Neocimicigenosides A and B, Cycloartane Glycosides from the Rhizomes of *Cimicifuga racemosa* and Their Effects on CRF-Stimulated ACTH Secretion from AtT-20 Cells

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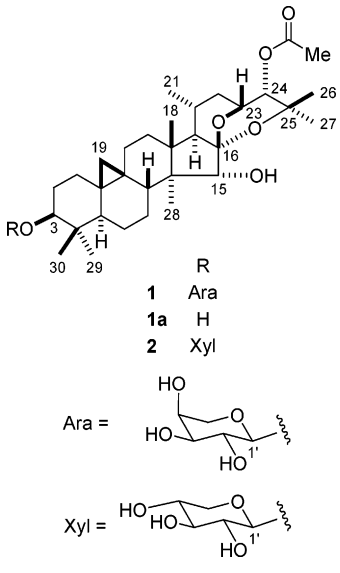
Two new cycloartane glycosides, named neocimicigenosides A (**1**) and B (**2**), were isolated from the rhizomes of *Cimicifuga racemosa*. The structures of **1** and **2** were determined on the basis of extensive spectroscopic analysis and enzymatic hydrolysis followed by chromatographic and spectroscopic analyses to be (16*S*,23*R*,24*S*)-24-acetoxy-16,23:16,25-diepoxy-15α-hydroxycycloartan-3*β*-yl α-L-arabinopyranoside (**1**) and (16*S*,23*R*,24*S*)-24-acetoxy-16,23:16,25-diepoxy-15α-hydroxycycloartan-3*β*-yl *β*-D-xylopyranoside (**2**), respectively. Neocimicigenosides A and B enhanced CRF-stimulated ACTH secretion from AtT-20 cells.

The rhizomes of *Cimicifuga racemosa* Nutt. (Ranunculaceae) are commonly called black cohosh and have long been used as a folk medicine by Native Americans to treat diarrhea, sore throat, and rheumatism.[1] Black cohosh has become a well-known alternative phytomedicine with reported health benefits in treating painful menstrual periods and menopausal disorders, both in the United States and in Europe. The chemical constituents of black cohosh have been investigated, and more than 20 highly oxygenated triterpene glycosides with the cycloartane skeleton,[2−13] isoflavones,[14] alkaloids,[15] and phenylpropanoids[16] have been isolated and identified. Among the secondary metabolites contained in black cohosh, triterpene glycosides have been suggested to contribute to its pharmacological effects.[17−19] The present investigation of the rhizomes of *C. racemosa* is part of a series of studies on the chemical constituents of herbal medicines[20−23] and has resulted in the isolation of two new cycloartane glycosides, which we named neocimicigenosides A (**1**) and B (**2**). The effects of **1** and **2** on CRF-stimulated ACTH secretion from AtT-20 cells are also reported.



A MeOH extract of the rhizomes of *C. racemosa* was passed through a porous-polymer polystyrene resin column, and the MeOH eluate portion was subjected to column chromatography on silica gel and octadecylsilanized (ODS) silica gel, as well as preparative HPLC, yielding **1** (45.0 mg) and **2** (16.4 mg).

