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A NEW PHENOLIC COMPOUND FROM *SALIX GLANDULOSA*

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Abstract – A new phenolic compound, saliglandol (**1**), was isolated from *Salix glandulosa* along with 22 known ones. Their chemical structures were established through analyzing spectroscopic data. Comparison of calculated and experimental ECD spectra of **1** resulted in assignment of its absolute configuration. All isolated compounds (**1**–**23**) were evaluated for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, an BT549), anti-inflammatory effects measuring nitric oxide (NO) levels in lipopolysaccharide (LPS)-stimulated murine microglia BV2 cells, and potential neuroprotective effects through induction of nerve growth factor (NGF) in C6 glioma cells.

Willows are deciduous trees and constitute the genus *Salix* (Salicaceae), which is well known for producing salicin, a precursor of the potent anti-inflammatory drug acetylsalicylic acid (aspirin). The bark extracts of willows have been used in traditional medicine for treating fever, pain, and inflammation.[1] According to the previous studies, several plants in the genus *Salix* have been reported to produce phenolic compounds, lignans, flavonoids, and terpenoids exhibiting cytotoxic, neuroprotective, and antiplasmodial activities.[2-6] However, there have been few investigations on the bioactive secondary metabolites from *Salix glandulosa* Seemen.[7] We previously reported salicin derivatives and phenolic compounds, and their biological activities from this source,[8,9] and herein, further 23 compounds were isolated including a new phenolic compound (**1**) (Figure 1). Their chemical structures were elucidated

through conventional spectroscopic data analysis coupled with computational methods. All these phytochemicals (**1**–**23**) were tested for the cytotoxic, anti-inflammatory, and neuroprotective activities.

