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Cytotoxic Trichilin-Type Limonoids from *Melia azedarach*

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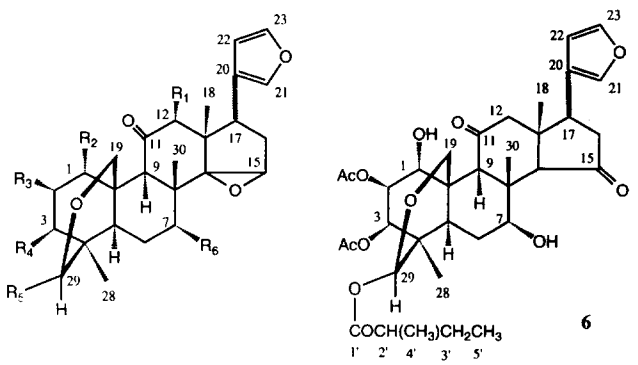
Abstract—Five new trichilin-type limonoids, named 12-deacetyltrichilin I (**1**), 1-acetyltrichilin H (**2**), 3-deacetyltrichilin H (**3**), 1-acetyl-3-deacetyltrichilin H (**4**), 1-acetyl-2-deacetyltrichilin H (**5**), together with four known trichilins, meliatoxin B_1 (**6**), trichilin H (**7**), trichilin D (**8**) and 1,12-diacetyltrichilin B (**9**) were isolated from the extract of the root bark of *Melia azedarach*. The structures were elucidated by spectroscopic means and their cytotoxic activities against P388 cells in vitro were tested by means of MTT assay.

Introduction

Limonoids have attracted much attention because of the marked insect antifeedant and growth regulating property,[1,2] cytotoxic[3] and antiviral activities.[4] Recently, we isolated two new azadirachtin derivatives and three highly cytotoxic sendanin analogues from the EtOH extract of the root bark of *Melia azedarach* (Meliaceae).[5,6] During our continuing study of the cytotoxic limonoids from the plant, five new trichilins (**1–5**) together with four known ones (**6–9**), were isolated from the plant. In this paper, we describe the isolation, structural elucidation and cytotoxic activity of these compounds.

Results and Discussion

The ethanolic extract of *M. azedarach* was suspended in water and then partitioned with dichloromethane and *n*-butanol, successively. The CH_2Cl_2 extract, the strongest cytotoxic part, was subjected to silica gel column chromatography by eluting with *n*-hexane:ethyl acetate gradient system (1:0–0:1) to give 14 fractions (fr. A–N). Further isolation and purification of the active fractions K [*n*-hexane:ethyl acetate (5:5) eluting part] and L (ethyl acetate eluting part) by means of open silica gel chromatography, ODS MPLC and ODS HPLC gave compounds **1–9**. The compounds **1–5** were new substances, while **6–9** were known ones which were confirmed to be meliatoxin B_1 (**6**), trichilin H (**7**), trichilin D (**8**) and 1,12-diacetyltrichilin B (**9**) by comparing the spectral and physical data with those in the literature.[7,8]



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1 $R_1 = R_2 = R_3 = R_6 = OH$, $R_4 = OAc$, $R_5 = OCOCH(CH_3)CH_2CH_3$
2 $R_1 = R_2 = R_3 = R_4 = OAc$, $R_6 = OH$, $R_5 = OCOCH(CH_3)_2$
3 $R_1 = R_3 = OAc$, $R_2 = R_4 = R_6 = OH$, $R_5 = OCOCH(CH_3)_2$
4 $R_1 = R_2 = R_3 = OAc$, $R_4 = R_6 = OH$, $R_5 = OCOCH(CH_3)_2$
5 $R_1 = R_2 = R_4 = OAc$, $R_3 = R_6 = OH$, $R_5 = OCOCH(CH_3)_2$
7 $R_1 = R_3 = R_4 = OAc$, $R_2 = R_6 = OH$, $R_5 = OCOCH(CH_3)_2$
8 $R_1 = H$, $R_2 = R_6 = OH$, $R_3 = R_4 = OAc$, $R_5 = OCOCH(CH_3)CH_2CH_3$
9 $R_1 = R_2 = R_3 = R_4 = OAc$, $R_6 = OH$, $R_5 = OCOCH(CH_3)CH_2CH_3$

