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Gusanlungionosides A−D, Potential Tyrosinase Inhibitors from *Arcangelisia gusanlung*

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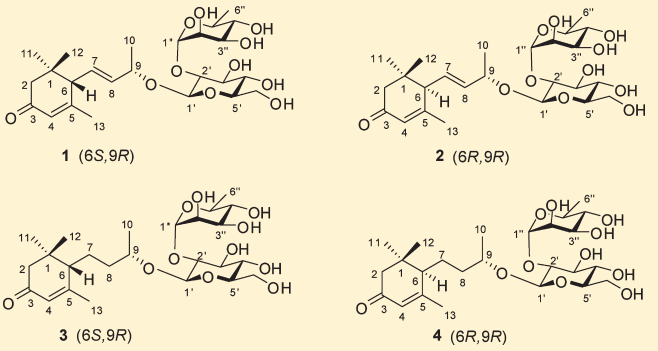
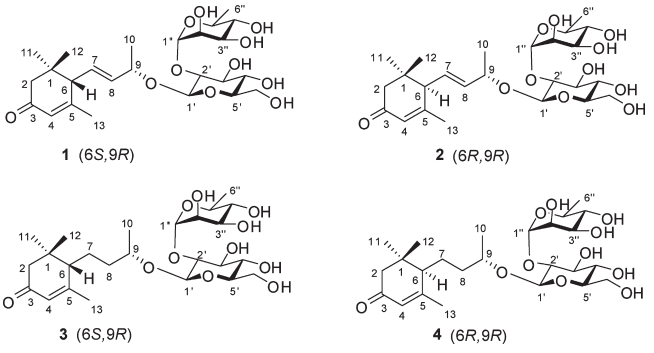
Supporting Information

ABSTRACT: Four new megastigmane glycosides, named gusanlungionosides A−D (**1**−**4**), together with 10 known compounds (**5**−**14**), were isolated from the stems of *Arcangelisia gusanlung*. The structures and absolute configurations of **1**−**4** were elucidated by comprehensive analysis of their NMR and CD data. Compounds **1**−**4** exhibited strong inhibitory effects not only on the mushroom tyrosinase activity in vitro but also on melanogenesis in cells.



Arcangelisia gusanlung H. S. LO (Menispermaceae) is a small shrub distributed in the south of China including Guangdong, Guangxi, and Hainan Provinces. Its whole stem has been used in China as an anti-inflammatory, antipyretic, and detoxication agent.[1] Previous chemical investigations of this plant revealed a number of protoberberine alkaloids from its stem bark.[2−4] Herein we report the isolation, structure elucidation, and antityrosinase activity of four new megastigane glycosides (**1**−**4**) and 10 known compounds from this species.



RESULTS AND DISCUSSION

The air-dried and smashed stems of *A. gusanlung* (18 kg) were extracted with MeOH (3 × 80 L). After removal of the solvent, the extract was partitioned successively with petroleum ether, CH_2Cl_2, EtOAc, and *n*-BuOH. The *n*-BuOH extract exhibited significant antityrosinase activity. By means of biassay- and HPLC-DAD-guided fractionation,[5,6] we isolated four new megastigane glycosides, named gusanlungionosides A−D (**1**−**4**), and 10 known compounds: *N-trans*-feruloyl-3-methoxytyramine (**5**),[7] thalifoine (**6**),[8,9] *p*-hydroxybenzyl alcohol (**7**),[10] *N*-methylcorydaldine (**8**),[8,11] syringaresinol (**9**),[12] *N-trans*-coumaroyltyramine (**10**),[13] (2*R*,3*S*,4*S*)-3α-[(β-D-glucopyranosyl)oxy]lyoniresinol (**11**),[14] (2*S*,3*R*,4*R*)-3α-[(β-D-glucopyranosyl)oxy]lyoniresinol (**12**),[15] (2*R*,3*S*,4*R*)-3α-[(β-D-glucopyranosyl)oxy]lyoniresinol (**13**),[16] and (2*S*,3*R*,4*S*)-3α-[(β-D-glucopyranosyl)oxy]lyoniresinol (**14**).[17]