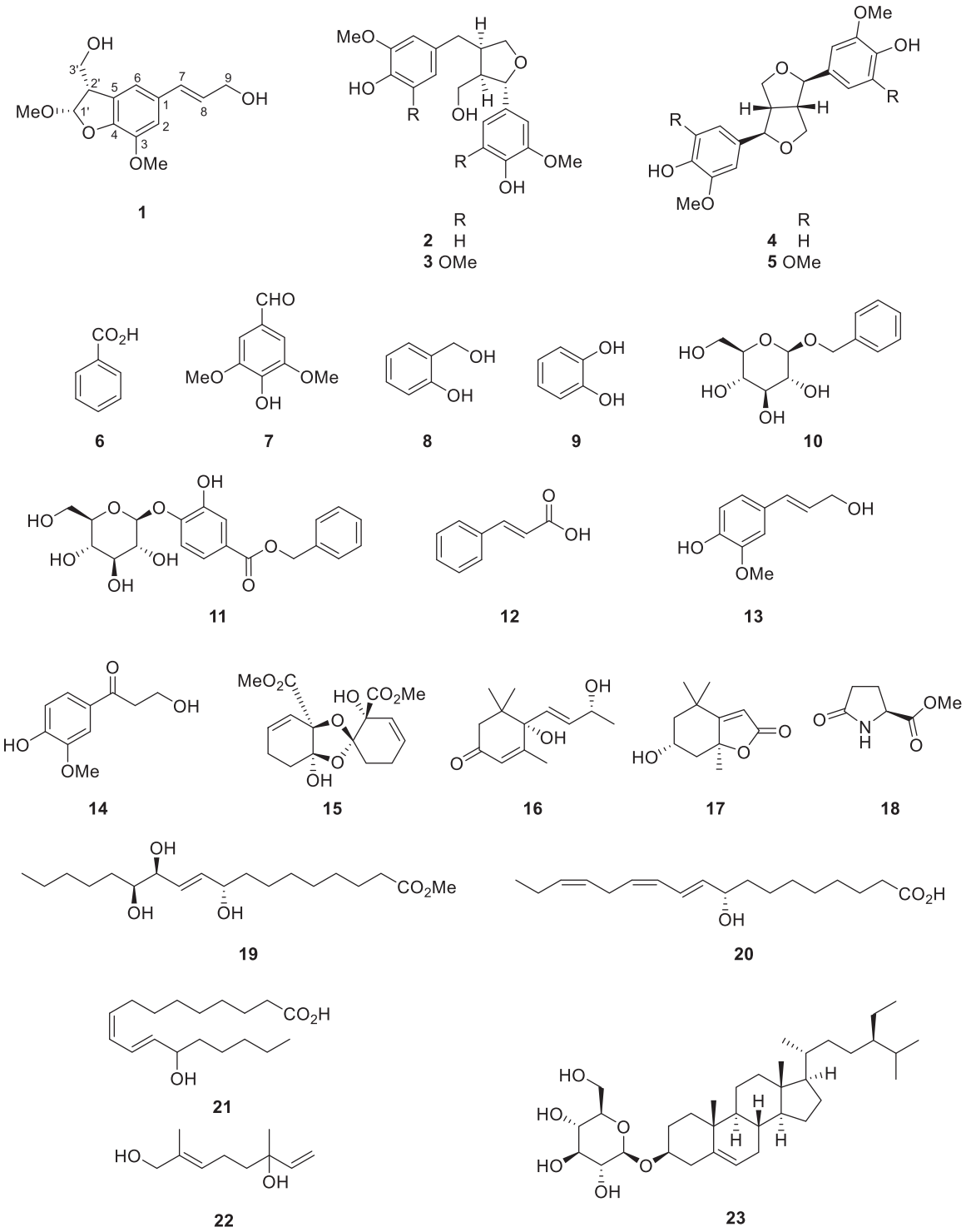

Figure 1. Structures of compounds **1**–**23**

Saliglandol (**1**) was isolated as a colorless gum and its molecular formula was deduced as $C_{14}H_{18}O_5$ from the $[M + H]^+$ ion peak at *m/z* 267.1222 $[M + H]^+$ (calcd. for $C_{14}H_{19}O_5^+$, 267.1227) on HRESIMS. The ^1H

NMR spectrum of **1** showed two aromatic [δ_H 6.86 and 6.84 (each 1H, brs)], two *trans*-coupled olefinic [δ_H 6.54 (1H, d, J = 15.7 Hz) and 6.22 (1H, dt, J = 15.7 and 5.9 Hz)], six methylene or methine [δ_H 5.77 (1H, d, J = 6.7 Hz), 4.29 (2H, d, J = 5.9 Hz), 4.04 (2H, m), and 3.66 (1H, overlap)], and two methoxy [δ_H 3.90 and 3.63 (each 3H, s)] protons. The ^{13}C NMR spectrum of **1** displayed total 15 peaks, eight of which peaks supporting the presence of an aromatic ring and a double bond (δ_C 110.3–146.3). These NMR data (Table 1) of **1** was almost identical with those of capstemol isolated from *Capsicum annuum* var. *angulosum*,[10] and extensive analysis of 2D NMR data confirmed that planar structures and relative configuration of **1** and capstemol were the same as shown in Figure 1. The relatively large chemical shift difference of the H-1′ in **1** (δ_H 5.77) and that in capstemol (δ_H 6.58) might be caused by different sample concentrations and/or NMR measuring temperatures.[11] For the assignment of the absolute configuration of **1**, the experimental ECD spectrum of **1** was compared to the calculated ECD spectra of two plausible enantiomers of **1** (Figure 2). The experimental ECD spectrum of **1** showed negative Cotton effect at 206, 217, and 227 nm, which corresponded to the calculated ECD spectrum of 1′*S*,2′*S* form of **1**. Consequently, the absolute configurations of C-1′ and C-2′ in **1** were assigned as both *S* and the full structure of **1** was thus established. Notably, since the specific optical rotation values of saliglandol (**1**, $[\alpha]_D^{25}$ –10) and capstemol ($[\alpha]_D^{25}$ +2.7) were opposite each other we proposed that capstemol is an enantiomer of saliglandol (**1**) possessing 1′*R*,2′*R* configuration.