12-Deacetyltrichilin I (1). 12-Deacetyltrichilin I (**1**) was obtained as colorless powder and assigned with a molecular formula $C_{33}H_{44}O_{12}$ from the $[M-(OCOCH(CH_3)CH_2CH_3)]^+$ ion at *m/z* 531 in the EIMS. The NMR data (Tables 1 and 2) indicated the presence of one 2-methylbutyryl and one acetyl group connected in a trichilin skeleton concluding three methyl (δ 1.14 s 3H, 1.12 s 3H and 0.95 s 3H) , a 14,15-epoxide (δ 3.76 s 15α-H, 58.9 d C-15, and 70.1 s C-14), a 19/29 bridged acyl acetal ester system (δ 4.33 d, 4.30 d 19–2H, 5.77 s 29-H, 64.0 t C-19, and 94.2 d C-29), a 11-ketone (213.5 s C-11) and a furyl moiety (δ 7.22 1H, δ 6.52 1H and δ 7.32 1H) such as trichilins.[9,10] It was also suggested that **1** has a free 1α-OH from the chemical shift of H-9 at δ 4.55, which will shift upfield to δ 4.0–4.2 in the 1-*O*-acetyl trichilins, due to the effect of the 1-hydroxyl in a 1,3-diaxial relationship.[11] The fact that the acetyl group was connected to C-3 in **1** was deduced from the ^1H NMR signal of H-3 (δ 5.45 d, $J = 4.7$ Hz). It should be a triplet in C-2, a singlet in C-12 and a multiplet in C-7. The deduction of 12α-OH group in **1** was carried out from the chemical shift (δ 3.00) of 17β-H, which was shifted downfield to δ 3.39 in trichilin A (12β-OH).[12] The 29-*exo*-configuration of **1** was assigned from the chemical shift of 3-H (δ 5.45 d), which appears at δ 5.3–5.6 in sendanins or trichilins with a 29-*exo*-configuration, but at δ 4.9–5.1 with a 29-*endo*-configuration.[8]

1-Acetyltrichilin H (2). 1-Acetyltrichilin H (**2**) was obtained as a colorless powder and assigned with a molecular formula $C_{38}H_{48}O_{15}$ from the $[M-OCOCH(CH_3)_2]^+$ ion at *m/z* 657 in the EIMS. The NMR data is quite similar to those of 1,12-diacetyltrichilin B (**9**),[8] except for the changing of the ester moiety of the 2-methylbutyryl group at C-29 to the 2-methylpropionyl group (δ 2.66 hept, 1.20 d, 1.21 d) in **2**.

3-Deacetyltrichilin H (3). 3-Deacetyltrichilin H (**3**) was obtained as a colorless powder and assigned with a molecular formula $C_{34}H_{44}O_{13}$ from the $[M-OCOCH(CH_3)_2]^+$ ion at *m/z* 573 in the EIMS. The structure of **3** was elucidated by comparison of the NMR data with those of trichilin H (**7**).[8] It was identical to trichilin H except for the signal due to the 3-acetyl group in **7**.

1-Acetyl-3-deacetyltrichilin H (4) and 1-acetyl-2-deacetyltrichilin H (5). 1-Acetyl-3-deacetyltrichilin H (**4**) and 1-acetyl-2-deacetyltrichilin H (**5**), obtained as a colorless powder, both had the same molecular formulas, $C_{36}H_{46}O_{14}$, as **7** from the $[M^+]$ ion at *m/z* 702 and $[M-OCOCH(CH_3)_2]^+$ ion at *m/z* 615 in the EIMS, respectively. The structures of **4** and **5** were also elucidated by comparison of the NMR data with those of **7**, that is, the ^1H NMR spectrum of **4** was identical to that of **7** except for the fact that the 1-hydroxyl-3-acetoxyl moiety in **7** was changed to the 1-acetoxyl-3-hydroxyl in **4**. The assignment of 1-acetoxyl in **4** was also supported by the upfield moving of H-9 (δ 4.13 s) in **4**, compared with that (δ 4.64 s, H-9) in **7**. The assignment of 1-acetoxyl in **5** was carried out in as the same manner as in **4**. The other acetoxyl group in **5** was deduced at C-3 from the doublet signals with $J=4.5$ Hz of H-3 (δ 5.47).