Gusanlungionoside A (**1**) was obtained as an amorphous powder, and its molecular formula was determined as $C_{25}H_{40}O_{11}$ by HRESIMS (*m*/*z* 539.2482 $[M + Na]^+$), indicating six degrees of unsaturation. The ^1H and ^{13}C NMR and HSQC spectroscopic data confirmed the presence of 25 carbons. The UV spectrum showed an α,β-unsaturated ketone absorption at λ_{max} 238.0 nm that was supported by the IR absorption band at 1653 cm^{-1}. Analysis of the ^1H, ^{13}C, and HSQC NMR spectroscopic data (Table 1) revealed, in addition to two sugar moieties, that there were four methyl, one methylene, two methine (one oxygenated), one quaternary, four olefinic (three of which were protonated), and one α,β-unsaturated carbonyl carbon (δ_C 202.2). Interpretation of the $^1H-^1H$ COSY

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Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectroscopic Data (methanol-d_4) of Gusanlungionosides A−D (1−4)

	1		2		3		4	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	37.4, C		37.3, C		37.6, C		37.5, C	
2	48.6, CH_2	2.42, 2.05 d (16.2)	48.6, CH_2	2.44, 2.04 d (16.8)	48.3, CH_2	2.47, 1.98 d (17.4)	48.3, CH_2	2.47, 1.98 d (17.4)
3	202.2, C		202.2, C		202.6, C		202.6, C	
4	126.3, CH	5.89 s	126.4, CH	5.89 s	125.7, CH	5.81 s	125.6, CH	5.81 s
5	166.2, C		166.0, C		170.2, C		170.2, C	
6	57.0, CH	2.67 d (9.6)	56.9, CH	2.68 d (9.6)	52.7, CH	2.01 d (4.8)	52.6, CH	1.97 m
7	128.9, CH	5.64 dd (15.6, 9.6)	129.0, CH	5.64 dd (15.6, 9.0)	27.3, CH_2	1.81, 1.64 m	26.9, CH_2	1.97, 1.63 m
8	138.6, CH	5.79 dd (15.6, 6.6)	138.4, CH	5.77 dd (15.6, 6.6)	38.3, CH_2	1.64, 1.64 m	38.0, CH_2	1.63, 1.52 m
9	76.6, CH	4.42 m	76.9, CH	4.42 m	75.3, CH	3.90 m	75.1, CH	3.84 m
10	21.2, CH_3	1.31 d (6.6)	21.2, CH_3	1.30 d (6.0)	20.0, CH_3	1.20 d (6.0)	20.0, CH_3	1.19 d (6.0)
11	27.6, CH_3	0.98 s	27.8, CH_3	1.01 s	27.8, CH_3	1.10 s	27.8, CH_3	1.10 s
12	28.2, CH_3	1.03 s	28.3, CH_3	1.03 s	29.2, CH_3	1.02 s	29.3, CH_3	1.01 s
13	24.2, CH_3	1.96 d (0.6)	24.0, CH_3	1.94 s	25.3, CH_3	2.05 d (0.6)	25.2, CH_3	2.05 s
1′	100.9, CH	4.43 d (7.8)	101.0, CH	4.43 d (7.8)	100.5, CH	4.41 d (7.8)	100.5, CH	4.41 d (7.8)
2′	78.8, CH	3.40 m	78.9, CH	3.39 m	78.6, CH	3.38 m	78.5, CH	3.37 m
3′	79.6, CH	3.48 t (9)	79.7, CH	3.46 t (9)	79.8, CH	3.47 t (9)	79.8, CH	3.47 t (9)
4′	71.9, CH	3.28 d (9)	71.9, CH	3.28 d (9.6)	72.2, CH	3.24 m	72.2, CH	3.25 m
5′	78.0, CH	3.21 m	78.0, CH	3.22 m	77.9,CH	3.24 m	78.0, CH	3.25 m
6′	62.9, CH_2	3.83 dd (12, 2.4), 3.66 m	62.9, CH_2	3.83 dd (11.4, 2.4), 3.65 m	63.2, CH_2	3.87, 3.63 m	63.1, CH_2	3.86, 3.64 m
1″	102.1, CH	5.24 d (1.2)	102.2, CH	5.25 br s	102.1, CH	5.23 d (1.2)	102.0, CH	5.24 s
2″	72.5, CH	3.91 m	72.5, CH	3.91 m	72.5, CH	3.90 m	72.5, CH	3.84 m
3″	72.4, CH	3.66 m	72.4, CH	3.65 m	72.5, CH	3.63 m	72.4, CH	3.64 m
4″	74.1, CH	3.38 m	74.1, CH	3.39 m	74.2, CH	3.38 m	74.1, CH	3.37 m
5″	69.9, CH	4.06 m	69.9, CH	4.04 m	69.8, CH	4.10 (m)	69.8, CH	4.09 m
6″	18.3, CH_3	1.20 d (6.6)	18.3, CH_3	1.22 d (6.6)	18.3, CH_3	1.20 d (6.0)	18.3, CH_3	1.19 d (6.0)