Table 1. ^{1}H [ppm, mult. (J in Hz)] and ^{13}C NMR spectroscopic data of compound **1** in methanol-d_4

position	δ_H	δ_C
1		131.4
2	6.84, brs	110.3
3		144.5
4		146.3
5		127.5
6	6.86, brs	114.8
7	6.54, d (15.7)	131.3
8	6.22, dt (15.7, 5.9)	126.6
9	4.29, d (5.9)	63.9
1′	5.77, d (6.7)	109.8
2′	3.66, overlap	48.2
3′	4.04, m	60.4
OCH_3-3	3.90, s	56.0
OCH_3-1′	3.63, s	57.1

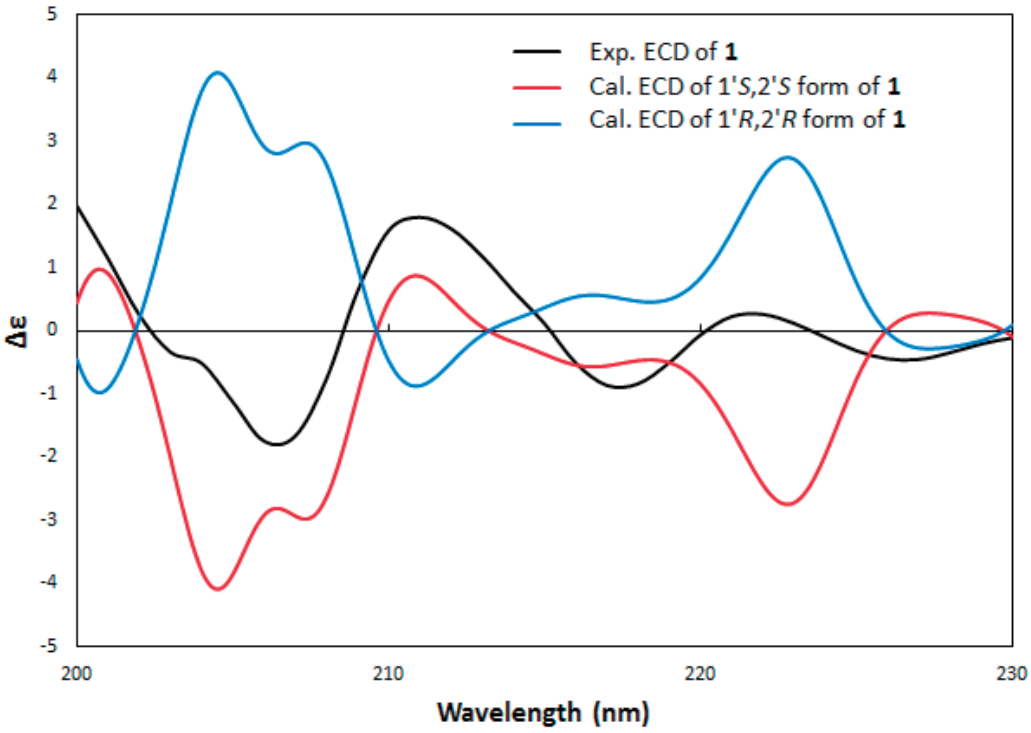

Figure 2. Experimental and calculated ECD spectra of **1**

The 22 known metabolites were characterized as (+)-lariciresinol (**2**),[12] (+)-5,5′-dimethoxylariciresinol (**3**),[13] (+)-pinoresinol (**4**),[14] (+)-syringaresinol (**5**),[15] benzoic acid (**6**),[16] syringaldehyde (**7**),[17] salicyl alcohol (**8**),[18] catechol (**9**),[19] benzyl-*O*-β-D-glucopyranoside (**10**),[20] glochierioside F (**11**),[21] (*E*)-cinnamic acid (**12**),[22] coniferyl alcohol (**13**),[23] ω-hydroxypropioguaiacone (**14**),[24] (–)-idesolide (**15**),[25,26] vomifoliol (**16**),[27] loliolide (**17**),[28] methyl (*R*)-pyroglutamate (**18**),[29] methyl (9*S*,12*S*,13*S*)-9,12,13-trihydroxy-10*E*-octadecenoate (**19**),[30] (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoic acid (**20**),[31] 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (**21**),[32] (*E*)-8-hydroxylinalool (**22**),[33] and sitosterol-3-*O*-β-D-glycoside (**23**)[34] by comparison of the spectroscopic data with those previously reported. Compound **15**, (–)-idesolide, was to the best of our knowledge isolated from the natural source for the second time except for the fruit of *Idesia polycarpa*.[25]

