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Limonoids from fruit of *Melia toosendan* and their cytotoxic activity

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Abstract

Two new limonoids, toosendanal and 12-*O*-methylvolkensin, were isolated from the fruits of *Melia toosendan* Sieb. et Zucc. along with three known limonoids, meliatoxin B_1, trichilin H, and toosendanin. The structures of the new limonoids were established by spectroscopic methods, with toosendanal having C-1/C-29 and C-19/C-29 acetal bridges. Both meliatoxin B_1 and toosendanin exhibit cytotoxic activity against KB cells. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Melia toosendan*; Meliaceae; Limonoid; Acetal bridge limonoid; Cytotoxic agent; Toosendanal; 12-*O*-methylvolkensin; Meliatoxin B_1; Toosendanin; Trichilin H

1. Introduction

Melia toosendan Sieb. et Zucc. (Meliaceae), the Chinaberry tree, has long been recognized as an insecticidal and medicinal plant in China, as discussed in Nakatani, Inada and Lavie (1986). Its fruits, with the names 'Chuan-Lian-Zi' in Chinese and 'Sen-Ren-Shi' in Japanese, are used for treatment of malaria, for stomach aches caused by roundworms, and even as an insecticide, see Inada, Kobayashi and Nakatani (1988). A number of triterpenes and limonoids have been isolated from the fruits, the most active constituents being the azadirachtin-type C-seco-limonoids (Warther, 1989; Ruo, Okumura, Iwagawa & Nakatani, 1994). Intact apo-euphol limonoids, such as the trichilins (Ochi, Kotsuki, Ishida & Tokoroyama, 1978) with a 14,15-epoxide and a C-19/C-29 lactol bridge are also active. Using a bioassay-guided fractionation procedure, we isolated five limonoids, including two new compounds, from a methanolic extract of *Melia toosendan* fruits. In this paper, we propose structures for these new limonoids and describe their levels of cytotoxicity against human KB tumor cell lines (Alley et al., 1988).

2. Results and discussion

An aqueous solution of the 90% methanolic extract obtained from *M. toosendan* was partitioned with diethyl ether and l-butanol, successively. The ether extract was highly cytotoxic against KB cells. This extract was then eluted through a Diaion HP-20 column using a H_2O/MeOH gradient to obtain fractions A–E. Fraction C (90% MeOH fraction), which was the most cytotoxic, was separated by silica gel column chromatography using C_6H_6-EtOAc. In addition, the active fraction (40% EtOAc) was separated by HPLC using a gel permeation column to give compounds **1**–**5**, which were detected using a bioassay-guided fractionation against KB cells. Compounds **1** and **2** were found to be new compounds, whereas compounds **3**–**5** were meliatoxin B_1 (Oelrichs, Hill, Vallely, MacLeod & Molinski, 1983), trichilin H (Nakatani, Ruo, Okumura, Naoki & Inagawa, 1994) and toosendanin (Xie & Yuan, 1985), based on comparison of their spectral and physical data with those in the literature.

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Table 1
^{1}H- and ^{13}C-NMR Spectral Data[a,b], for Toosendanal (**1**) and 12-*O*-Methylvolkensin (**2**)