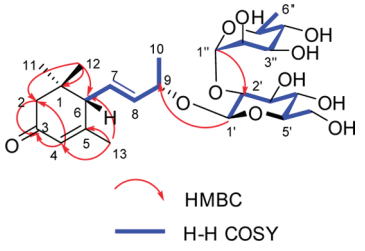

Figure 1. Key HMBC and COSY correlations of **1**.

NMR data of **1** identified three isolated proton spin-systems corresponding to C-6−C-10, C-1′−C-6′, and C-1″−C-6″ units, and the remaining connections were established by analysis of HMBC correlations. The HMBC correlations from Me-11 and Me-12 to C-1, C-2, and C-6 indicated that C-11, C-12, C-2, and C-6 were all connected with C-1, whereas correlations from H-4 to C-2 and C-3 and from H-2 to C-3 led to the connection of C-3 with C-2 and C-4. The cross-peaks of Me-13 with C-4, C-5, and C-6 in the HMBC spectrum demonstrated that C-5 was connected with C-4, C-6, and C-13, which completed a cyclohexanone unit (Figure 1). The fragment C-7−C-10 connected to the cyclohexanone ring in **1** was established by ^{1}H−^{1}H COSY correlations, which revealed that **1** possessed a 3-oxo-α-ionol moiety.[18−20]

Acid hydrolysis of **1** afforded D-glucose and L-rhamnose, which were identified by GC analysis with authentic samples.[21] This was confirmed by daughter ions at m/z 393 ($[M + Na - 146]^+$) from m/z 539 and at m/z 231 $[M + Na - 146 - 162]^+$ from m/z 393 in the tandem MS data. The J values of the anomeric protons (H-1′ and H-1″) established the sugar units as β-D-glucopyranosyl (J = 7.8 Hz) and α-L-rhamnopyranosyl (J = 1.2 Hz) in **1**. The connection of the sugar residues in **1** was determined by the HMBC correlations. The correlations from H-1′ to C-9, and in turn from H-9 to C-1′, established the connection of the glucopyranosyl moiety to C-9 of the aglycone, whereas those from H-2′ to C-1″, and in turn from H-1″ to C-2′, demonstrated that the rhamnopyranosyl unit was linked to C-2′ of the glucopyranosyl moiety. Thus, the planar structure of **1** was characterized as shown.

The absolute configuration of **1** was determined by analysis of its coupling constant, CD spectrum, and chemical shift values compared to those of its analogues. The large coupling constant (J = 16 Hz) between H-7 and H-8 indicated the E configuration of the C-7−C-8 double bond. The CD spectrum of **1** showed a negative Cotton effect at 244 nm, which was similar to that of physanoside B, indicating the 6S configuration.[19,20] It was reported that the chemical shifts of C-9 and C-10 were indicative for 9R (ca. δ_9 77.3−79.1, δ_{10} 21.2−21.8) and 9S (ca. δ_9 74.7−76.3, δ_{10} 22.3−22.6) configurations for $\Delta^{7,8}$ types of 9-hydroxymegastigamane 9-O-β-D-glucopyranosides.[22−26] Thus, the absolute configuration at C-9 was tentatively assigned as R on the basis of the diagnostic chemical shifts of C-9 (δ 76.6) and C-10 (δ 21.2) in the ^{13}C NMR spectrum. Hence, the structure of **1** was elucidated as (6S,9R)-3-oxo-α-ionyl-9-O-α-L-rhamnopyranosyl-(1″→2′)-β-D-glucopyranoside.