The cytotoxic activities of the isolated compounds (**1**–**23**) were evaluated against four human cancer cell lines such as A549 (non-small-cell adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and BT549 (breast carcinoma). As shown in Table 2, compounds **5**, **9**, and **15** exhibited moderate to potent cytotoxicity with IC_{50} values ranging from 1.86 to 23.76 μM. Catechol (**9**), the degradation product of salicortin,[35] which was also isolated from the same source,[8] showed potent cytotoxicity against SK-MEL-2 cells (IC_{50} 1.86 μM) comparable with that of cisplatin, the positive control (IC_{50} 1.47 μM). This compound exhibited moderate activity against the other three cancer cell lines (IC_{50} 10.38–16.54 μM). (–)-Idesolide (**15**) exhibited potent and mild activity against SK-MEL-2

(IC_{50} 8.57 μM) and SK-OV-3 (IC_{50} 23.76 μM) cells, respectively. The other compounds tested were inactive (IC_{50} > 30 μM).

Table 2. Cytotoxicity of compounds **5**, **9**, and **15** against four cultured human cancer cell lines in the SRB bioassay

compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	BT549
5	>30.0	13.81	12.29	>30.0
9	13.62	10.38	1.86	16.54
15	>30.0	23.76	8.57	>30.0
Cisplatin[b]	2.64	2.90	1.47	5.68

[a]50% inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth. [b]Cisplatin was used as a positive control.

The anti-inflammatory effects of the isolates (**1**–**23**) were assessed through the measurement of NO levels in LPS-stimulated BV-2 cells. Compounds **5**, **6**, **9**, **15**, and **22** inhibited NO production with IC_{50} values in the range of 10.39–19.34 μM, which were stronger than that of L-NMMA (IC_{50} 21.82 μM), the positive control, without significant cell toxicity at 20 μM (Table 3). The strong inhibitory activity of (–)-idesolide (**15**) is consistent with those in the previous studies.[25,36] Interestingly, (+)-pinoresinol (**4**) displayed weak activity (IC_{50} 71.66 μM), whereas its dimethoxylated derivative **5** showed potent activity (IC_{50} 16.21 μM). Compounds **2**–**4**, **13**, **14**, and **16** exhibited moderate or weak activity (IC_{50} 43.39–81.66 μM) and the others were inactive (IC_{50} > 100 μM).

Table 3. Inhibitory effect of selected compounds on NO production in LPS-activated BV-2 cells

compound	IC_{50} (μM)[a]	cell viability (%)[b]
2	76.66	111.58 ± 9.46
3	43.39	130.51 ± 8.36
4	71.66	97.20 ± 7.97
5	16.21	84.19 ± 10.72
6	13.82	93.00 ± 17.74
9	10.39	112.00 ± 10.00
13	81.66	96.66 ± 6.59
14	45.37	85.36 ± 8.95
15	14.68	125.74 ± 3.66
16	52.70	102.44 ± 6.45

22	19.34	101.73 ± 5.75
L-NMMA[c]	21.82	114.08 ± 2.96

[a]IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b]Cell viability following treatment with 20 μM of each compound was determined using the MTT assay and is expressed as a percentage (%). Data are expressed as the mean ± SD of three independent experiments.
[c]Positive control substance.

The neuroprotective activities of the metabolites (**1**–**23**, each 20 μM) were also tested by assessing their induction potentials on NGF secretion in C6 cells (Table 4). Catechol (**9**) was a powerful stimulant of NGF release (234.66 ± 10.35%), when compared with 6-shogaol, the positive control (168.58 ± 7.16%). Salicyl alcohol (**8**) and (–)-idesolide (**15**) also exhibited strong NGF secretion (162.35 ± 9.58% and 168.83 ± 9.23%) and the other compounds showed moderate activity (102.44 ± 11.25% – 150.67 ± 9.64%).