Cytotoxic activities

The cytotoxic activities of compounds **1–9** against P388 lymphocytic leukemia cells in vitro were examined and their IC_{50} values were determined as shown in Table 3. As can be seen from Table 3, all of those trichilins with a 14,15-epoxide, a 19/29 bridged acyl acetal ester system, a 11-ketone and furanyl moiety showed significant cytotoxic activities. Especially the compounds **1**, **3** and **8**, which have one or two acetyl groups in the structures, exhibited strong activities. The activities of compounds **2**, **4**, **5**, **7** and **9**, which have more than three acetyl groups in the structures, decreased slightly. When the 14,15-epoxide structure was broken, as in compound **6**, the cytotoxic activity decreased greatly.

Experimental

General methods

Melting points were uncorrected. Analytical instruments were used as follows, $[\alpha]_D$: JASCO DIP-4; MS:

Table 1. ^1H NMR data of trichilin type compounds (400 MHz, δ, $CDCl_3$)

	1	2	3	4	5
1	4.51 br t (4.2)	5.70 d (4.3)	4.44 br t (4.4)	5.78 d (4.3)	5.70 d (4.4)
2	4.69 m	5.97 t (4.4)	5.79 t (4.0)	5.93 t (4.4)	4.83 dd (9.2, 4.6)
3	5.45 d (4.7)	5.45 d (4.3)	4.13 br t (4.3)	4.05 br d (3.2)	5.47 d (4.5)
5	2.78 dd (13.9, 3.9)	2.86 dd (14.0, 4.0)	2.76 dd (13.9, 3.9)	2.84 dd (13.9, 4.0)	2.81 dd (14.2, 4.4)
6α	1.74 dt	1.73 dt (14.0, 4.0)	1.74 dt (14.5, 3.8)	1.75 dt (14.7, 4.2)	1.73 dt (14.4, 3.9)
6β	2.00 m	2.11 m	2.04 m	2.05 m	2.03 m
7	3.65 br s	3.70 br s	3.68 br s	3.70 br s	3.70 br s
9	4.55 s	4.18 s	4.66 s	4.13 s	4.20 s
12β	4.09 s	5.46 s	5.21 s	5.45 s	5.45 s
15	3.76 s	3.74 s	3.74 s	3.71 s	3.73 s
16α	2.32 dd	2.24 dd (13.4, 6.4)	2.24 dd (13.0, 6.2)	2.23 dd (13.6, 6.3)	2.25 dd (13.4, 6.4)
16β	1.91 d	1.89 dd (13.5, 11.2)	1.92 dd (13.5, 11.3)	1.89 dd (13.4, 11.3)	1.90 dd (13.3, 11.2)
17	3.00 dd (11.0, 5.4)	2.97 dd (11.0, 6.0)	2.97 dd (11.1, 6.4)	2.96 dd (11.0, 6.6)	2.97 dd (11.2, 6.3)
18	1.14 s	1.24 s	1.28 s	1.23 s	1.25 s
19a	4.33 d (8.0)	4.45 d (13.6)	4.33 ABq (9.9)	4.43 d (13.6)	4.47 d (13.3)
19b	4.30 d (8.7)	4.34 d (13.6)		4.33 d (13.4)	4.34 d (13.3)
21	7.22	7.12	7.13	7.11	7.12
22	6.52	6.09	6.16	6.08	6.09
23	7.32	7.33	7.33	7.32	7.33
28	0.95 s	0.87 s	0.99 s	1.01 s	0.84 s
29	5.77 s	5.87 s	5.78 s	5.77 s	5.75 s
30	1.12 s	1.14 s	1.15 s	1.14 s	1.15 s
OAc	2.15 s	1.89 s	1.98 s	1.97 s	1.99 s
		1.98 s	2.15 s	2.02 s	2.06 s
		2.05 s		2.08 s	2.11 s
		2.11 s			
2′	2.47 sext	2.66 hept (7.3)	2.62 hept (7.0)	2.61 help (7.0)	2.63 help (7.0)
3′	1.53 m, 1.70 m	1.20 d (7.0)	1.19 d (7.0)	1.19 d (7.0)	1.21 d (7.0)
4′	1.17 d (7.0)	1.21 d (7.0)	1.20 d (7.0)	1.19 d (7.0)	1.19 d (7.0)
5′	0.93 t (7.4)				