Gusanlungionoside B (**2**) was obtained as an amorphous powder and displayed similar UV and IR profiles to those of **1**. The HRESIMS of **2** gave a pseudomolecular ion peak at m/z 539.2484 $[M + Na]^+$. The ^{1}H, ^{13}C NMR, HSQC, ^{1}H−^{1}H COSY, and HMBC spectra revealed that **2** had the same planar structure as **1** and was a stereoisomer of **1**. The β-D-glucopyranosyl and α-L-rhamnopyranosyl units were also determined via tandem MS, ^{1}H and ^{13}C NMR, and acid hydrolysis. The chemical shift values of C-9 (δ 76.9) and C-10 (δ 21.2) in **2** were similar to those of **1**, indicating the 9R configuration in **2**. Thus, **2** must have a different absolute configuration at C-6 compared to **1**. The CD spectrum of **2** showed a positive Cotton effect at 242 nm, similar to that of eriojaposide A[24] and (6R,9S)-3-oxo-α-ionol β-D-glucopyranoside.[22] This confirmed the 6R absolute configuration in **2**. Thus, the structure of **2** was

Table 2. Mushroom Tyrosinase Inhibitory Activity of Gusanlungionosides A−D (1−4)

compound	IC_{50} (mM)[a]
1	0.15 ± 0.01
2	0.16 ± 0.02
3	0.20 ± 0.02
4	0.19 ± 0.01
kojic acid[b]	0.37 ± 0.02

[a] Each value is expressed as the mean ± SD (n = 3). [b] Positive control.

established as (6*R*,9*R*)-3-oxo-α-ionyl-9-*O*-α-L-rhamnopyranosyl-(1′′→2′)-β-D-glucopyranoside.

Gusanlungionosides C (**3**) and D (**4**) were isolated as two amorphous powders and displayed similar UV and IR profiles to those of **1** and **2**. Their HRESIMS [m/z 541.2653 ($[M + Na]^+$)] showed that **3** and **4** had the same molecular formula, $C_{25}H_{42}O_{11}$, with two hydrogen atoms more than those of **1** and **2**. This implied that there was one unsaturation degree less in **3** and **4**. Analysis of the ^1H and ^{13}C NMR data revealed that **3** and **4** had similar structural fragments to those of **1** and **2** except for two more methylene units instead of a C-7/C-8 double bond. This observation was further confirmed by analysis of the $^1H-^1H$ COSY and HMBC data. Thus, the planar structures of **3** and **4** were determined as shown.

Similarly, the absolute configuration of C-9 in **3** and **4** was elucidated as *R* due to their diagnostic chemical shifts of C-9 and C-10 in the ^{13}C NMR spectra.[25] The CD spectra of **3** and **4** assigned the absolute configuration of C-6. The CD spectrum of **4** showed a positive Cotton effect at 210 nm ($\Delta\varepsilon$ + 25.9 mdeg), which was similar to that of byzantionoside B (6*R*),[25] whereas the CD spectrum of **3** showed a negative Cotton effect at 210 nm ($\Delta\varepsilon$ −15.6 mdeg). Thus, the absolute configuration of C-6 was identified as 6*S* for **3** and 6*R* for **4**. The β-D-glucopyranosyl and α-L-rhamnopyranosyl units were also determined via ^1H and ^{13}C NMR and acid hydrolysis. Thus, the structures of **3** and **4** were assigned as (6*S*,9*R*)-9-hydroxymegastigman-4-en-3-one 9-*O*-α-L-rhamnopyranosyl-(1′′→2′)-β-D-glucopyranoside and (6*R*,9*R*)-9-hydroxymegastigman-4-en-3-one 9-*O*-α-L-rhamnopyranosyl-(1′′→2′)-β-D-glucopyranoside, respectively.

Most megastigmane glycosides isolated previously from diverse plants have the 1′′→6′ linkage in their disaccharide unit. Gusanlungionosides A−D (**1**−**4**) have a rare α-L-rhamnopyranosyl-(1′′→2′)-β-D-glucopyranoside disaccharide moiety. Only one analogue was reported with a (1′′→2′) disaccharide connection, (6*S*,9*R*)-vomifoliol-9-*O*-α-L-rhamnopyranosyl-(1′′→2′)-β-D-glucopyranoside, and was isolated from *Zizyphi fructus*.[27] Furthermore, this is the first report of the isolation of 9-hydroxymegastigman-4-en-3-one with a 6*S* configuration, even though the corresponding diastereomer with 6*R* configuration (blumenol C) was previously isolated from the aerial parts of *Stachys byzantina*.[28]

All isolated compounds were evaluated against the mushroom tyrosinase activity in vitro with kojic acid as a positive control. Compounds **1**−**4** displayed strong inhibitory activity against tyrosinase with IC_{50} values of 0.15, 0.16, 0.2, and 0.19 mM, respectively, whereas the IC_{50} value of kojic acid was 0.37 mM (Table 2). However, compounds **5**−**14** were inactive (IC_{50} > 1 mM).

