New Constituents from the Leaves of *Morinda citrifolia*

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A new iridoid glycoside, citrifoside (1), and a new anthraquinone, 1,5,15-trimethylmorindol (2), together with 24 known compounds, were isolated from the leaves of *Morinda citrifolia*. The structures of the new compounds were elucidated by spectral data. 1,5,15-Trimethylmorindol (2) did not show significant cytotoxic activity by itself but showed cytotoxicity when combined with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), while citrifoside (1) did not show any activity even with TRAIL.

Key words *Morinda citrifolia*; Rubiaceae; iridoid glycoside; anthraquinone

Morinda citrifolia L. (Rubiaceae), commonly called as "noni" in the Hawaiian and Tahitian islands, is a small evergreen tree originating in tropical Asia or Polynesia. In Japan, it is called as "Yaeyama-aoki" and grows in Okinawa Prefecture and the Ogasawara Islands. The roots, stems, bark, leaves, and fruits have been used traditionally as folk herbal remedies for many diseases including diabetes, hypertension, and cancer.[1)] In the course of our investigation for active compounds from tropical plants, we have studied the leaves of this plant. In this report, we describe the isolation and structure elucidation of a new iridoid glycoside, citrifoside (**1**), and a new anthraquinone, 1,5,15-trimethylmorondol (**2**), together with those of 24 known compounds from the leaves of *M. citrifolia*.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an important member of the TNF superfamily.[2)] TRAIL selectively induces apoptosis of a wide variety of cancer and transformed cells without damaging most normal cells. However, the potential application of TRAIL in cancer therapy is limited as many cancer cells are found to be resistant to the cytotoxicity of TRAIL. Therefore, a natural product with synergistic activity with TRAIL but minimal toxicity would be a new tool for cancer therapy. In this study, we examined the enhancement effects of the new compounds (**1**, **2**) on the cytotoxisity of TRAIL. 1,5,15-Trimethylmorindol (**2**) did not show a significant cytotoxic activity by itself but it showed cytotoxicity when combined with TRAIL, while citrifoside (**1**) did not show any activity even with TRAIL.

Results and Discussion

The MeOH extract from the leaves of *M. citrifolia* was partitioned between acidic water (pH 3.5) and EtOAc. The aqueous part was then basified (pH 9.5) and extracted again with EtOAc.[3)] Both EtOAc extracts were fractionated using ODS columns. Further purification of reversed-phase HPLC afforded two new compounds (**1**, **2**) and 24 known compounds.

Compound **1** was obtained as a colorless amorphous solid. Its molecular formula was determined as $C_{16}H_{22}O_{10}$ by HR-ESI-MS (*m*/*z* 397.1115 $[M+Na]^+$). Its IR spectrum showed the presence of a conjugated ester (1700, 1635 cm^{-1}). The ^{13}C-NMR spectral data of **1** exhibited 16 carbon signals (Table 1), with nine representing the aglycon, one methoxy group (δ 51.03), and six for the glucopyranose unit (δ 98.66, d, C-1′; δ 72.97, d, C-2′; δ 76.66, d, C-3′; δ 69.88, d, C-4′; δ 77.23, d, C-5′; δ 60.89, d, C-6′). The absolute configuration of the glucose was determined to be a D type by LC/MS analysis following conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxysorbitol acetate derivative.[4—6)] The β-anomeric configuration for the glucose was judged from its large $^3J_{H1,H2}$ coupling constant (J=7.9 Hz). HMBC correlations (between C-1/H-1′ and H-1/C-1′) and a NOESY correlation (H-1/H-1′) (Fig. 2) suggested that the β-glucopyranose unit was attached at the C-1 position of aglycon. The HMBC correlations also revealed the presence of an iridoid aglycon together with a carbomethoxy group at C-4. These data displayed a signal pattern similar to that of citrifolinin A, an iridoid glucoside previously isolated from the same plant.[7)] The ^{13}C-NMR of **1**, however, lacked the signals of a

Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1** in DMSO-d_6

	^{1}H	^{13}C
1	5.28 (1H, d, J=4.0 Hz)	95.23
3	7.37 (1H, s)	150.85
4		109.92
5	3.53 (1H, d, J=8.1 Hz)	37.08
6	5.97 (1H, d, J=5.2 Hz)	133.98
7	5.72 (1H, d, J=5.2 Hz)	134.25
8	4.57 (1H, m)	76.33
8-OH	5.17 (1H, d, J=6.7 Hz)	
9	2.22 (1H, m)	49.50
11		166.57
11-COOMe	3.64 (3H, s)	51.03
1′	4.48 (1H, d, J=7.9 Hz)	98.66
2′	2.99 (1H, ddd, J=8.0, 7.9, 5.0 Hz)	72.97
2′-OH	5.00 (1H, d, J=5.0 Hz)	
3′	3.16 (1H, m)	76.66
3′-OH	4.96 (1H, d, J=4.9 Hz)	
4′	3.07 (1H, m)	69.88
4′-OH	4.93 (1H, d, J=5.3 Hz)	
5′	3.13 (1H, m)	77.23
6′	3.67 (1H, m)	60.89
	3.45 (1H, ddd, J=11.8, 5.9, 5.7 Hz)	
6′-OH	4.51 (1H, t, J=5.7 Hz)	