VG AutoSpec; IR: Perkin–Elmer 1710; ^1H and ^{13}C NMR: Bruker AM 400 and 500 MHz at 303 K. NOESY experiments were made with mixing time of 0.6 s and processed on a Bruker data station with an Aspect 3000 computer. Silica gel column chromatography was carried out on Merk Kieselgel 60 (70–230 mesh) at amounts equivalent to 100 times the sample amount. MPLC was performed with a column (22 mm i.d. × 300 mm) packed with 40 μm silica gel or 20 μm ODS. HPLC was performed with a Hibar RT RP-18 column (20 mm i.d. × 250mm) packed with 7 μm ODS. The NMR coupling constants (J) are given in Hz.

Plant material

The root bark of *M. azedarach* L. was collected at Jiangsu, China in 1993. The species was identified by Professor Zhi-Yu Zhang (Second Military Medical University, Shanghai, China). A reference specimen has been deposited in Herbarium of the Tokyo University of Pharmacy & Life Science.

Extraction and isolation

The fresh root bark of *M. azedarach* (5 kg) was cut into slices and extracted with 24 L 70% EtOH at 70 °C three times. The concentrated extract (241 g) was partitioned between CH_2Cl_2 and H_2O, followed between *n*-butanol and H_2O. The CH_2Cl_2 soluble fraction (56 g) was subjected to silica gel column chromatography using an *n*-hexane:EtOAc (1:0–0:1) gradient system to give 14 fractions (fr. A–N). Fraction L (15 g) was further chromatographed on silica gel column, eluted with CH_2Cl_2:MeOH (80:1–10:1) and purified by ODS MPLC and ODS HPLC with MeOH:H_2O eluting systems to give compounds **1** (t_R = 6.89 min in 70% MeOH) 17 mg, **2** (t_R = 6.94 min in 70% MeOH) 4.5 mg, **3** (t_R = 10.30 min in 65% MeOH) 3.8 mg, **4** (t_R = 9.30 min in 65% MeOH) 2.5

Table 2. ^{13}C NMR of trichilin-type compounds (100 MHz, δ, $CDCl_3$)