	1		**2**	
Position	δ_H (*J*: Hz)	δ_C [c]	δ_H (*J*: Hz)	δ_C
1	4.73 brs	70.7 d	4.73 t-like	70.9 d
2	α 3.29 dt (15.7, 4.7)	37.2 t	α 2.15 dt (16.6, 2.2)	27.7 t
	β 2.32 d-like (15.7)		β 2.22 dt (16.6, 2.9)	
3	5.99 t-like	74.3 d	4.93 d (2.9)	71.6 d
4		40.5 s		42.7 s
5	3.63 dt (11.4, 4.0)	28.8 d	2.89 d (12.5)	38.5 d
6	α 2.35 m	24.3 t	4.04 dd (12.5, 2.9)	74.0 d
	β 2.02 dt (14.3, 4.0)			
7	4.55 brs	69.4 d	4.37 d (2.9)	73.4 d
8		45.1 s		46.1 s
9	4.70 s	47.5 d	3.13 d (9.3)	34.8 d
10		42.9 s		40.7 s
11		208.8 s	α 1.75 m	37.9 t
			β 1.58 m	
12	5.48 s	79.9 d	4.61 s	98.0 d
13		47.9 s		139.1 s
14	4.11 s	59.3 d		144.5 s
15		217.3 s	4.95 m	77.0 d
16	α 2.75 m	44.6 t	α 2.58 m	31.5 t
	β 2.76 m		β 1.60 m	
17	3.53 m	38.4 d	3.44 d (9.5)	46.8 d
18	1.17 s	21.8 q	1.75 s	16.1 q
19	α 4.64 d (12.1)	64.8 t	0.96 s	16.0 q
	β 4.73 d (12.1)			
20		123.9 s		128.7 s
21	7.24 s	141.1 d	7.28 s	139.0 d
22	6.43 s	111.3 d	6.42 s	110.5 d
23	7.55 s	143.6 d	7.31 s	142.8 d
28	1.18 s	20.0 q	α 3.59 d (8.1)	78.0 t
			β 3.62 d (8.1)	
29	5.39 s	96.6 d	1.20 s	19.7 q
30	1.34 s	20.6 q	1.35 s	20.7 q
1'				166.8 s
2'				129.4 s
2'-CH_3			1.93 d (5.9)	11.9 q
3'			6.96 dd (7.3, 5.9)	136.5 d
3'-CH_3			1.82 d (7.3)	14.3 q
OAc	1.95 s	170.6 s, 20.7 q	1.95 s	170.3 s, 20.8 q
	2.09 s	170.7 s, 28.8 q		
OCH_3			3.06 s	53.9 q

[a] Spectra were recorded at 400 MHz for ^{1}H and at 100 MHz for ^{13}C in $CDCl_3$.
[b] TMS was the internal standard.
[c] Multiplicities were determined by DEPT and ^{1}H-^{13}C COSY spectra.

Compound **1**, toosendanal, was obtained as colorless needles, m.p. 272.0–273.5°C, as well as having an $[\alpha]_D$ −32.7° ($c=0.1$, MeOH); it also gave a positive Ehrlich test. The molecular formula of **1** derived from its molecular ion at m/z 556.6084 in EIMS, and NMR spectral data, was $C_{30}H_{36}O_{10}$. Its UV spectrum gave a maximal absorption at 212 nm, whereas its IR spectrum had absorbances at 3495 (OH), 1750, 1725, and 1708 (ester), 1697 and 879 cm^{-1} (furan ring), respectively. Moreover, the absorption at 879 cm^{-1}, and the three olefinic proton signals at δ6.43, 7.24 and 7.55 (each 1H, s) in the ^{1}H-NMR spectrum suggested the presence of a furan ring. Furthermore, the ^{1}H-NMR spectrum suggested three tertiary methyl groups (δ1.17, 1.18 and 1.34) and two acetyl groups (δ1.95 and 2.09) were present in the molecule. ^{13}C-NMR and DEPT spectra of **1** revealed the presence of five methyl groups, four methylene groups, eight methine groups, four methine groups attached to an oxygen moiety, give quaternary carbons, three carbonyl carbons containing a carbonyl carbon (δ217.3) and two ester carbonyl carbons (δ170.6 and 170.7) (Table 1).

The similarity of the NMR spectra of **1** to that of toosendanin (**5**) suggested that they differed only with