The cellular melanin contents and tyrosinase activity in α-MSH-stimulated B16F10 mouse melanoma cells treated with compounds **1**−**4** were evaluated (Figure 2) All the compounds exhibited significant inhibition on melanogenesis but no cytotoxic effects on B16F10 mouse melanoma cells at concentrations up to 200 μg mL^{-1} (data not shown).

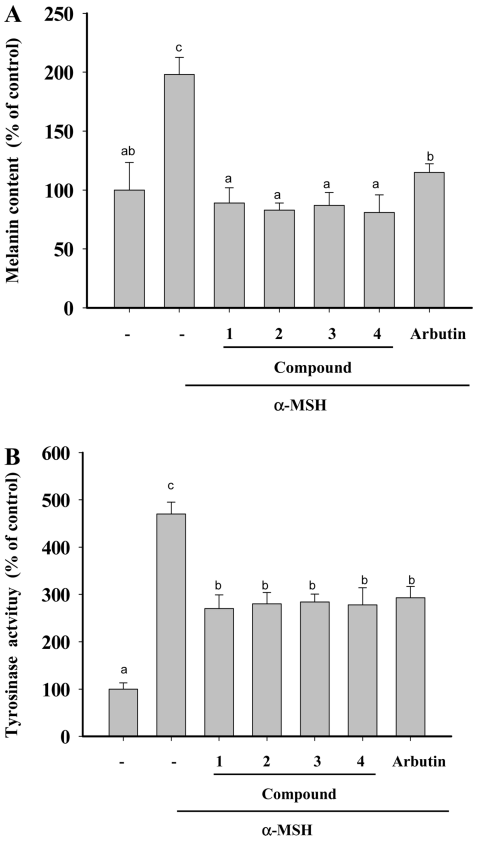

Figure 2. Effects of compounds **1**−**4** on melanin content and cellular tyrosinase activity of B16F10 mouse melanoma cells. Each value is expressed as the mean ± SD (n = 3). Values marked by the same letter are not significantly different (p > 0.05). Values marked "a", "b", and "c" mean significantly different (p < 0.05) compared with each other. Value marked "ab" means that group is not significantly different from the groups marked "a" and "b", but significantly different from the group marked "c".

The gene expression in the α-MSH-stimulated B16F10 mouse melanoma cells in the presence of test sample was analyzed by RT-PCR (Figure 3). The result indicated that the level of tyrosinase mRNA was strongly decreased by treatment with compounds **1**−**4**, suggesting that their antimelanogenic activity was probably associated with suppression of tyrosinase gene expression.

In conclusion, gusanlungionosides A−D (**1**−**4**) suppress not only mushroom tyrosinase activity in vitro but melanogensis in cells. This is the first report of megastigmane derivatives displaying significant inhibitory activity against tyrosinase.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR spectra were recorded on a Shimadzu FTIR-8400s. UV spectra were run on a Shimadzu UV-2550 UV−vis spectrophotometer. CD spectra were measured on a JASCO J-810 spectrometer. 1D and 2D NMR spectra were measured in methanol-d_4 (δ_H 3.30/δ_C 49.5) on a Bruker Avance III 600 spectrometer (^1H: 600 MHz, ^{13}C: 150 MHz). HRESIMS were obtained using a LTQ Orbitrap XL spectrometer. GC was performed on an Agilent 6890N (FID, NPD). Analytical HPLC was performed on a Waters 600 with a Waters 2996 photodiode array detector. Semipreparative HPLC was performed on a Shimadzu LC-6AD with a Shimadzu SPD-6AD spectrophotometric detector.

