80→30 : 70→40 : 60→50 : 50→60 : 40→80 : 20)→MeOH] to give 13 fractions [Fr. 14-1, Fr. 14-2, Fr. 14-3, Fr. 14-4 (230 mg), Fr. 14-5 [=ecdysterone (**9**, 20.6 g)], Fr. 14-6 (422 mg), Fr. 14-7, Fr. 14-8 (325 mg), Fr. 14-9, Fr. 14-10, Fr. 14-11, Fr. 14-12, Fr. 14-13]. Fraction 14-4 (230 mg) was purified by HPLC [MeOH–H_2O (40 : 60, v/v)] to give 2β,3β,14α,17β-tetrahydroxy-5β-androst-7-en-6-one (**13**, 23 mg). Fraction 14-6 (422 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give taxisterone (**10**, 100 mg) and pterosterone (**11**, 80 mg). Fraction 14-8 (325 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give pfaffiaglycoside B (**3**, 11.6 mg). Fraction 15 (100.67 g) was separated by reversed-phase silica gel column chromatography [80 g, MeOH : H_2O (20 : 80→30 : 70→40 : 60→50 : 50→60 : 40→80 : 20, v/v)→MeOH] to give 16 fractions [Fr. 15-1, Fr. 15-2, Fr. 15-3, Fr. 15-4, Fr. 15-5, Fr. 15-6, Fr. 15-7, Fr. 15-8, Fr. 15-9 (1.90 g), Fr. 15-10, Fr. 15-11, Fr. 15-12 (6.10 g), Fr. 15-13]. Fraction 15-9 (1.90 g) was furthermore purified by reversed-phase silica gel column chromatography [50 g, MeOH–H_2O (20 : 80→40 : 60→60 : 40→80 : 20, v/v)] and HPLC [MeOH–H_2O (40 : 60, v/v)] to give pfaffiaglycoside C (**4**, 3.8 mg), pfaffiaglycoside D (**5**, 2.0 mg), and pfaffiaglycoside E (**6**, 2.0 mg). Fraction 15-12 (6.10 g) was furthermore purified by reversed-phase silica gel column chromatography [50 g, MeOH–H_2O (20 : 80→40 : 60→60 : 40→80 : 20, v/v)] and HPLC [MeOH–H_2O (45 : 55, v/v)] to give boussingoside A_2 (**8**, 46 mg) and pfaffoside C (**14**, 160 mg). The known compounds were identified by comparison of their physical data ($[\alpha]_D$, ^{1}H-NMR, ^{13}C-NMR, and MS) with reported values.

Pfaffianol A (**1**): A white powder; $[\alpha]_D^{26}$ +33.5° (c=0.30, MeOH); IR (KBr) $\nu_{\mu\alpha\xi}$ 3420, 2943, 1710, 1655 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 0.85 (1H, dd like, H-5), 0.87, 1.00, 1.01, 1.24, 1.36 (3H each, all s, H_3-25, 24, 26, 23, 27), 2.25 (1H, dd like, H-19β), 2.69 (1H, dd, J=12.5, 12.5 Hz, H-19α), 3.35 (1H, dd, J=4.1, 12.5 Hz, H-18), 3.45 (1H, dd, J=4.0, 11.5 Hz, H-3), 4.66 (1H, dd, J=4.6, 11.5 Hz, H-16), 4.77, 4.81 (1H each, both s like, H_2-29), 5.52 (1H, dd like, H-12); ^{13}C-NMR data see Table 1; EI-MS m/z: 456 $[M]^+$; HR-EI-MS m/z: 456.3241 (Calcd for $C_{29}H_{44}O_4$ $[M]^+$, 456.3239).

Pfaffiaglycoside A (**2**): A white powder; $[\alpha]_D^{23}$ +27.9° (c=0.50, MeOH); IR (KBr) ν_{max} 3430, 2936, 1718, 1655, 1072 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 0.85 (1H, m, H-5), 0.91, 0.98, 1.06, 1.19, 1.28 (3H each, all s, H_3-25, 24, 26, 23, 27), 2.25 (1H, dd like, H-19β), 2.69 (1H, dd, J=12.5, 12.5 Hz, H-19α), 3.16 (1H, dd, J=4.1, 12.5 Hz, H-18), 3.40 (1H, dd, J=4.1, 11.5 Hz, H-3), 4.60 (1H, dd, J=4.0, 11.5 Hz, H-16), 4.73, 4.77 (1H each, both s like, H_2-29), 5.43 (1H, dd like, H-12), 6.10 (1H, d, J=8.0 Hz, H-1′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z: 641 $[M+Na]^+$; HR-FAB-MS m/z: 641.3672 (Calcd for $C_{35}H_{54}O_9Na$ $[M+Na]^+$, 641.3666).