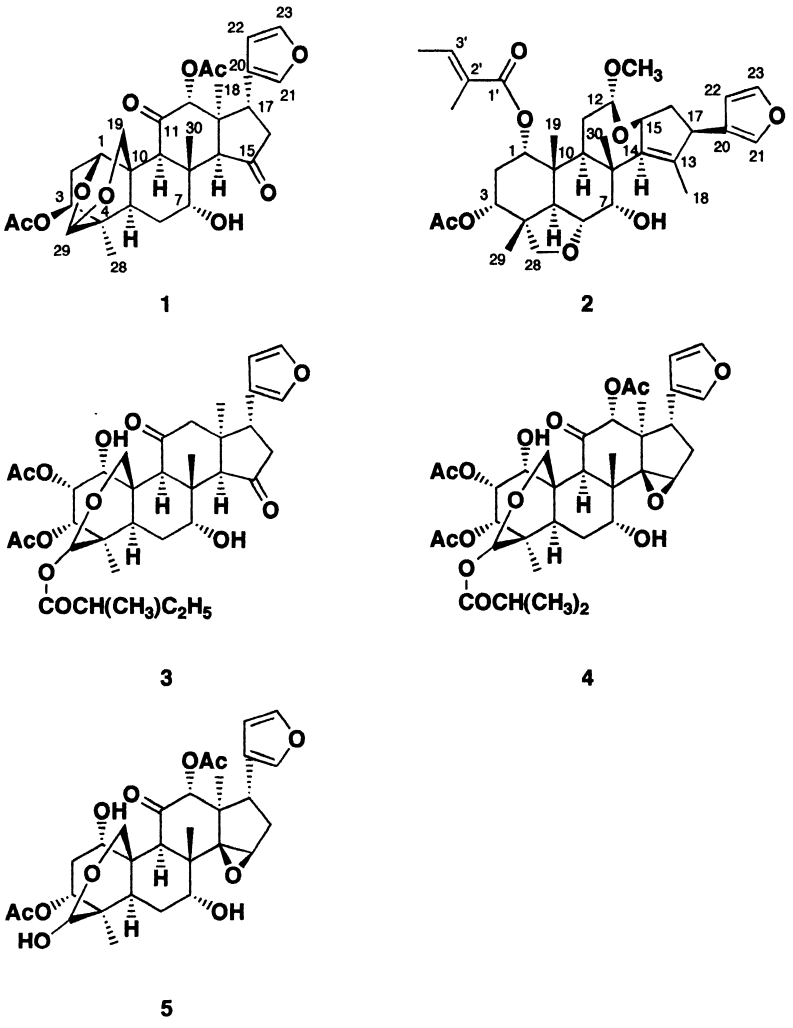

Fig. 1. Structure of compounds 1–5.

respect to an epoxy ring on C14/15 in **5** and a ketone on the D ring in **1**. However, **1** was eighteen mass units smaller than **5**. Hence, **1** appeared to be a dehydrated analog of **5**. The heteronuclear multiple bond connectivity (HMBC) spectrum of **1** showed ^{1}H-^{13}C long-range correlations between methylene protons at δ4.64 (H-19α) and 4.73 (H-19β) and among δ42.9 (C-10), 28.8 (C-5), 70.7 (C-1) and 96.6 (C-29), which in turn was coupled to δ1.18 (Me-28). On the other hand, the signal of H-1 (δ4.73) showed long-range correlations with δ28.8 (C-5), 42.9 (C-10) and 96.6 (C-29). These findings revealed acetal bridges at C-1/C-29 and C-19/C-29. Accordingly, we propose that toosendanal (**1**) has a planar structure (Fig. 1) following long-range spin networks from Me-18 (δ1.17) and Me-30 (δ1.34).

The relative stereochemistry of **1** was obtained from the results of the nuclear Overhauser effect spectroscopy (NOESY) (Fig. 1). These results suggested an A/B *trans*, B/C *trans* and C/D *cis* structure. The NOEs between H-14/18-Me and H-17/30-Me indicated an α configuration of the furan ring. NOEs were observed between H-3/H-2α and H-2α/28-Me, and the H-3 signal appeared triplet-like in the ^{1}H-NMR spectrum. A Dreiding model of **1** suggested that the A ring formed a twisted boat like other trichilin type limonoids (Ochi,

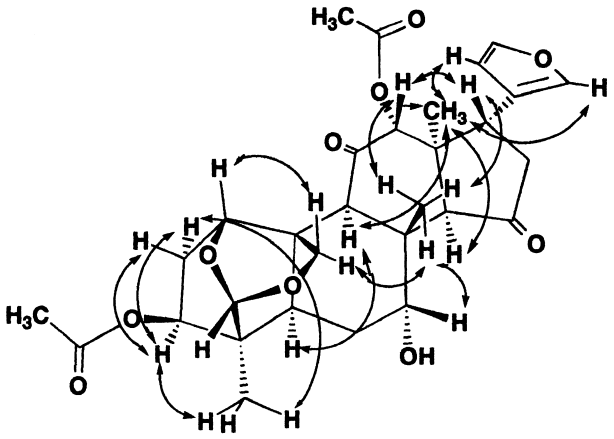

Fig. 2. NOEs of toosendanal (**1**)

Table 2
Growth Inhibitory Concentration (IC_{50}) of Limonoids from *Melia toosendan* against KB Cells

Compound	IC_{50} value (μg/ml)
Toosendanal (**1**)	>10
12-*O*-Methylvolkensin (**2**)	8.72
Meliatoxin B_1 (**3**)	>10
Trichilin H (**4**)	0.11
Toosendanin (**5**)	3.82
Adriamycin (HCl salt)	0.066

Kotsuki, Ishida & Tokorayama, 1978). Therefore, H-3 in **1** assumes an α position on the basis of the NOEs, with the small coupling value indicative of triplet-like (see Fig. 2). The relative stereochemistry of **1** is shown in Fig. 1. Toosendanal (**1**) is the first report of a limonoid having C-1/C-29 and C-19/C-29 acetal bridges.