Neocimicigenoside A (**1**), isolated as an amorphous solid, showed an $[M + H]^+$ ion at *m*/*z* 663.4110 in the positive-ion HRESIMS, corresponding to the empirical molecular formula $C_{37}H_{58}O_{10}$. The glycosidic nature of **1** was shown by strong IR absorptions at 3443, 1070, and 1042 cm^{-1}. A positive coloration in the Liebermann-Burchard reaction, as well as six three-proton singlet signals, a three-proton doublet signal, and an anomeric proton signal at δ 4.78 (d, $J = 7.0$ Hz) in the ^{1}H NMR spectrum suggested that **1** was a triterpene monoglycoside. Enzymatic hydrolysis with naringinase gave a new triterpene aglycon (**1a**: $C_{32}H_{50}O_6$), named neocimigenol, and a monosaccharide, which was identified as L-arabinose by HPLC analysis of the H_2O-soluble portion of the hydrolysate. The ^{1}H NMR spectrum of **1a** in C_5D_5N showed signals for two characteristic cyclopropane protons at δ 0.60 and 0.35 (each d, $J = 3.9$ Hz), six tertiary methyl groups at δ 1.77, 1.40, 1.22, 1.21, 1.16, and 1.11 (each s), a secondary methyl group at δ 1.00 (d, $J = 6.3$ Hz), an acetyl methyl group at δ 2.09 (s), and two exchangeable protons at δ 5.72 (d, $J = 4.5$ Hz) and 4.46 (d, $J = 10.4$ Hz), which disappeared with the addition of HCl vapor. These ^{1}H NMR data and the ^{13}C NMR features of **1a** were essentially analogous to those of cimigenol, a representative cycloartane type triterpene, widely found in plants of the genus *Cimicifuga*.[24] The hydroxymethine protons at δ 4.00 (s) and 3.54 (dd, $J = 11.5$, 4.4 Hz), cyclopropane protons at δ 0.57 and 0.33, and four singlet methyl protons at δ 1.20, 1.19, 1.14, and 1.08 were assigned to H-15, H-3, H_2-19, Me-18, Me-29, M-28, and Me-30, respectively, by comparison with the ^{1}H and ^{13}C NMR spectra of **1a** with those of previously reported cimigenol derivatives,[5,12] as well as analysis of its ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. However, the ^{1}H and ^{13}C NMR signals arising from the modified side-chain moiety of **1a** did not agree with those of any other cimigenol derivatives reported up to the present. To assign the structure of the modified side-chain moiety, the ^{1}H−^{1}H COSY and 2D TOCSY spectra of **1a** were carefully inspected with the three-proton doublet signal at δ 0.98 attributable to Me-21 as the starting point for analysis. The Me-21 protons showed a spin-coupling correlation with a broad multiplet proton centered at δ 1.81, which was unambiguously assigned to H-20 and exhibited correlations with a pair of methylene protons at δ 1.91 and 1.63 (H_2-22) and also with a methine proton at δ 1.59 (H-17). The methylene protons

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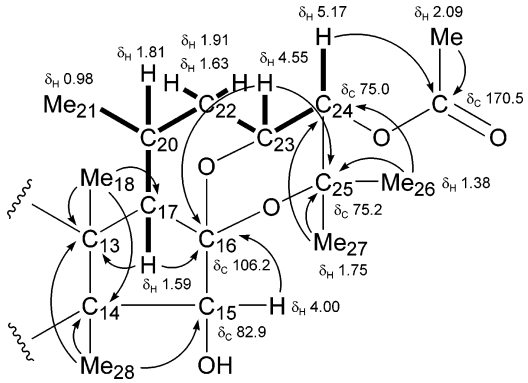

Figure 1. Plane structure of the modified side-chain moiety of **1a**. Bold lines indicate the ^{1}H−^{1}H spin-couplings traced by the ^{1}H−^{1}H COSY spectrum, and arrows indicate ^{1}H/^{13}C long-range correlations observed in the HMBC spectrum.

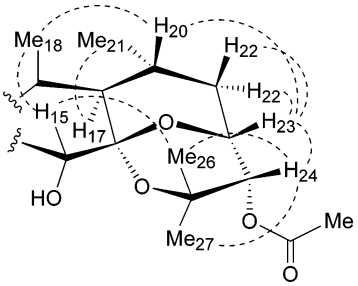

Figure 2. NOE correlations of the modified side-chain moiety of **1a**.

(H_2-22), in turn, displayed a correlation with an oxymethine proton at δ 4.55 (H-23), which was correlated to a vicinal oxymethine proton at δ 5.17 (H-24). The H-17 and H-24 signals did not show further spin-coupling correlations. In the HMBC spectrum of **1a**, a pair of geminal methyl protons at δ 1.75 (Me-27) and 1.38 (Me-26) exhibited long-range correlations not only with their attached carbon at δ 75.2 (C-25) but also with a carbon resonance at δ 75.0. The latter was associated with a one-bond coupled proton at δ 5.17 (H-24) by the HMQC spectrum. These HMBC correlations implied a linkage between C-24 and C-25. The oxymethine proton H-23 showed HMBC correlations with an acetalic carbon at δ 106.2 and the quaternary carbon H-25. H-15 and H-17 showed $^2J_{C,H}$ correlations with the acetalic carbon, resulting in its assignment to C-16. These data were indicative of linkages between C-16 and C-23 and between C-16 and C-25, each through an oxygen atom. An HMBC correlation between H-24 and the carbonyl carbon of an acetyl group at δ 170.5 gave evidence for the locus of an acetoxy group at C-24. Thus, the planar structure of **1a** was determined as shown in Figure 1. In the phase-sensitive NOESY experiment, NOE correlations between H-15 and Me-26; H-17 and Me-21; Me-18 and H-15/H-20; H-23 and H-20/H_2-22/H-24; and H-24 and Me-26/Me-27 provided evidence for the 15α, 16*S*, 23*R*, and 24*S* configurations (Figure 2). The linkage position of the α-L-arabinopyranosyl moiety to the aglycon **1a** was determined to be the C-3 hydroxy group by a long-range correlation between the anomeric proton of the arabinosyl group at δ 4.78 and the C-3 oxymethine carbon at δ 88.5. Thus, the gross structure of **1** was determined to be (16*S*,23*R*,24*S*)-24-acetoxy-16,23:16,25-diepoxy-15α-hydroxy-cycloartan-3β-yl α-L-arabinopyranoside.