Pfaffiaglycoside B (**3**): A white powder; $[\alpha]_D^{23}$ +35.3° (c=0.30, MeOH); IR (KBr) ν_{max} 3430, 2945, 1710, 1655, 1070 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 0.85 (1H, m, H-5), 0.87, 1.00, 1.01, 1.24, 1.36 (3H each, all s, H_3-25, 24, 26, 23, 27), 2.25 (1H, dd like, H-19β), 2.69 (1H, dd, J=12.5, 12.5 Hz, H-19α), 3.35 (1H, dd, J=4.1, 12.5 Hz, H-18), 3.45 (1H, dd, J=4.0, 11.5 Hz, H-3), 4.66 (1H, dd, J=4.6, 11.5 Hz, H-16), 4.78, 4.82 (1H each, both s like, H_2-29), 5.52 (1H, dd like, H-12), 4.98 (1H, d, J=8.0 Hz, H-1′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z: 655 $[M+Na]^+$; HR-FAB-MS m/z: 655.3463 (Calcd for $C_{35}H_{52}O_{10}Na$ $[M+Na]^+$, 655.3458).

Pfaffiaglycoside C (**4**): A white powder; $[\alpha]_D^{23}$ −16.1° (c=0.13, MeOH); IR (KBr) ν_{max} 3430, 2926, 1670, 1074 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 1.05, 1.12, 1.34, 1.37, 1.50 (3H each, all s, H_3-19, 18, 26, 27, 21), 2.55 (1H, ddd, J=4.1, 13.1, 13.1 Hz, H-12), 2.81 (1H, dd, J=8.9, 8.9 Hz, H-17), 2.98 (1H, dd, J=4.0, 14.0 Hz, H-5), 3.56 (1H, m, H-9), 4.15 (1H, m, H-2), 4.21 (1H, m, H-3), 4.96 (1H, d, J=8.0 Hz, H-1′), 6.19 (1H, d, J=2.0 Hz, H-7); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z: 649 $[M+Na]^+$; HR-FAB-MS m/z: 649.3571 (Calcd for $C_{33}H_{54}O_{11}Na$ $[M+Na]^+$, 649.3564).

Pfaffiaglycoside D (**5**): A white powder; $[\alpha]_D^{23}$ +23.8° (c=0.10, MeOH); IR (KBr) ν_{max} 3430, 2934, 1670, 1072 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 1.03 (3H, t, J=7.4 Hz, H_3-29), 1.05, 1.21, 1.31, 1.47, 1.59 (3H each, all s, H_3-19, 18, 26, 27, 21), 2.55 (1H, m, H-12), 2.98 (1H, dd, J=9.0, 9.0 Hz, H-17), 3.04 (1H, dd, J=4.0, 12.4 Hz, H-5), 3.60 (1H, m, H-9), 3.98 (1H, m, H-22), 4.15 (1H, m, H-2), 4.20 (1H, m, H-3), 5.09 (1H, d, J=8.0 Hz, H-1′), 6.29 (1H, d, J=2.0 Hz, H-7); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z: 693 $[M+Na]^+$; HR-FAB-MS m/z: 693.3829 (Calcd for $C_{35}H_{58}O_{12}Na$ $[M+Na]^+$, 693.3826).

Pfaffiaglycoside E (**6**): A white powder; $[\alpha]_D^{23}$ +24.6° (c=0.10, MeOH); IR (KBr) ν_{max} 3430, 2926, 1675, 1075 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz) δ: 1.42, 1.43, 1.47, 1.58, 1.74 (3H each, all s, H_3-18, 26, 27, 21, 19), 2.25 (1H, dd, J=9.0, 9.0 Hz, H-17), 3.11 (1H, dd, J=11.0, 12.0 Hz, H-4a), 3.84 (1H, m, H-22), 4.08 (1H, m, H-3), 4.46 (1H, m, H-2), 7.47 (1H, dd like, H-15), 5.06 (1H, d, J=8.0 Hz, H-1′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z: 661 $[M+Na]^+$; HR-FAB-MS m/z: 661.3196 (Calcd for $C_{33}H_{50}O_{12}Na$ $[M+Na]^+$, 661.3200).