12-*O*-methylvolkensin (**2**), was also obtained as colorless needles with m.p. 236.5–238.0°C. It has an $[\alpha]_D$ −52.0° ($c=0.1$, $CHCl_3$), and gave a positive Ehrlich test. Mass spectral analysis suggested a molecular formula of $C_{33}H_{42}O_{10}$ (HRMS m/z 598.3118 $[M]^+$). The IR spectrum gave absorbances at 3513 (OH), 1739 (C═O), 1697 (conj. C═O) and 898 cm^{-1} (furan ring). Of these, the absorption at 898 cm^{-1}, combined with ^{1}H-NMR signals of three olefinic protons at δ6.42, 7.28 and 7.31 (each 1H, s) indicated the compound possessed a furan ring. Seven singlet methyl groups were also suggested from the ^{1}H-NMR spectrum (δ0.96, 1.20, 1.35, 1.75, 1.93, 1.95 and 3.06) as well as three aliphatic methyl groups at δ0.96, 1.20, 1.35, and 1.93, two olefinic methyl groups (δ1.75 and 1.93), an acetyl group (δ1.95), and a methoxy group (δ3.06). Absorbances at 1739 and 1697 cm^{-1} and ^{13}C-NMR signals at δ166.8 and 170.3 also suggested two carbonyl ester carbons. The HMBC spectrum of **2** showed ^{1}H-^{13}C long-range correlations between methyl protons at δ1.20 (H-29) and δ71.6 (C-3), 42.7 (C-4), 38.5 (C-5) and 78.0 (C-28), and between the two methylene signals at δ3.59 (H-28α) with those at 3.62 (H-28β) and δ1.20 (C-29), 38.5 (C-5), 42.7 (C-4) and 74.0 (C-6). Thus, an ether bridge was indicated by the ^{1}H-^{13}C long-range correlations between C-28 and C-6. The presence of an ether bridge between C-12 and C-15 was also indicated by the ^{1}H-^{13}C long-range correlations between C-12 and H-15. The positions of the acetyl, methoxyl and the tigloyl groups were also suggested from ^{1}H-^{13}C long-range correlations in the HMBC spectrum.

The relative stereochemistry of **2** has an A/B *trans*, B/C *trans* structure, based on the fact that NOEs were observed between 19-Me/H-6, 29-Me and 30-Me and H-5/28-Me and H-9 in NOESY experiment. Further, the NOEs between 19-Me/H-1, H-1/H-3, H-3/H-29, 30-Me/H-6 and 30-Me/H-7 indicated a β configuration of the furan ring. On the other hand, the stereochemistries of H-15, 12-Me, H-9 and H-17 are α, because NOEs appeared between H-15/H-9 and 12-Me/H-17. Based upon these findings, the stereochemistry of **2** is shown in Fig. 1. The structure of **2** was confirmed as 12-*O*-methyl ether of volkensin (Kraus & Bokel, 1981; Ara, Siddiqui, Faizi & Siddiqui, 1989).

The cytotoxic activity of compounds **1**–**5** against KB cells is shown in Table 2. Trichilin H (**4**) and toosendanin (**5**) were highly cytotoxic against KB cells in vitro. However, toosendanal (**1**) and meliatoxin B_1 (**3**) did not show any significant level of toxicity. Trichilin H and toosendanin have C-14/C-15-epoxide structures, whereas toosendanal and meliatoxin B_1 have C-15-keto structures, suggesting perhaps that the cytotoxic activity against KB cells requires the C-14/C-15-epoxide structure. 12-*O*-methyl volkensin (**2**) was not significantly cytotoxic.

3. Experimental

Melting points were determined on a Yanagimoto micro-melting-point apparatus and were uncorrected. The UV spectra were obtained using a Hitachi 200-10 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-300 spectrophotometer. NMR spectra were obtained with a JEOL JNM GX-400 instrument (400 MHz for ^{1}H-NMR). Chemical shifts are given in ppm relative to internal tetramethylsilane (TMS). Mass spectra were recorded with a Hitachi M-80B spectrometer.