Neocimicigenoside B (**2**), isolated as an amorphous solid, showed an $[M + H]^+$ ion at *m*/*z* 663.4108 in the positive-ion HRESIMS, indicating that the molecular formula of **2** was the same as that of **1** ($C_{37}H_{58}O_{10}$). Furthermore, the ^{1}H and ^{13}C NMR signals of **2** were superimposable with those of **1**, except for the signals due to the sugar moiety. In the ^{1}H NMR spectrum of **2**, the large coupling constants (>7.4 Hz) between H-1 and H-2, between H-2 and H-3, and between H-3 and H-4 suggested that the monosaccharide attached to C-3 of the aglycon was β-xylopyranose. Enzymatic hydrolysis of **2** with naringinase gave **1a** and D-xylose. Thus, the structure of **2** was characterized as (16*S*,23*R*,24*S*)-24-acetoxy-16,23:16,25-diepoxy-15α-hydroxyl-cycloartan-3β-yl β-D-xylopyranoside.

Table 1. Effects of **1** and **2** on Basal and CRF-Stimulated ACTH Secretion from AtT-20 Cells[a]

	ACTH (ng/mL)
control	7.0 ± 0.7
1	7.2 ± 0.5
2	7.7 ± 0.2
CRF	9.9 ± 0.4**
CRF + **1**	13.7 ± 1.5**
CRF + **2**	14.9 ± 2.9**

[a] Data are expressed as means ± SEM of four replicate samples. Statistical significance as compared to the control is indicated as ** ($P < 0.01$) as determined by Dunnett's multiple comparison tests.

The cimigenol derivatives reported up to the present all have two epoxy rings between C-16 and C-23 and between C-16 and C-24.[24] Neocimicigenosides A and B differ from the reported cimigenol derivatives in forming epoxy rings between C-16 and C-23 and between C-16 and C-25 and in having an acetoxy group at C-24.

The efficacy of an alcoholic extract of *C. racemosa* rhizomes on climacteric complaints has been well documented.[25−28] Furthermore, our preliminary examinations suggested that a *C. racemosa* extract was also useful for the treatment of stress-related disorders,[29] which prompted us to evaluate neocimicigenosides A and B (**1** and **2**) for their effects on pituitary activity by using AtT-20 cells (Table 1).[30] As compared with the control, the amount of ACTH secreted from AtT-20 cells was significantly increased by corticotrophin releasing factor (CRF) stimulation. Although **1** or **2** did not effect ACTH secretion by itself, co-incubation of **1** or **2** with CRF significantly enhanced the ACTH secretion from AtT-20 cells. Thus, neocimicigenosides A and B appear to promote hypothalamo-pituitary-adrenal (HPA) activity, which may lead to stress resistance being recovered.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. ESIMS data were obtained on a Micromass LCT mass spectrometer (Manchester, UK). Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and octadecylsilanized (ODS) silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. HPLC was performed by using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh) or a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. A Capcell Pak C_{18} UG80 column (10 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan) was employed for preparative HPLC. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gland Island, NY); FBS (Gibco); BSA (Serologicals-Proteins, Kankakee, IL); CRF (Sigma, St. Louis, MO). All other chemicals used were of biochemical reagent grade.