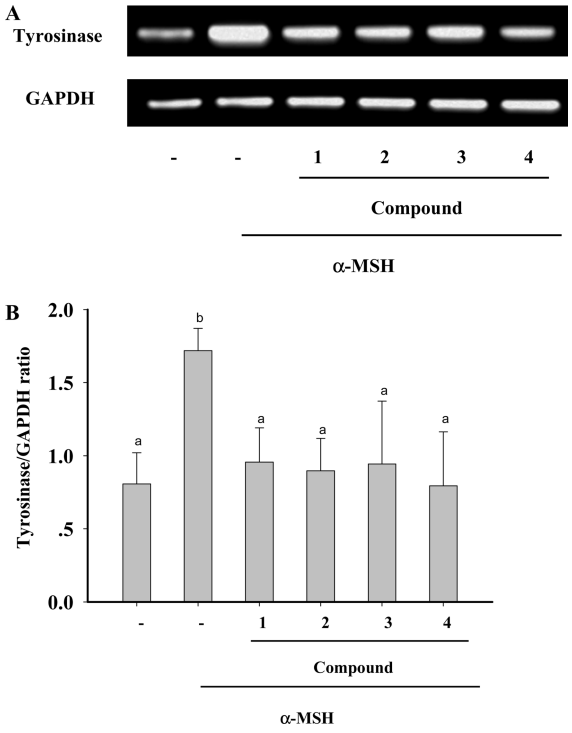

Figure 3. RT-PCR analysis of tyrosinase mRNA expression in B16F10 mouse melanoma cells. GAPDH was used as an internal control. (A) Typical results of agarose gel electrophoresis. (B) The ratio of tyrosinase to GAPDH expression was calculated as the proportion of the tyrosinase PCR product intensity to that of the GAPDH product by densitometric measurement. This experiment was repeated separately three times. Values with the same superscript letters are not significantly different from each other at $p < 0.05$.

α-Melanocyte-stimulating hormones (α-MSH), L-3,4-dihydroxyphenylalanine (L-DOPA), mushroom tyrosinase, and Triton X-100 were purchased from Sigma (St, Louis, MO, USA). Arbutin was purchased from Alfa Aesar. The primer sequences of the investigated genes were obtained from Bioneer (Seoul, Korea). DMEM medium without phenol-red, trypsin-EDTA, and fetal bovine serum (FBS) were acquired from Gibco BRL (Grand Island, NY, USA). The culture supplies were obtained from SPL Brand Products (SPL, Suwon, Korea). All other chemicals were of analytical grade. B16F10 mouse melanoma cells were purchased from the Korean Cell Line Bank (Seoul, Korea).

Plant Material. The stems of *A. gusanlung* were collected from Wanning city in Hainan Province of People's Republic of China in August 2008. The sample was identified by Prof. Guobiao Chen from the Institute for Drug Control of Hainan Province. A voucher specimen (No. 200808) was deposited in the herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing.

Extraction and Isolation. The air-dried and smashed stems of *A. gusanlung* (18 kg) were extracted with MeOH (3 × 80 L) and afforded a crude extract of 880 g after evaporation of the solvent under vacuum. The extract was suspended in H_2O (2.0 L) and partitioned sequentially with petroleum ether (3 × 3.0 L), EtOAc (3 × 3.0 L), and *n*-BuOH (3 × 3.0 L). The *n*-BuOH extract (630 g) was subjected to column chromatography over macroporous resin D101 and eluted successively with EtOH−H_2O (1:9, 3:7, 6:4, and 1:0) to yield four fractions (B1 to B4). Fraction B2 (40 g) was subjected to chromatography over silica gel (400 g, 100−200 mesh) and eluted with CH_2Cl_2−MeOH to yield 12 fractions (B2-1 to B2-12) on the basis of TLC and HPLC-DAD analyses. Fraction B2-10 (3.0 g) was subjected to chromatography over silica gel (120 g, 200−300 mesh) and eluted with CH_2Cl_2−MeOH to yield 15 subfractions (B2-10-1 to B2-10-15). Subfraction B2-10-11 (300 mg) was purified further by semipreparative HPLC (40% aqueous MeOH) to afford **1** (51 mg), **2** (82 mg), **3** (32 mg), and **4** (21 mg). Subfraction B2-10-10 (800 mg) was chromatographed over silica gel (40 g, 200−300 mesh) and eluted with CH_2Cl_2−MeOH (100:1 to 1:1). The CH_2Cl_2−MeOH (6:4) elute was further separated on Sephadex LH-20 (MeOH) followed by semipreparative HPLC (35% aqueous MeOH) to give compounds **11** (11 mg), **12** (16 mg), **13** (23 mg), and **14** (27 mg). Fraction B1 (30 g) was chromatographed over silica gel (300 g, 100−200 mesh), using a gradient of CH_2Cl_2−MeOH (from 100:0 to 0:100), to yield 12 fractions (B1-1 to B1-10). Subfraction B1-3 (700 mg) was further chromatographed over silica gel (70 g, 200−300 mesh) and eluted with CH_2Cl_2−Me_2CO (100:0 to 10:1). The CH_2Cl_2−Me_2CO (100:5) elute was further purified on Sephadex LH-20 (MeOH) followed by semipreparative HPLC (50% aqueous MeOH) to give **6** (52 mg), **7** (11 mg), **8** (8 mg), **9** (17 mg), and **10** (16 mg). Repeated crystallization of fraction B1-7 (MeOH) yielded **5** (10 mg).