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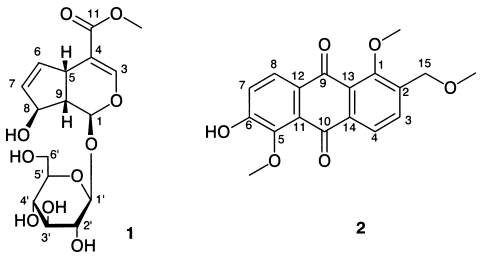

Fig. 1. Structures of Citrifoside (**1**) and 1,5,15-Trimethylmorindol (**2**)

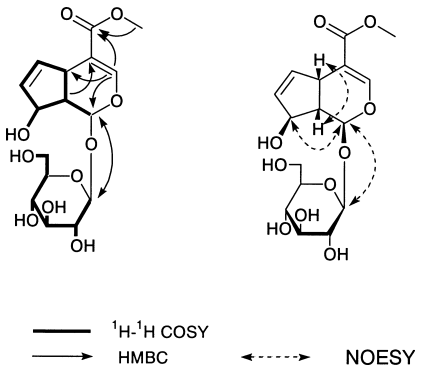

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC, and NOESY Correlations for Citrifoside (**1**)

C-8 oxygenated quaternary carbon, a C-10 carbonyl carbon, and the rearranged ferulic acid moiety in citrifolinin A, and instead showed the signal of an oxygenated C-8 methine carbon (δ_C 76.33, δ_H 4.57). Moreover, the ^{1}H–^{1}H COSY spectrum of **1** showed that H-8 (δ_H 4.57) was coupled with H-9 (δ_H 2.22) and a hydroxyl proton (δ_H 5.17). The NOESY correlation between H-1 and H-8 showed 8-β-hydroxyl configuration (Fig. 2). Thus, the structure of compound **1** was established to be 8-dehydroxymethylgardenoside, an iridoid glucoside lacking C-10 carbon (Fig. 1) and we designated it citrifoside. Full assignments of ^{1}H- and ^{13}C-NMR signals were accomplished using ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY experiments (Table 1).

Compound **2**, a yellow amorphous powder, had a $[M]^+$ peak at *m/z* 328.0945 in HR-EI-MS, corresponding to the molecular formula $C_{18}H_{16}O_6$. Its IR spectrum showed the presence of carbonyl groups (1667, 1656 cm^{-1}). ^{1}H- and ^{13}C-NMR spectral data of **2** (Table 2) revealed the presence of two 1,2,3,4-tetrasubstituted aromatic rings with two pairs of *ortho*-coupled proton signals [δ_H 7.95 and 7.83 (J=8.0 Hz); 7.86 and 7.32 (J=8.5 Hz)], three methoxy groups (δ_C 61.70, 60.52, 58.13; δ_H 3.82, 3.82, 3.40), one oxygenated methylene group (δ_C 68.24, δ_H 4.58), two carbonyl groups (δ_C 181.87, 180.76), and one phenolic hydroxy group (δ_H 10.66). These partial structures could be put together by its HMBC and NOESY correlations (Fig. 3). The data led us to conclude that compound **2** is 6-hydroxy-1,5-dimethoxy-2-methoxymethylanthraquinone and we named it 1,5,15-trimethylmorindol. The 1-demethyl compound, 5,15-dimethylmorindol (**11**), which was previously isolated from the fruits of this plant,[8] was also isolated from the leaves in our present experiment.

The other 24 compounds were identified to be roseoside II (**3**),[9] deacetyl asperuloside (**4**),[10] kaempferol-3-*O*-α-L-rhamnopyranosyl-1(1→6)-β-D-glucopyranoside (**5**),[11] ursolic acid (**6**),[12] quercetin-3-*O*-α-L-rhamnopyranosyl-1(1→6)-β-D-glucopyranoside (**7**),[11] 13-hydroxy-9,11,15-octadecatrienoic acid (**8**),[13] pteryxin (**9**),[14] peucedanocoumarin III (**10**),[14] 5,15-dimethylmorindol (**11**),[8] barbinervic acid (**12**),[15] clethric acid (**13**),[16] rotungenic acid (**14**),[17] hederagenin (**15**),[18] oleanolic acid (**16**),[12] pheophorbide a (**17**),[19] methyl pheophorbide b (**18**),[20] methyl pheophorbide a (**19**),[19] 15^1(*S*)-hydroxypurpurin-7 lactone dimethyl ester (**20**),[21] 13^2(*R*)-hydroxypheophorbide a methyl ester (**21**),[21] 15^1(*R*)-hydroxypurpurin-7 lactone dimethyl ester (**22**),[21] 13^2(*S*)-hydroxypheophorbide a methyl ester (**23**),[21] 3-*O*-acetylpomolic acid (**24**),[22] 13-epi-phaeophorbide a methyl ester (**25**),[23] and phytol (**26**)[24] by comparison with literature spectral data.