Table 4. Effects of selected compounds on NGF secretion in C6 cells

compound	NGF secretion[a] (%)	cell viability[b] (%)
1	141.89 ± 6.35	106.06 ± 4.56
2	131.68 ± 6.98	96.93 ± 7.90
3	149.07 ± 8.46	97.72 ± 5.99
4	102.44 ± 11.25	89.39 ± 0.32
5	114.88 ± 7.89	86.85 ± 1.96
6	119.12 ± 11.35	99.13 ± 0.12
7	136.71 ± 10.57	108.08 ± 0.51
8	162.35 ± 9.58	100.44 ± 0.38
9	234.66 ± 10.35	101.99 ± 2.39
10	110.96 ± 8.46	99.63 ± 1.63
11	112.20 ± 7.61	100.32 ± 0.41
12	111.99 ± 5.74	93.51 ± 0.63
13	137.22 ± 5.26	91.87 ± 5.99
14	133.00 ± 10.34	102.40 ± 6.18
15	168.83 ± 9.23	116.17 ± 1.22
16	144.90 ± 8.21	101.56 ± 1.50
17	122.05 ± 13.42	99.61 ± 0.01
18	134.61 ± 12.05	101.08 ± 6.40
19	124.74 ± 8.69	97.78 ± 0.17

20	139.27 ± 10.35	98.74 ± 0.21
21	150.67 ± 9.64	99.84 ± 2.25
22	124.14 ± 7.29	103.49 ± 2.00
23	122.28 ± 2.53	107.03 ± 10.65
6-shogaol[c]	168.58 ± 7.16	125.80 ± 0.93

[a]C6 cells were treated with 20 μM of each compound. After 24 h, the content of NGF secreted in the C6-conditioned medium was measured by ELISA. The level of secreted NGF is expressed as the percentage of the untreated control (set as 100%).
[b]Cell viability after treatment with 20 μM of each compound was determined by an MTT assay and is expressed as a percentage (%). Results are the means of three independent experiments, and the data are expressed as mean ± SD.
[c]Positive control substance.

Since (–)-idesolide (**15**) was first isolated from the fruit of *Idesia polycarpa* in 2005,[25] there have been several reports on its biological effects such as anti-inflammatory, anti-adipogenic, and anti-apoptotic activities *in vitro* and enhancement of recognition memory *in vivo*,[25,36-39] which are consistent with our current study on anti-inflammatory and neuroprotective activities of (–)-idesolide (**15**). However, the cytotoxic activities of this compound against cancer cell lines were not reported previously.

In this study, we have demonstrated the isolation and characterization of 23 compounds, including a new phenolic compound (**1**) from the twigs of *S. glandulosa* and their biological activities. The potent cytotoxic activity of (–)-idesolide (**15**) against human cancer cell line (SK-MEL-2), along with its previously reported bioactivities, suggest that this compound may be a good drug candidate.

EXPERIMENTAL

General. Optical rotation data were recorded utilizing a JASCO P-1020 polarimeter (JASCO, Easton, MD, USA). IR spectra were obtained utilizing a Bruker IFS-66/S Fourier-transform IR spectrometer (Bruker, Karlsruhe, Germany). UV spectra were garnered using a Shimadzu UV-1601 UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). NMR studies were accomplished employing a Bruker AVANCE III 700 NMR spectrometer and resultant spectra were processed using MestReNova (Mnova) 10.0 with default weighting functions. HRESIMS data were obtained using an Agilent iFunnel 6550 Q-TOF MS instrument. Semi-preparative HPLC was conducted utilizing a Gilson 306 pump (Gilson, Middleton, WI, USA) and a Shodex refractive index detector (Shodex, New York, NY, USA) with a Phenomenex Luna C18 10 μm column (10 × 250 mm, Phenomenex, Torrance, CA, USA) at a flow rate 2 mL/min. LPLC was accomplished employing a LiChroprep Lobar-A C-18 column (240 × 10 mm, 40–63 μm, Merck, Darmstadt, Germany) equipped with an FMI QSY-0 pump (Teledyne Isco, Lincoln, NE, USA). Open column chromatography was implemented with silica gel 60 (70–230 and 230–400 mesh;