Gusanlungionoside A (***1***): amorphous powder; $[\alpha]^{20}_D$ −125.9 (*c* 0.06, MeOH); UV (MeOH) λ_{max} 238.0 nm; IR ν_{max} (KBr) 3409 (OH), 2933, 1653 (C=O), 1070, 1043 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (nm) −188.9 (244); ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 539 $[M + Na]^+$, 393 $[M - rha + Na]^+$, 231 $[M - rha - glc + Na]^+$; HRESIMS *m/z* 539.2484 $[M + Na]^+$ (calcd for $C_{25}H_{40}O_{11}Na$, 539.2468).

Gusanlungionoside B (***2***): amorphous powder; $[\alpha]^{20}_D$ +79.3 (*c* 0.09, MeOH); UV (MeOH) λ_{max} 237.5 nm; IR ν_{max} (KBr) 3407 (OH), 2933, 1654 (C=O), 1070, 1043 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (nm) +126.8 (242); ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 539 $[M + Na]^+$, 393 $[M - rha + Na]^+$, 231 $[M - rha - glc + Na]^+$; HRESIMS *m/z* 539.2484 $[M + Na]^+$ (calcd for $C_{25}H_{40}O_{11}Na$, 539.2468).

Gusanlungionoside C (***3***): amorphous powder; $[\alpha]^{20}_D$ −7.4 (*c* 0.09, MeOH); UV (MeOH) λ_{max} 240.5 nm; IR ν_{max} (KBr) 3409 (OH), 2933, 1653 (C=O), 1077, 1044 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (nm) −15.6 (212); ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 541 $[M + Na]^+$, 395 $[M - rha + Na]^+$, 233 $[M - rha - glc + Na]^+$; HRESIMS *m/z* 541.2642 $[M + Na]^+$ (calcd for $C_{25}H_{42}O_{11}Na$, 541.2641).

Gusanlungionoside D (***4***): amorphous powder; $[\alpha]^{20}_D$ −3.2 (*c* 0.09, MeOH); UV (MeOH) λ_{max} 240.5 nm; IR ν_{max} (KBr) 3407 (OH), 2933, 1651 (C=O), 1077, 1044 cm^{-1}; CD (MeOH) $\Delta\varepsilon$ (nm) +25.9 (210); ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 541 $[M + Na]^+$, 395 $[M - rha + Na]^+$, 233 $[M - rha - glc + Na]^+$; HRESIMS *m/z* 541.2642 $[M + Na]^+$ (calcd for $C_{25}H_{42}O_{11}Na$, 541.2641).

Acid Hydrolysis of 1−4 (ref 21). A solution of each compound (5 mg) in 0.21 N HCl (5 mL) was heated at 90 °C for 5 h. After extraction with EtOAc (3 × 5 mL), each reaction mixture was diluted with H_2O and extracted with EtOAc (3 × 5 mL). The aqueous layer was evaporated and cryodesiccated, and the residue was analyzed by TLC over silica gel ($CHCl_3$−MeOH−H_2O, 6:4:1) and comparison with authentic samples. The sugar residue was dissolved in pyridine (1 mL), and then 1 mL of HMDS−TMCS (hexamethyldisilazane−trimethylchlorosilane, 2:1) was added. The mixture was stirred at 60 °C for 30 min. After centrifugation, the supernatant was analyzed by GC under the following conditions: capillary column, Agilent DB-5 (0.25 mm × 30 m × 0.25 μm); detection, FID; detector temperature, 280 °C; injection temperature, 250 °C; carrier, N_2 gas. By comparing the retention time (t_R) of the monosaccharide derivatives with those of the standard samples, the absolute configuration of the monosaccharides in **1**−**4** was confirmed to be L-rhamnose (t_R 6.17 min) and D-glucose (t_R 12.73 min).

