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Identification of a New Hexenoic Acid Glycoside and the RSV Principles from *Vitis vinifera* cv. Muscat of Alexandria

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Respiratory syncytial virus (RSV) is the most common worldwide cause of lower respiratory tract infections (LRI) in infants, elderly people, and immune-compromised individuals.[1-3] RSV can be reinfected and previous infection does not prevent subsequent re-infection,[4,5] but there is no effective vaccine against RSV.[6] Ribavirin has been approved as an antiviral agent against RSV infection, however, its efficacy and clinical benefits are still controversial.[7,8]

Vitis vinifera L. cv. Muscat of Alexandria (Vitaceae) is one of the cultivars of *V. vinifera*. There have been several phytochemical reports on this cultivar including flavonoid,[9] stilbenoid,[10] and monoterpenes[11] with biological activities of antioxidant,[12] *anti*-inflammation,[13] and antiproliferation.[14] In the present study, the EtOAc extracts of the combined stems and leaves of *V. vinifera* cv. Muscat of Alexandria showed activity (IC_{50} = 0.644 μg/mL) during the screening to discover new *anti*-RSV agents from higher plants. To the best of our knowledge, this cultivar was never previously reported as *anti*-RSV agents. Thus, it was subjected to detailed phytochemical investigation, affording a new compound **1** together with four known glycosides **2-5** which were isolated from this cultivar for the first time (Fig. 1). Herein, the structure identifications of the isolates are reported as well as the evaluation on their *anti*-RSV activity.

Compound **1** isolated from the combined stems and leaves of *V. vinifera* cv. Muscat of Alexandria, was obtained as a translucent and amorphous solid. The molecular formula of **1** was deduced to be $C_{12}H_{20}O_7$ by HR-ESI-MS, which exhibited a molecular ion peak at *m/z* 299.1098 ([*M* + Na]$^+$). The IR spectrum indicated the presence of hydroxyl group(s) at 3373 cm^{-1} and conjugated C=O group(s) at 1724 cm^{-1}.[15] In the ^{1}H NMR spectrum of **1**, the protons of an olefinic functionality appeared at δ_H 5.89 and 7.09 with a 15.8 Hz of coupling constant, indicating their *trans*-configuration.[15] In the ^{13}C-NMR spectrum, a signal at δ_C 166.7 revealed the presence of an esteric C=O group in **1**. The DEPT 135° spectrum demonstrated two methylene groups at δ_C 35.3 (C-4) and 22.4 (C-5), along with one methyl group at δ_C 14.0 (C-6). Additionally, the ^{1}H, ^{1}H-COSY correlations of H-6/H-5, H-5/H-4, H-4/H-3, and H-3/H-2 and the HMBC correlations of H-3/C-1 and H-2/C-1 clearly confirmed the structure of a hexenoic acid moiety in **1**. The glucose moiety appeared at δ_H 5.52 (1H, d, *J* = 7.6 Hz)/δ_C 95.8 (C-1'), 3.83 (1H, dd, *J* = 12.1, 2.0 Hz) & 3.67 (1H, dd, *J* = 12.1, 4.6 Hz)/62.4 (C-6'), 3.45-3.34 (4H, m)/78.9 (C-5'), δ_C 78.1 (C-3'), 74.0 (C-2'), and 71.1 (C-4'). The ^{1}H- and ^{13}C-NMR signals of the glucose were similar to those of the known compound, tiglic acid 1-*O*-β-D-glucopyranoside.[16] The ester linkage between the anomeric carbon, C-1' of the glucose moiety and C-1 of the hexenoic acid moiety was deduced by the HMBC cross peak of H-1'/C-1. The β configuration of glucose was concluded from the *J* value (*J* = 7.6 Hz) of the anomeric proton signal.[16] Further detailed analysis of ^{1}H,^{1}H-COSY, NOESY, HSQC, and HMBC data (Fig. 2) allowed unambiguous assignments for all ^{1}H and ^{13}C NMR signals of **1**. As a result, compound **1** was elucidated as the new compound, (*E*)-2-hexenoic acid 1-*O*-β-D-glucopyranoside.