Table 2. ^{1}H- and ^{13}C-NMR Data of Compound **2** in Acetone-d_6

	^{1}H	^{13}C
1		157.22
1-OMe	3.82[a] (3H, s)	61.70
2		139.49
3	7.83 (1H, d, J=8.0 Hz)	133.29
4	7.95 (1H, d, J=8.0 Hz)	122.52
5		146.82
5-OMe	3.82[a] (3H, s)	60.52
6		156.82
6-OH	10.66 (1H, s)	
7	7.32 (1H, d, J=8.5 Hz)	121.47
8	7.86 (1H, d, J=8.5 Hz)	126.02
9		180.76
10		181.87
11		126.02
12		127.17
13		124.56
14		135.85
15	4.58 (2H, s)	68.24
15-OMe	3.40 (3H, s)	58.13

a) 1-OMe at δ 3.818 and 5-OMe at δ 3.822 were distinguishable in the ^{1}H- and 2D NMR experiments of compound **2**.

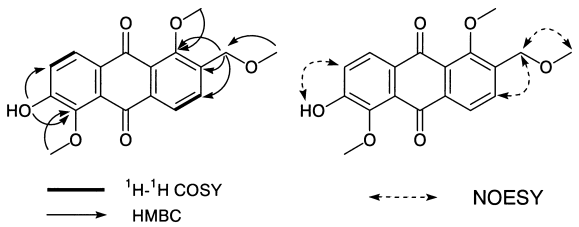

Fig. 3. Key ^{1}H–^{1}H COSY, HMBC, and NOESY Correlations for 1,5,15-Trimethylmorindol (**2**)

In the cell proliferation assay, 25 μg/ml of 1,5,15-trimethylmorindol (**2**) did not show significant cytotoxic activity on the human T-cell leukemia cell line, Jurkat, by itself but it showed cytotoxicity (IC_{50} 14.5—15.0 μg/ml) when combined with 0.5—1.5 μg/ml of TRAIL (Table 3). On the other hand, 50 μg/ml of citrifoside (**1**) did not show any significant growth inhibition on Jurkat cells either with or without TRAIL. These results suggest that combined treatment with 1,5,15-trimethylmorindol (**2**) and TRAIL might be a new strategy for cancer therapy.

Experimental

General Procedures ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AMX 500 spectrometer using TMS as the internal standard. Mass

Table 3. Cell Proliferation Assay Results (T/C%) of Compound **2**

2 (μg/ml)	TRAIL (μg/ml)			
	0	0.5	1.0	1.5
25.0	87	32	34	37
6.3	92	79	75	73
1.56	97	88	79	81
0	100	90	85	80
IC_{50} (μg/ml)	>25	14.6	14.5	15.0

spectra were obtained on a JEOL JMS-700 spectrometer. Optical rotations were determined on a JASCO P-1020 polarimeter. UV and IR spectra were obtained on a Shimadzu UV-2500PC UV–VIS recording spectrophotometer and a Perkin Elmer Spectrum One FT-IR spectrometer, respectively.

Plant Material The dried leaves of *M. citrifolia* cultivated in Okinawa were purchased from Sunlife Limited, Okinawa, Japan, in November, 2004. A voucher specimen (J-020) is deposited in the Institute of Biomaterials & Bioengineering, Tokyo Medical & Dental University.