Mushroom Tyrosinase Assay. The activity evaluation of compounds on mushroom tyrosinase was carried out by a method described previously, with minor modifications.[29] Briefly, 80 μL of phosphate buffer (pH 6.8), 40 μL of 25 mM L-DOPA, and 40 μL of test samples were added to a 96-well microplate, followed by mixing, and then 40 μL of mushroom tyrosinase (500 U mL^{-1}) was added. After incubation at

25 °C for 10 min, the amount of dopachrome was determined by measuring the absorbance at 490 nm in an ELISA reader (ELx800TM, BioTek, USA). Kojic acid was used as a positive control. The extent of tyrosinase inhibition is expressed as the concentration necessary for 50% inhibition (IC_{50}), which was calculated for each substance from the concentration−response curve. Experiments were repeated three times ($n = 3$), and the average IC_{50} was reported. Data were calculated as the percentage of inhibition by the following formula:

Inhibition (%) = $[(A_{\text{test sample}} - A_{\text{blank}})/A_{\text{control}}) \times 100]$ %, where A_{control} is the OD value at 490 nm with enzyme, but without test sample; A_{blank} is the OD value at 490 nm with test sample, but without enzyme; and $A_{\text{test sample}}$ is the OD value at 490 nm with test sample and enzyme.

Determination of Melanin Content in B16F10 Mouse Melanoma Cells. B16F10 mouse melanoma cells were grown in DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. B16F10 mouse melanoma cells (2×10^5 cells) were incubated in a six-well plate. The intracellular melanin contents were measured by a previously established procedure, with minor modifications.[30] After 16 h incubation, cells were pretreated with compounds at a concentration of 100 μg mL^{-1} for 30 min before treatment with α-MSH (250 nM, final concentration) and incubation for 48 h. After incubation, the supernatant medium of each well was removed and washed twice with cold phosphate-buffered saline (PBS), and the cell pellet was dissolved in 0.5 mL of 1 M NaOH at 90 °C for 60 min. The absorbance at 405 nm was determined in an ELISA reader (ELx800TM, BioTek, USA). Arbutin was used as a positive standard.

Assay of Cellular Tyrosinase Activity. Tyrosinase activity in B16F10 mouse melanoma cells was determined by measuring the rate of oxidation of L-DOPA.[31] B16F10 mouse melanoma cells (4×10^5 cells) were treated in the same way as described above. The cell pellet was lysed in 0.2 mL of PBS buffer (pH 6.8) containing 1% (w/v) Triton X-100. Each cell extract (100 μL) was placed in a 96-well plate, and the enzymatic assay was commenced by adding 100 μL of L-DOPA (2 mM); the amount of dopachrome formed was determined by measuring the absorbance at 490 in an ELISA reader (ELx800TM, BioTek, USA) after 60 min at 37 °C. Results from samples were analyzed as a percentage of the control.

RNA Preparation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). RT-PCR was used to analyze gene expression in the B16F10 mouse melanoma cells after stimulation with α-MSH in the presence of test sample for 48 h. B16F10 mouse melanoma cells (2×10^5 cells) were treated in the same way as described above. Total RNA was isolated with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from the total RNA (2 μg) containing oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen). The primer sequences for GAPDH and tyrosinase were as follows: GAPDH sense 5′-CAC TCA CGG CAA ATT CAA CGG CAC-3′, antisense 5′-GAC TCC ACG ACA TAC TCA GCA C-3′; tyrosinase sense 5′-GGC CAG CTT TCA GGC AGA GGT-3′, antisense 5′-TGG TGC TTC ATG GGC AAA ATC-3′. The PCR reaction involved an initial 5 min denaturation at 94 °C, followed by 25 cycles at 94 °C, 30 s; 55−60 °C, 30 s; 72 °C, 1 min; and a final 7 min extension. Aliquots of individual PCR products were separated on 1% agarose gel, stained with ethidium bromide, and imaged by a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). Densitometric analysis was done using image analysis software (GelQuant, DNR Bio-Imaging Systems Ltd.).

Data Analysis. All tests were carried out independently in triplicate ($n = 3$). The data are expressed as the mean ± standard derivation (SD). One-way analysis of variance (ANOVA) was performed to test the difference between the means. All analyses were performed using SPSS 16 (SPSS Institute, Cary, NC, USA).

■ ASSOCIATED CONTENT

Supporting Information. NMR and CD spectra of gusanlungionosides A−D. This information is available free of charge via the Internet at http://pubs.acs.org.

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