Compounds **2-5** were identified as the known flavonoid glycosides, kaempferol 3-*O*-β-D-glucuronopyranosyl methyl ester,[17] kaempferol 3-*O*-β-D-glucuronopyranoside,[18] quercetin

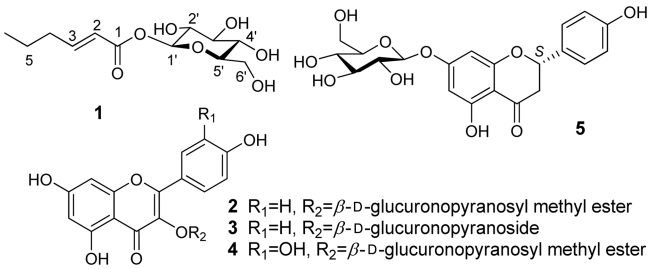

Figure 1. Structures of **1-5** from the stems and leaves of *V. vinifera* cv. Muscat of Alexandria.

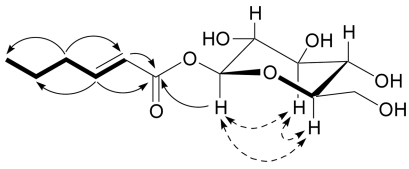

Figure 2. Important ^{1}H, ^{1}H-COSY (▬), ^{1}H,^{1}H-NOESY (-----) and ^{1}H, ^{13}C-HMBC (→) Correlations of **1**.

[a]These authors contributed equally to this work.

Table 1. Antiviral Activities of Compounds **1-5** against Respiratory Syncytial Virus (RSV) as Propagated in HEp-2 Cells

No.	Name	IC_{50} [μM][a]
1	(*E*)-2-hexenoic acid 1-*O*-β-D-glucopyranoside	>100
2	kaempferol 3-*O*-β-D-glucuronopyranosyl methyl ester	2.7
3	kaempferol 3-*O*-β-D-glucuronopyranoside	>100
4	quercetin 3-*O*-β-D-glucuronopyranosyl methyl ester	54.9
5	naringenin 7-*O*-β-D-glucopyranoside	10.0
	Ribavirin[b]	48.8

[a]Drug concentration required to inhibit RSV activity by 50%. [b]Positive control.

3-*O*-β-D-glucuronopyranosyl methyl ester,[19] and naringenin 7-*O*-β-D-glucopyranoside,[20,21] respectively, by comparison of their physical and spectroscopic data with those in the literature.[17-21] To the best of our knowledge, **2-5** were isolated from *V. vinifera* cv. Muscat of Alexandria for the first time. Moreover, this is the first report on compound **2** from the family Vitaceae.

Although there were several previous reports on the RSV inhibitory activity of flavonoids including quercetin,[22,23] studies of flavonoid glycosides relevant to RSV inhibitory activity have not been done so much. Thus we performed plaque assay to evaluate the inhibitory effects of the compounds **1-5** on the RSV propagated HEp-2 cells.[24,25] The antiviral drug, ribavirin[26] was used as a positive control (IC_{50} = 48.8 μM). As a result, **2** and **5** showed potent *anti*-RSV activities with IC_{50} values of 2.7 and 10.0 μM, respectively. Compound **4** exhibited moderate activity (IC_{50} = 54.9 μM). The isolates **1** and **3** did not show inhibitory activity against the RSV (Table 1). The only structural difference between the potent compound **2** and the inactive compound **3** was the presence of a methyl ester in **2**. This result indicated that the presence of the methyl ester seems to be important for the RSV activity. However, further study with more various derivatives is necessary to conclude the structure activity relationship.

Experimental

General Method. Column chromatography (CC): Silica gel (230-400 mesh; Merck, Germany) and YMC RP-18 gel (ODS-A, 12 nm, S-150 μm; YMC, Kyoto). Thin-layer chromatography (TLC): Silica gel 60 F_{254} and RP-18 F_{254s} silica gel plates (Merck, Germany); spot observation under UV light and visualization by spraying with 10% aq. H_2SO_4 solution, followed by heating at 120 °C for 5 min. HPLC: Preparative HPLC Acme 9000 (Young Lin, Republic of Korea) equipped with 5-ODS-H column (S-5 μm, 250 mm × 20 mm; Chemco, Japan); t_R in min. Optical rotations: JASCO P-1010 polarimeter. Circular dichroism (CD): JASCO J-715 CD/ORD spectropolarimeter. UV spectra: Hitachi U-3000 spectrophotometer. IR spectra: Bio-Rad FTS 135 FT-IR spectrometer. 1D and 2D NMR spectra: Varian Unity INOVA 400 MHz FT-NMR spectrometer, at 400 and 100 MHz, resp.; δ in ppm rel. to Me_4Si (TMS), *J* in Hz. HR-ESI-MS: Agilent 6220 Accurate-Mass TOF LC/MS system; in *m/z*.