Extraction and Isolation The dried leaves (1.03 kg) were extracted three times with MeOH (5.0 l) at room temperature for one week. The MeOH solutions were combined and evaporated to give 236.2 g of MeOH extract. The extract was suspended in saturated aqueous NaCl (5.0 l), acidified with hydrochloric acid to pH 3.5, and extracted twice with EtOAc (5.0, 2.5 l). The EtOAc solutions were combined and evaporated to give 43.71 g of acid EtOAc extract. The aqueous solution was then alkalized with aqueous NaOH to pH 9.0, extracted twice with EtOAc (5.0, 2.5 l), and evaporated to give 3.33 g of alkaline EtOAc extract. The alkaline EtOAc extract (3.33 g) was subjected to reversed-phase column chromatography (50 g of Wakogel 50C18, Wako Pure Chemical Industry Ltd., step gradient with water, MeOH, and THF) to obtain six fractions. These fractions were further purified repeatedly by preparative HPLC (Capcellpak C18 UG 80, Shiseido, 30×250 mm) to give **1** (9.7 mg), **3** (11.7 mg), **4** (1.9 mg), **5** (1.1 mg), and **6** (28.7 mg). The acid EtOAc extract (43.71 g) was also subjected to reversed-phase column chromatography (200 g of Wakogel 50C18, step gradient with water, MeOH, and THF) to obtain twelve fractions. These fractions were also purified repeatedly by preparative HPLC (Capcellpak C18 UG 80, 30×250 mm) to give **2** (12.3 mg), **5** (16.8 mg), **7** (7.6 mg), **8** (2.7 mg), **9** (4.3 mg), **10** (3.7 mg), **11** (4.1 mg), **12** (4.4 mg), **13** (8.0 mg), **14** (9.8 mg), **15** (7.4 mg), **16** (76.6 mg), **17** (7.7 mg), **18** (5.6 mg), **19** (15.8 mg), **20** (2.7 mg), **21** (4.7 mg), **22** (7.5 mg), **23** (11.1 mg), **24** (10.5 mg), **25** (1.3 mg), and **26** (5.2 mg).

Citrifoside (**1**): Colorless amorphous solid; $[\alpha]_D^{20}$ −56.8° (c=0.105, MeOH); HR-ESI-MS: m/z $[M+Na]^+$ 397.1115 (Calcd for $C_{16}H_{22}O_{10}Na$: 397.1111); IR ν_{max} cm^{-1}: 3350, 1700, 1635, 1439, 1369, 1286, 1180, 1156, 1072, 1022, 1004; UV (MeOH) λ_{max} nm (log ε): 235.4 (3.86); ^{1}H- and ^{13}C-NMR (500 MHz and 125 MHz, DMSO-d_6): see Table 1.

1,5,15-Trimethylmorindol (**2**): Yellow amorphous powder; HR-EI-MS: m/z $[M]^+$ 328.0945 (Calcd for $C_{18}H_{16}O_6$: 328.0947); IR ν_{max} cm^{-1}: 3380, 1667, 1656, 1574, 1374, 1319, 1269, 1200, 1126, 1082, 1046, 1023, 994, 961; UV (MeOH) λ_{max} nm (log ε): 340.8 (3.37), 270.0 (3.88), 248.4 (3.92); ^{1}H- and ^{13}C-NMR (500 MHz and 125 MHz, acetone-d_6): see Table 2.

Determination of the Absolute Configuration of the Glucose Unit of Citrifoside (1)[4—6] A solution of **1** (5 mg) in 1 M HCl (dioxane–water, 1 : 1, 2 ml) was heated at 100 °C for 2 h. The reaction mixture was concentrated, dissolved in water, passed through a Waters Sep-Pak Light C18 cartridge, and evaporated to furnish the glucose residue. After dissolving in water (1 ml), the solution of (*S*)-α-methylbenzylamine (5 mg) and 2-picoline-borane complex[25] (14 mg) in EtOH (1 ml) was added. The mixture was allowed to stand overnight, then acidified by addition of acetic acid (0.2 ml), and evaporated to dryness. The residue was acetylated with acetic anhydride (0.3 ml) in pyridine (0.3 ml) at 100 °C for 1 h. After co-distillation with toluene, DMSO (0.2 ml) was added to the residue and the crude mixture was identified by LC/MS analysis with the standard D- or L-glucose derivatives prepared under the same conditions. HPLC conditions: column, Cadenza CD-C18 (4.6 mm I.D.×75 mm, Imtact Co., Kyoto, Japan); flow rate, 0.8 ml/min; eluent, CH_3CN–H_2O (35 : 65) containing 0.1% formic acid; oven temperature, 40 °C; detection, MS (Agilent 1100 MSD, SIM, posi, m/z 538). The derivatives of standard D- and L-glucoses were detected with t_R (min) of 14.2 and 13.1, respectively. The derivative of the glucose of citrifoside (**1**) was detected with with t_R (min) of 14.2 and determined to be a D type.

Cell Proliferation Assay Jurkat cells (3.5×10^5) were seeded in 95 μl of culture medium per well in 96-well microtiter plates and were treated with 5 μl of various concentrations of samples in the absence or presence of 0.5, 1.0, or 1.5 μg/ml recombinant human soluble TRAIL (Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.), and then incubated for 42 h at 37 °C in a 5% CO_2–95% air atmosphere. Cell proliferation (percentage of control cells) was determined with the colorimetric assay using Almar Blue (Biosource International, Camarillo, CA, U.S.A.). The IC_{50} values of apigenin and vincristine were 30 μg/ml and 3.1 ng/ml, respectively.

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