Merck, Darmstadt, Germany) and RP-C18 silica gel (230–400 mesh, Merck, Darmstadt, Germany). Sephadex LH-20 (Pharmacia Co. Ltd.) was used as a packing material for molecular sieve column chromatography. TLC was carried out with precoated silica gel F254 plates and RP-18 F254s plates (Merck, Darmstadt, Germany).

Plant material. Twigs of *S. glandulosa* were collected in Gunwi, Korea in March 2013, and the plant was identified by one of the authors (K. R. L.). A voucher specimen (SKKU-NPL 1304) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

Extraction and isolation. The *S. glandulosa* twigs (10 kg) were extracted with 80% aqueous MeOH under reflux and filtered. The filtrate was evaporated under reduced pressure to yield a MeOH extract (630 g), which was suspended in distilled H_2O and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-butanol, yielding 7, 13, 65 and 100 g of each residue, respectively. The $CHCl_3$-soluble phase (13 g) was subjected to a silica gel open column ($CHCl_3$-MeOH, 20:1 → 1:1) to furnish 11 fractions (C1–C11). Fraction C2 (70 mg) was purified using semi-preparative HPLC, eluting with an isocratic system of 50% aqueous MeOH, to give compounds **4** (6 mg), **7** (6 mg), and **15** (15 mg). Compound **17** (3 mg) was isolated through purification of fraction C3 (300 mg) using semi-preparative HPLC (50% aqueous MeOH). Fraction C4 (1.8 g) was separated via an RP-C_{18} silica gel open column (60% aqueous MeOH) to yield 10 subfractions (C4-1–C4-10). Fraction C4-1 (500 mg) was applied to a Sephadex LH-20 column (CH_2Cl_2-MeOH, 1:1) to give five subfractions (C4-1-1–C-4-1-5). Fractions C4-1-2–C4-1-5 (80, 80, 90, and 270 mg, respectively) were purified by semi-preparative HPLC (35–40% aqueous MeOH) to yield compounds **1** (2 mg), **2** (5 mg), **3** (4 mg), **5** (2 mg), **8** (40 mg), **9** (5 mg), **13** (20 mg), and **14** (3 mg). Compound **16** (5 mg) was isolated from the fraction C4-2 (160 mg) via LPLC (45% aqueous MeCN) followed by semi-preparative HPLC (45% aqueous MeOH). Fraction C4-3 (200 mg) was separated successively using a Sephadex LH-20 column (CH_2Cl_2-MeOH, 1:1) and semi-preparative HPLC (25% aqueous MeCN) to give compounds **6** (3 mg) and **22** (3 mg). Compound **12** (5 mg) was purified via a Sephadex LH-20 column (CH_2Cl_2-MeOH, 1:1) followed by semi-preparative HPLC (48% aqueous MeCN) from the fraction C4-4 (80 mg). Compounds **19** (3 mg), **20** (2 mg), and **21** (3 mg) were isolated from the fractions C4-6–C4-8 (30 mg each) via semi-preparative HPLC (70% aqueous MeCN). Fraction C8 (1.8 g) was separated via an RP-C_{18} silica gel open column (60% aqueous MeOH) to yield two subfractions (C8-1–C8-2) and compound **23** (50 mg) was acquired by recrystallization from fraction C8-1 (300 mg). The EtOAc-soluble fraction (20 g) was separated over a silica gel open column ($CHCl_3$-MeOH-H_2O, 4:1:0.1) to give 11 fractions (E1-E11). Compound **18** (3 mg) was isolated from