Plant Material. The plant material defined as rhizomes of *C. racemosa* was provided by Tokiwa Phytochemical Co., Ltd., Chiba, Japan, and authenticated by one of the authors (Y.S.). A small amount of the sample is preserved in our laboratory (00-CR-011).

Extraction and Isolation. The plant material (dry weight, 5.2 kg) was extracted with hot MeOH (21 L) for 3 h twice. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (445 g) was passed through a Diaion HP-20 column, successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The MeOH eluate portion (181 g) was subjected to column chromatography on silica gel and was eluted with stepwise gradient mixtures of $CHCl_3$−MeOH (19:1; 9:1; 4:1; 2:1) and finally with MeOH to give seven fractions (I−VII). Fraction V was subjected to a silica gel column eluted with $CHCl_3$−MeOH (19:1), then an ODS silica gel column eluted with MeCN−H_2O (1:1; 5:8), and finally subjected to preparative HPLC using MeCN−H_2O (1:1), to yield **1** (45.0 mg) and **2** (16.4 mg).

Neocimicigenoside A (1): amorphous solid; $[\alpha]_D^{27}$ +58.0 (*c* 0.10, C_5H_5N); IR (film) ν_{max} 3443 (OH), 2933 and 2871 (CH), 1732 (C═O), 1070, 1042 cm^{-1}; ^1H NMR (C_5D_5N + HCl vapor) δ 5.14 (1H, d, J = 7.7 Hz, H-24), 4.78 (d, J = 7.0 Hz, H-1′), 4.53 (1H, ddd, J = 9.5, 8.5, 7.7 Hz, H-23), 4.42 (1H, dd, J = 8.8, 7.0 Hz, H-2′), 4.33 (1H, br s, H-4′), 4.27 (1H, dd, J = 12.2, 2.9 Hz, H-5′a), 4.18 (1H, dd, J = 8.8, 3.5 Hz, H-3′), 3.96 (1H, s, H-15), 3.77 (1H, dd, J = 12.2, 1.7 Hz, H-5′b), 3.46 (1H, dd, J = 11.6, 4.3 Hz, H-3), 2.08 (3H, s, Ac), 1.85 (1H, dd, J = 12.5, 8.5 Hz, H-22a), 1.78 (1H, m, H-20), 1.73 (3H, s, Me-27), 1.60 (1H, dd, J = 12.5, 9.5 Hz, H-22b), 1.56 (1H, d, J = 9.9 Hz, H-17), 1.36 (3H, s, Me-26), 1.24 (3H, s, Me-29), 1.15 (3H, s, Me-18), 1.10 (3H, s, Me-28), 0.99 (3H, s, Me-30), 0.96 (3H, d, J = 6.2 Hz, Me-21), 0.48 (1H, d, J = 3.8 Hz, H-19a), 0.25 (1H, d, J = 3.8 Hz, H-19b); ^{13}C NMR (C_5D_5N + HCl vapor) δ 32.3 (C-1), 29.7 (C-2), 88.5 (C-3), 41.2 (C-4), 47.4 (C-5), 21.0 (C-6), 26.3 (C-7), 48.8 (C-8), 19.9 (C-9), 26.5 (C-10), 26.3 (C-11), 34.0 (C-12), 40.7 (C-13), 46.7 (C-14), 82.8 (C-15), 106.1 (C-16), 61.7 (C-17), 19.8 (C-18), 30.8 (C-19), 26.0 (C-20), 20.4 (C-21), 29.7 (C-22), 67.5 (C-23), 74.9 (C-24), 75.1 (C-25), 26.5 (C-26), 33.8 (C-27), 11.8 (C-28), 25.6 (C-29), 15.3 (C-30), 107.1 (C-1′), 72.7 (C-2′), 74.5 (C-3′), 69.3 (C-4′), 66.5 (C-5′), 170.5 (Ac, C═O), 20.5 (Ac, Me); HRESIMS *m/z* 663.4110 $[M + H]^+$ (calcd for $C_{37}H_{59}O_{10}$, 663.4108).