Plant Material. The stems and leaves of *V. vinifera* cv. Muscat of Alexandria were collected from the Oha Farm in Ansung, South Korea, in September 2010 and were identified by one of the authors, Nam Sook Lee. A voucher specimen (No. EA312) was deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, Ewha Womans University, Korea.

Extraction and Isolation. The stems and leaves of *V. vinifera* cv. Muscat of Alexandria (2.66 kg) were extracted with MeOH (28 L × 3) for 24 h by percolation. The solvent was evaporated *in vacuo* to afford a MeOH extract (103.12 g). The residue was suspended in water (2 L), and partitioned with *n*-hexane (1.5 L × 7), EtOAc (1.5 L × 4), and *n*-BuOH (1.5 L × 4), sequentially. The EtOAc extract (19.18 g) was separated by silica gel (200 g) liquid column chromatography using gradient mixtures of *n*-hexane-acetone-MeOH (19:1:0 → MeOH 100% v/v) as the mobile phase to give 20 fractions (Frs. 1-20). Fr. 16 and Fr. 17 was combined (1.62 g) and then fractionated by CC (ODS-A, 100 g; H_2O-MeOH = 2:1 → 1:2) leading to 15 fractions (Frs. 16.1-16.15). Fr. 16.4 (90.05 mg) was subjected to CC (Sephadex LH-20; MeOH 100%) affording two subfractions (Frs. 16.4.1-16.4.2). Compound **1** (t_R 22 min, 18.2 mg, 0.00068% w/w) was obtained from subfraction Fr. 16.4.1 (60.95 mg) after purification by prep. HPLC (5-ODS-H, 20 × 250 mm, 5 μm, 2 mL/min; MeOH-H_2O = 2:3). Fr. 16.11 (11.2 mg) was purified by CC (Sephadex LH-20; MeOH 100%) to afford **2** (6.63 mg, 0.00025% w/w). Fr. 20 was chromatographed over CC (ODS-A, 100 g; MeOH-H_2O = 3:7 → 1:1) to provide 11 fractions (Frs. 20.1-20.11). Fr. 20.7 (158.10 mg) was subjected to further CC (Sephadex LH-20; MeOH 100%), to produce compound **3** (28.26 mg, 0.00106% w/w). Fr. 18 was fractionated by CC (SiO_2, 200g; $CHCl_3$-MeOH = 99:1 → 7:1), leading to 10 fractions (Frs. 18.1-18.10). A mixture of Fr. 18.10 (1.26 g) and Fr. 19 (1.08 g) was subjected to CC (ODS-A, 100 g; MeOH-H_2O = 3:7 → 1:1) to provide 14 fractions (Frs. 18.10.1-18.10.14). Compound **4** (9.55 mg, 0.00036% w/w) was obtained from Fr. 18.10.10 upon purification by CC (Sephadex LH-20; MeOH 100%). Fr. 18.10.8 (198.8 mg) was chromatographed on a sephadex LH-20 column with MeOH to provide 8 subfractions (Frs. 18.10.8.1-18.10.8.8). TLC analysis of Fr. 18.10.8.2 and Fr. 18.10.8.5 indicated the presence of only one compound in both cases. Each fraction was dried under reduced pressure, and afforded **5** (13.23 mg, 0.00050% w/w).