fraction E1 (500 mg) via a RP-C_{18} silica gel open column (20% aqueous MeCN) and followed by semi-preparative HPLC (15% aqueous MeCN). Fraction E3 (2.5 g) was applied on an RP-C_{18} silica gel open column (50% aqueous MeOH) to yield seven subfractions (E3-1–E3-7). Fraction E3-2 (1.4 g) was subjected to a silica gel column ($CHCl_3$-MeOH-H_2O, 6:1:0.05) and further purified by semi-preparative HPLC (20% aqueous MeCN) to yield compound **10** (5 mg). Compound **11** (3 mg) was isolated from fraction E3-4 (90 mg) via semi-preparative HPLC (25% aqueous MeCN).

Saliglandol (1). Colorless gum; $[\alpha]_D^{25}$ –10 (*c* 0.05, MeOH); IR (KBr) ν_{max} cm^{-1}: 3430, 2950, 2843, 1672, 1610; UV (MeOH) λ_{max} (log ε) 263 (1.53) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 206 (–1.74), 211 (1.79), 217 (–0.86), 227 (–0.45) nm; ^1H (700 MHz) and ^{13}C NMR (175 MHz) data, see Table 1; HRESIMS (positive-ion mode) *m/z* 267.1222 $[M + H]^+$ (calcd. for $C_{14}H_{19}O_5^+$, 267.1227).

ECD simulation analysis. All conformers addressed in the study were found using the Macromodel (version 2015-2, Schrodinger LLC) module with "mixed torsional/low mode sampling" in the MMFF94 force field. The searches were initially implemented in the gas phase with a 50 kJ/mol energy window limit and 10,000 maximum number of steps to exhaustively explore all potential conformers. The Polak–Ribiere conjugate gradient protocol was utilized to minimize conformers with 10,000 maximum iterations and a 0.001 kJ (mol Å)$^{-1}$ convergence threshold on the rms gradient. 1′*S*,2′*S*- and 1′*R*,2′*R*-**1** conformers within 5 kJ/mol found in the MMFF force field were considered and the geometry of the conformers was optimized at the B3LYP/6–31+G(d,p) level in the gas phase and ECD calculations were performed at the identical theory level and basis sets. The generated excitation energies and rotational strengths were Boltzmann-averaged on the basis of the calculated Gibbs free energy of each conformer (Tables S1 and S2, Supplementary Information) and used for ECD visualization utilizing SpecDis with a sigma/gamma value of 0.05 eV for a best fitting.[40]

Cytotoxicity assessment. The cytotoxicity of the compounds addressed in this study against the cultured human tumor cell lines A549, SK-OV-3, SK-MEL-2, and BT549 was evaluated using the SRB assay.[41] Cells addressed in the current study were purchased from the American Type Culture Collection (Manassas, VA), and maintained at the Korea Research Institute of Chemical Technology.

NO production and viability in LPS-stressed BV-2 cells. The inhibitory effect of the test compounds on LPS-stimulated NO production was scrutinized using BV2 cells. The target cells were seeded on a 96-well plate at 4×10^4 cells/well and treated with/without different concentrations of the purified molecules. LPS (100 ng/mL) was added to BV2 cells and they were incubated for 24 h. Nitrite (NO_2,

soluble oxidation product of NO) concentrations present in the culture medium were measured using the Gries reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The supernatant (50 μL) was mixed with an identical volume of the Gries reagent. After 10 min, absorbance at 570 nm was measured utilizing a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA). Generated nitrite concentrations were gauged using graded sodium nitrite solution as a standard. Cell viability was evaluated using MTT assay.

NGF and cell viability assays. The C6 glioma cells (the Korean Cell Line Bank, Seoul, Republic of Korea) were used to assess the release of NGF into the culture medium. The test cells were seeded onto 24-well plates at a density of 1×10^5 cells/well. After 24 h, the cells were treated with serum-free DMEM and incubated with various concentrations of compounds for an additional 24 h. The medium supernatant was collected from the culture plates and NGF levels were evaluated using an ELISA development kit. Cell viability was also assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in which the results were expressed as a percentage of the control group (untreated cells).

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