Enzymatic Hydrolysis of 1. Compound **1** (30.6 mg) was treated with naringinase (EC 232-962-4; Sigma) (240 mg) in AcOH/AcOK buffer (pH 4.3, 20 mL) at room temperature for 168 h. The reaction mixture was passed through a combination of Sep-Pak C_{18} cartridge (Waters, Milford, MA) and Toyopak IC-SP M cartridge (Tosoh) eluted with MeOH−H_2O (1:9) followed by MeOH. The MeOH eluate fraction was purified by silica gel column chromatography eluted with $CHCl_3$−MeOH (70:1) to afford neocimigenol (**1a**) (5.8 mg). The MeOH−H_2O (1:9) eluate fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. × 250 mm, 5 μm, Shiseido); solvent, MeCN−H_2O (17:3); flow rate, 1.0 mL/min; detection, RI and OR. Identification of L-arabinose was carried out by comparison of its retention time and optical rotation with those of an authentic sample: t_R (min) 9.19 (positive optical rotation).

Neocimigenol (1a): amorphous solid; $[\alpha]_D^{23}$ +30.0 (*c* 0.10, $CHCl_3$); IR ν_{max} (film) 3443 (OH), 2925 and 2855 (CH), 1731 (C═O), 1069, 1043, 1027 cm^{-1}; ^1H NMR (C_5D_5N) δ 5.72 (1H, d, J = 4.5 Hz, 3-OH), 5.19 (1H, d, J = 7.7 Hz, H-24), 4.57 (1H, q-like, J = 8.4 Hz, H-23), 4.46 (1H, d, J = 10.4 Hz, 15-OH), 4.02 (1H, d, J = 10.4 Hz, H-15), 3.55 (1H, m, H-3), 2.09 (3H, s, Ac), 1.77 (3H, s, Me-27), 1.40 (3H, s, Me-26), 1.22 (3H, s, Me-18), 1.21 (3H, s, Me-29), 1.16 (3H, s, Me-28), 1.11 (3H, s, Me-30), 1.00 (3H, d, J = 6.3 Hz, Me-21), 0.60 (1H, d, J = 3.9 Hz, H-19a), 0.35 (1H, d, J = 3.9 Hz, H-19b); ^1H NMR (C_5D_5N + HCl vapor) δ 5.17 (1H, d, J = 7.7 Hz, H-24), 4.55 (1H, ddd, J = 9.6, 8.5, 7.7 Hz, H-23), 4.00 (1H, s, H-15), 3.54 (1H, dd, J = 11.5, 4.4 Hz, H-3), 2.09 (3H, s, Ac), 1.91 (1H, dd, J = 12.5, 8.5 Hz, H-22a), 1.81 (1H, m, H-20), 1.75 (3H, s, Me-27), 1.63 (1H, dd, J = 12.5, 9.6 Hz, H-22b), 1.59 (1H, d, J = 9.9 Hz, H-17), 1.38 (3H, s, Me-26), 1.20 (3H, s, Me-18), 1.19 (3H, s, Me-29), 1.14 (3H, s, Me-28), 1.08 (3H, s, Me-30), 0.98 (3H, d, J = 6.3 Hz, Me-21), 0.57 (1H, d, J = 3.9 Hz, H-19a), 0.33 (1H, d, J = 3.9 Hz, H-19b); ^{13}C NMR (C_5D_5N + HCl vapor) δ 32.6 (C-1), 31.2 (C-2), 78.0 (C-3), 41.0 (C-4), 47.4 (C-5), 21.4 (C-6), 26.4 (C-7), 49.0 (C-8), 19.9 (C-9), 26.9 (C-10), 26.5 (C-11), 34.2 (C-12), 40.8 (C-13), 46.9 (C-14), 82.9 (C-15), 106.2 (C-16), 61.8 (C-17), 20.0 (C-18), 31.1 (C-19), 26.1 (C-20), 20.5 (C-21), 29.8 (C-22), 67.6 (C-23), 75.0 (C-24), 75.2 (C-25), 26.6 (C-26), 33.9 (C-27), 11.9 (C-28), 26.1 (C-29), 14.8 (C-30), 170.5 (Ac, C═O), 20.6 (Ac, Me); HRESIMS *m/z* 531.3664 $[M + H]^+$ (calcd for $C_{32}H_{51}O_6$, 531.3686).