3.1. Plant material

Seeds of *Melia toosenndan* Sieb. et Zucc. (Meliaceae) were collected in December 1994 in Szechwan, China. A voucher specimen is deposited in the Herbarium of College of Pharmacy, Nihon University, Chiba, Japan.

3.2. Extraction and isolation

Crushed fruit of *M. toosendan* (5.0 kg) were sonicated in 90% MeOH (15 l × 3) for 30 min. The MeOH extract was concentrated in vacuo to give a brown residue (946.0 g), which was suspended in H_2O (2 l), and partitioned with Et_2O (2 l × 3). The aqueous layer was partitioned l-butanol (2 l × 3). The evaporated combined Et_2O soluble extract (125.5 g) was next subjected to Diaion HP-20 column chromatography (15 cm i.d. × 30 cm), eluted with MeOH-H_2O (0:1 → 1:0, each 20 l). The evaporated extract (18.9 g) of 90% methanolic fraction was then fractionated by silica gel column chromatography with C_6H_6–EtOAc (1:0 → 0:1). The C_6H_6–EtOAc (8:2) fraction (1.02 g) was chromatographed on silica gel ($CHCl_3$–MeOH/99:1) to

afford 12-*O*-methyl volkensin (**2**) (26.0 mg) and toosendanin (**5**) (124.5 mg). The C_6H_6–EtOAc (8:2) fraction (4.89 g) was next subjected to silica gel column chromatography ($CHCl_3$–MeOH/98:2) to afford toosendanal (**1**) (35.8 mg), meliatoxin B_1 (**3**) (32.8 mg) and trichilin H (**4**) (20.6 mg).

*3.2.1. Toosendanal (**1**).*

Colorless needles, m.p. 272.0–273.5°, $[\alpha]_D^{18}$ −32.7° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm: 212.IR ν_{max}^{KBr} cm^{-1}: 3550, 3495, 2964, 1750, 1725, 1708, 1376, 1274, 1232, 1179, 1122, 1071, 1054, 879, 790, 607. HRMS *m*/*z*: Calcd. for $C_{30}H_{36}O_{10}$:556.2307. Found: 556.2309. EIMS *m*/*z* (rel. int.): 556 $[M]^+$ (6.0), 514 (31.9), 496 (16.6), 468 (8.1). ^1H- and ^{13}C-NMR data are shown in Table 1.

*3.2.2. 12-O-methylvolkensin (**2**).*

Colorless needles, m.p. 236.5–238.0°, $[\alpha]_D^{18}$ −52.0° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm (log ϵ): 205 (3.01), 271 (2.89). IR ν_{max}^{KBr} cm^{-1}: 3513, 2936, 1739, 1697, 1648, 1437, 1377, 1268, 1154, 1058, 898, 791, 729. HRMS *m*/*z*: Calcd. for $C_{34}H_{46}O_9$: 598.3138. Found: 598.3115. EIMS *m*/*z* (rel. int.): 598 $[M]^+$ (8.9), 566 (11.8), 548 (6.2), 435 (11.3), 259 (18.7), 147 (100), 83 (78.7), 43 (36.2). ^1H- and ^{13}C-NMR data are shown in Table 1.

3.3. Bioassay of cytotoxic activity against KB cells

The MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in 96-well plates. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product which can be measured spectrophotometrically. Human KB cells (10^{-4} cells/ml) were seeded into each well in 100 μl of Eagle's minimum essential medium (MEM) (Gibco Co. Ltd.) containing 10% fetal bovine serum (FBS) (Gibco Co. Ltd.), 1% glutamine (Nissui Co. Ltd.) and gentamycin (100 μg/ml) (Wako Chemical Co. Ltd.), plates were then incubated at 37°C in a humidified atmosphere containing 5% CO_2. Various drug concentrations (10 μl) were added to the cultures 1 day after subculture. At 4 days, 20 μl of MTT solution (5 mg/ml) was added to each well. After a further 4 h incubation 100 μl of dimethylsulfoxide was added to the wells and formazan crystals in each were dissolved by vibration. Optical density was measured using a microplate reader (BIO RAD) at two wavelengths (590 nm and 700 nm). Data was obtained from triplicate wells. Adriamycin (HCl salt) was used as a control treatment.

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