Acid Hydrolyses of Pfaffiaglycosides A—E (2—6) A solution of **2**—**6** (4.0 mg each for **2** and **3**, 1.0 mg each for **4**—**6**) in 1.0 M aqueous HCl (1 ml) and 1,4-dioxane (1 ml) was heated under reflux for 3 h, respectively. After cooling, the reaction mixture was poured into ice-water and neutralized with Amberlite IRA-400 (OH^- form), and the resin was removed by filtration. On removal of the solvent from the filtrate, the residue was passed through a Sep-Pack C_{18} cartridge by elution with H_2O and then MeOH. The H_2O eluate obtained from **2**, **4**—**6** was concentrated and the residue was subjected to HPLC analysis to identify the D-glucose under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d.×250 mm; detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, MeCN–H_2O (75 : 25, v/v); flow rate, 0.80 ml/min; column temperature, room temperature. Identification of D-glucose present in the H_2O eluate was carried out by comparison of its retention time and optical rotation with that of an authentic sample [t_R: 9.8 min (positive optical rotation)]. The H_2O eluate obtained from **3** was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (3 mg) in pyridine (0.5 ml) at 60 °C for 2 h. After reaction, the solution was treated with N,O-bis(trimethylsilyl)trifluoroacetamide (0.1 ml) at 60 °C for 2 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucuronic acid under the following conditions: GLC column, Supeluco STB^{TM}-1, 0.25 mm i.d.×30 m; column temperature, 230 °C; detector temperature, 230 °C; injector temperature, 230 °C; carrier gas, N_2. Identification of D-glucuronic acid present in the H_2O eluate obtained from **3** was carried out by comparison of its retention time with that of an authentic sample [t_R: 23.9 min]. The MeOH eluate obtained from **2** and **3** was concentrated and the residue was purified by reversed-phase silica gel column chromatography [MeOH : H_2O (50 : 50→70 : 30) to give pfaffianol A (**1**, 1.9 mg from **2**, 1.8 mg from **3**), was identified by comparison of their physical data ($[\alpha]_D$, ^{1}H-NMR, ^{13}C-NMR, and MS) with those of isolated pfaffianol A (**1**).

Reagents for Bioassay Methods Dulbecco's modified Eagle's medium (DMEM, 4500 mg/l glucose) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.); fetal bovine serum (FBS), penicillin, and streptomycin were

purchased from Gibco (Invitrogen, Carlsbad, CA, U.S.A.); the Cell Counting Kit-8™ was from Dojindo Lab. (Kumamoto, Japan); and the other chemicals were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Cell Culture Murine B16 melanoma 4A5 cells (RCB0557) were obtained from Riken Cell Bank (Tsukuba, Japan), and the cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO_2/air. The cells were harvested by incubation in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin for *ca.* 5 min at 37 °C and were used for the subsequent bioassays.

Melanogenesis The melanoma cells (2.0×10^4 cells/400 μl/well) were seeded into 24-well multiplates. After 24 h of culture, a test compound and theophylline 1 mM were added and incubated for 72 h. The cells were harvested by incubating with PBS containing 1 mM EDTA and 0.25% trypsin, and then the cells were washed with PBS. The cells were treated with NaOH 1 M (120 μl/tube, 80 °C, 30 min) to yield a lysate, an aliquot (100 μl) of the lysate was transferred to a 96-well microplate, and the optical density of each well was measured with a microplate reader (Model 550, Bio-Rad Laboratories) at 405 nm (reference: 655 nm). The test compound was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the medium was 0.1%. The production of melanin was corrected based on cell viability. Inhibition (%) was calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{inhibition (\%)}=[(A-B)/A]/(C/100)\times100$$

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively, and C indicates cell viability (%).

Cell Viability The melanoma cells (5.0×10^3 cells/100 μl/well) were seeded into 96-well microplates and incubated for 24 h. After 70 h incubation with theophylline 1 mM and a test compound, 10 μl of WST-8 solution (Cell Counting Kit-8™) was added to each well. After a further 2 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 450 nm (reference: 655 nm). The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. Cell viability (%) was calculated using the following formula.

$$\text{cell viability (\%)}=A/B\times100$$

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively.

Statistical Analyses Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analyses.

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