Neocimicigenoside B (2): amorphous solid; $[\alpha]_D^{27}$ +18.0 (*c* 0.10, C_5H_5N); IR (film) ν_{max} 3347 (OH), 2928 and 2870 (CH), 1732 (C═O), 1069, 1049 cm^{-1}; ^1H NMR (C_5D_5N + HCl vapor) δ 5.15 (1H, d, J = 7.5 Hz, H-24), 4.84 (d, J = 7.4 Hz, H-1′), 4.53 (1H, ddd, J = 9.6, 8.5, 7.5 Hz, H-23), 4.34 (1H, dd, J = 11.3, 4.8 Hz, H-5′a), 4.21 (1H, ddd, J = 10.3, 8.7, 4.8 Hz, H-4′), 4.17 (1H, dd, J = 8.7, 8.3 Hz, H-3′), 4.02 (1H, dd, J = 8.3, 7.4 Hz, H-2′), 3.97 (1H, s, H-15), 3.72 (1H, dd, J = 11.3, 10.3 Hz, H-5′b), 3.49 (1H, dd, J = 11.7, 4.2 Hz, H-3), 2.08 (3H, s, Ac), 1.86 (1H, dd, J = 12.5, 8.5 Hz, H-22a), 1.79 (1H, m, H-20), 1.74 (3H, s, Me-27), 1.59 (1H, dd, J = 12.5, 9.6 Hz, H-22b), 1.57 (1H, d, J = 10.0 Hz, H-17), 1.37 (3H, s, Me-26), 1.29 (3H, s, Me-29), 1.16 (3H, s, Me-18), 1.11 (3H, s, Me-28), 1.04 (3H, s, Me-30), 0.96 (3H, d, J = 6.2 Hz, Me-21), 0.50 (1H, d, J = 3.9 Hz, H-19a), 0.27 (1H, d, J = 3.9 Hz, H-19b); ^{13}C NMR (C_5D_5N + HCl vapor) δ 32.3 (C-1), 30.0 (C-2), 88.5 (C-3), 41.3 (C-4), 47.5 (C-5), 21.0 (C-6), 26.3 (C-7), 48.9 (C-8), 19.9 (C-9), 26.5 (C-10), 26.3 (C-11), 34.1 (C-12), 40.8 (C-13), 46.8 (C-14), 82.8 (C-15), 106.1 (C-16), 61.8 (C-17), 19.9 (C-18), 30.9 (C-19), 26.1 (C-20), 20.5 (C-21), 29.8 (C-22), 67.5 (C-23), 75.0 (C-24), 75.1 (C-25), 26.5 (C-26), 33.8 (C-27), 11.8 (C-28), 25.6 (C-29), 15.4 (C-30), 107.4 (C-1′), 75.5 (C-2′), 78.5 (C-3′), 71.2 (C-4′), 66.9 (C-5′), 170.5 (Ac, C═O), 20.6 (Ac, Me); HRESIMS *m/z* 663.4108 $[M + H]^+$ (calcd for $C_{37}H_{59}O_{10}$, 663.4108).

Enzymatic Hydrolysis of 2. Compound **2** (7.4 mg) was subjected to enzymatic hydrolysis using naringinase as described for **2** to give **1a** (1.3 mg) and a sugar fraction. HPLC analysis of the sugar fraction under the same conditions as for **1** showed the presence of D-xylose: t_R (min) 9.62 (positive optical rotation).

AtT-20 Cell Culture Assay. AtT-20 mouse anterior pituitary tumor cells were grown and cultured in DMEM supplemented with 10% (v/v) FBS.[31] Cells were placed in each well of 24-well (16 mm diameter) multiwell plates (Sumitomo Bakelite, Tokyo, Japan) at an initial density of 10^5 cells per well and were subcultured for 7−9 days (80−90% confluence). The culture medium was removed from the multiwell plates, and cells adhering to the well surface were washed twice with 1 mL of DMEM supplemented with 0.1% (w/v) BSA (DMEM/BSA) and then incubated in 1 mL of DMEM/BSA in a humidified atmosphere of 10% CO_2/air for 1 h at 37 °C. Neocimicigenosides A and B (1 μg/mL) were independently dissolved in DMEM/BSA supplemented with 0.1% (v/v) DMSO (DMEM/BSA/DMSO). An aliquot of 1 mL of either neocimicigenoside A or B solution was added to each well, and preincubation followed at 37 °C. After 30 min of culture, CRF (10 ng/mL) was added to each well, and the plates were incubated for an additional 2 h. The ACTH content of the supernatant was measured by EIA (Peninsula Laboratories, San Carlos, CA). Four replicate samples of each were analyzed.

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