(*E*)-2-Hexenoic acid 1-*O*-β-D-glucopyranoside (1): Translucent, amorphous solid. $[\alpha]_D^{25}$: –21.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm: 235 (3.25); IR (KBr) ν_{max} cm^{-1}: 3373, 1724, 1650, 1074; ^{1}H-NMR (CD_3OD, 400 MHz) δ 7.09 (1H, dt, *J* = 15.8, 7.3 Hz, H-3), 5.89 (1H, dt, *J* = 15.8, 1.6 Hz, H-2), 5.52 (1H, d, *J* = 7.6 Hz, H-1'), 3.83 (1H, dd, *J* = 12.1, 2.0 Hz, H-6'b), 3.67 (1H, dd, *J* = 12.1, 4.6 Hz, H-6'a), 3.45-3.34 (4H, m, H-2', 3', 4' and 5'), 2.23 (2H, qd, *J* = 7.3, 1.6 Hz, H-4), 1.51 (2H, sext, *J* = 7.3 Hz, H-5), 0.95 (3H, t, *J*

= 7.3 Hz, H-6); ^{13}C-NMR (CD_3OD, 100 MHz) δ 166.7 (C-1), 152.7 (C-3), 121.8 (C-2), 95.8 (C-1'), 78.9 (C-5'), 78.1 (C-3'), 74.0 (C-2'), 71.1 (C-4'), 62.4 (C-6'), 35.3 (C-4), 22.4 (C-5), 14.0 (C-6); HRESIMS *m/z* = 299.1098 $[M+Na]^+$ (calcd for $C_{12}H_{20}NaO_7$: 299.1101).

Kaempferol 3-*O*-β-D-glucuronopyranosyl Methyl Ester (2): Pale yellow powder. $[\alpha]_D^{25}$: –38.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm: 350 (2.00), 265 (2.40); IR (KBr) ν_{max} cm^{-1}: 3254, 1734, 1606; ^{1}H- and ^{13}C-NMR: data are in agreement with literature values;[17] HRESIMS *m/z* = 477.1034 $[M+H]^+$ (calcd for $C_{22}H_{21}O_{12}$: 477.1028).

Kaempferol 3-*O*-β-D-glucuronopyranoside (3): Pale yellow powder. $[\alpha]_D^{25}$: –28.8 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm: 350 (1.79), 265 (2.26); IR (KBr) ν_{max} cm^{-1}: 3302, 1655, 1650, 1179; ^{1}H- and ^{13}C-NMR: data are in agreement with literature values;[18] HRESIMS *m/z* = 463.0879 $[M+H]^+$ (calcd for $C_{21}H_{19}O_{12}$: 463.0871).

Quercetin 3-*O*-β-D-glucuronopyranosyl Methyl Ester (4): Yellow powder. $[\alpha]_D^{25}$: –48.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm: 360 (2.37), 255 (2.90); IR (KBr) ν_{max} cm^{-1}: 3254, 1654, 1605; ^{1}H- and ^{13}C-NMR: data are in agreement with literature values;[19] HRESIMS *m/z* = 493.0982 $[M+H]^+$ (calcd for $C_{22}H_{21}O_{13}$: 493.0977).

Naringenin-7-*O*-β-D-glucopyranoside (5): Yellow powder. $[\alpha]_D^{25}$: –29.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm: 280 (1.54), 230 (2.32); CD (*c* 0.2, MeOH) nm: 284 (–14.8), 328 (+2.1);[20] IR (KBr) ν_{max} cm^{-1}: 3346, 1644, 1075; ^{1}H- and ^{13}C-NMR: data are in agreement with literature values;[21] HRESIMS *m/z* = 435.1290 $[M+H]^+$ (calcd for $C_{21}H_{23}O_{10}$: 435.1286).

Plaque Assay Method. This experiment was carried out with a slight modification of the method reported by Kim *et al.*[24] HEp-2 cells were seeded in 6-well plates at a density of 500,000 cells per well. When the cells reached 95% confluence, culture media were removed and 800 μL of serum-free MEM media (Gibco, USA) was added to each well. Total 100 μL of diluted viral suspension (200 PFU) and 100 μL of serially diluted compounds **1-5** (0.05, 0.5, 5 and 50 μg/mL) were added to each well. The plates were incubated for 2 h at 37 °C with shaking every 15 min. The virus-mixed samples were removed from each well, and then the cells were overlaid with 0.6% agarose (Lonza, Switzerland) in MEM media containing 10% FBS (Wisent, Canada). After incubation for 96 h at 37 °C, the cell monolayers were stained by neutral red (Sigma, USA) to visualize the viral plaques. The inhibitory effects of the compounds on RSV were evaluated by the reduction of plaque numbers compared to the control (without compounds **1-5**). Ribavirin, an antiviral drug, was used as a positive control.

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Supporting Information. The NMR spectral data of compound **1** are available as supporting information.

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