	1	2	3	4	5	6	9
1	70.9 d[a]	72.0 d[a]	70.2 d[a]	70.2 d[a]	70.2 d[a]	69.3 d[a]	70.1 d[a]
2	66.7 d	66.8 d	66.7 d	68.8 d	66.7 d	68.8 d	66.8 d
3	72.9 d[a]	72.2 d[a]	74.6 d[a]	73.5 d[a]	74.6 d[a]	71.9 d[a]	72.1 d[a]
4	40.6 s	40.6 s	40.4 s	41.1 s	40.4 s	40.7 s	40.6 s
5	41.1 d	34.1 d	34.1 d	34.2 d	34.1 d	41.0 d	40.1 d
6	25.5 t[b]	26.2 t	25.9 t	26.4 t	25.9 t	23.0 t	26.6 t[b]
7	70.0 d[a]	71.3 d[a]	73.8 d[a]	71.9 d[a]	73.8 d[a]	73.4 d[a]	72.2 d[a]
8	42.4 s	43.0 s	43.1 s	43.0 s	43.0 s	43.1 s	43.1 s
9	47.8 d	47.9 d	48.0 d	47.9 d	48.0 d	51.4 d	47.9 d
10	41.5 s	40.9 s	40.9 s	41.5 s	40.8 s	42.7 s	41.0 s
11	213.5 s	205.5 s	205.6 s	205.5 s	205.6 s	209.9 s	205.6 s
12	78.9 d	77.2 d	77.2 d	77.0 d	77.2 d	50.5 t	77.0 d
13	46.2 s	45.3 s	45.3 s	45.3 s	45.3 s	44.8 s	45.3 s
14	70.1 s	70.1 s	71.4 s	71.4 s	71.4 s	62.4 d	71.3 s
15	58.9 d	57.9 d	58.1 d	58.0 d	58.1 d	219.4 s	57.9 d
16	33.1 t	33.4 t	33.4 t	33.4 t	33.4 t	43.3 t	33.4 t
17	38.9 d	38.7 d	38.6 d	38.7 d	38.6 d	41.9 d	38.7 d
18	22.6 q	22.0 q	22.3 q	22.0 q	22.2 q	20.9 q	21.9 q
19	64.0 t	63.7 t	64.1 t	63.7 t	64.1 t	63.9 t	63.7 t
20	123.5 s	122.3 s	122.3 s	122.3 s	122.3 s	121.8 s	122.3 s
21	142.3 d	142.6 d	142.6 d	142.5 d	142.6 d	143.4 d	142.6 d
22	112.7 d	111.7 d	111.7 d	111.7 d	111.7 d	110.3 d	111.7 d
23	140.7 d	140.6 d	140.6 d	140.6 d	140.6 d	140.2 d	140.6 d
28	19.3 q	19.0 q	20.8 q	19.3 q	18.9 q	20.2 q	19.1 q
29	94.2 d	93.4 d	93.5 d	94.2 d	93.5 d	93.1 d	93.5 d
30	16.5 q	15.7 q	15.7 q	15.7 q	15.7 q	18.5 q	15.6 q
$CH_3\underline{C}O$	170.1 s	169.0 s	170.2 s	168.9 s	170.2 s	168.9 s	169.0 s
		169.2 s	170.8 s	169.0 s	170.4 s	169.9 s	169.2 s
		170.1 s		170.1 s	170.8 s		170.0 s
		170.2 s					170.1 s
$\underline{C}H_3CO$	21.2 q	20.8 q	21.0 q	20.7 q	20.7 q	20.8 q	20.4 q
		20.8 q	21.1 q	20.7 q	20.9 q	20.9 q	20.7 q
		20.8 q		21.8 q	21.1 q		20.8 q
		20.8 q					20.9 q
1′	175.0 s	175.8 s	175.6 s	175.7 s	175.6 s	175.3 s	175.3 s
2′	27.9 d	29.1 d	29.3 d	27.8 d	29.3 d	28.1 d	29.0 d
3′	26.5 t[b]	18.7 q	18.6 q	18.6 q	18.6 q	26.5 t	26.3 t[b]
4′	16.3 q	18.7 q	19.0 q	18.8 q	18.7 q	16.3 q	16.3 q
5′	11.5 q					11.3 q	11.3 q

[a,b] May be interchangable.

Table 3. Cytotoxic activity of compounds **1–9** against P388 cells

Compound	IC_{50} values (μg/mL)
12-Deacetyltrichilin I (**1**)	0.011
1-Acetyltrichilin H (**2**)	0.47
3-Deacetyltrichilin H (**3**)	0.045
1-Acetyl-3-deacetyltrichilin H (**4**)	0.40
1-Acetyl-2-deacetyltrichilin H (**5**)	0.66
Meliatoxin B_1 (**6**)	5.4
Trichilin H (**7**)	0.16
Trichilin D (**8**)	0.055
1,12-Diacetyltrichilin B (**9**)	0.46

mg, **5** (t_R = 9.10 min in 65% MeOH) 3 mg, **6** (t_R = 7.69 min in 70% MeOH) 9 mg, **7** (t_R = 8.42 min in 60% MeOH) 45.4 mg and **9** (t_R = 8.13 min in 70% MeOH) 12.6 mg. Fraction K (1.5 g) was chromatographed on silica gel column eluted with *n*-hexane:acetone (8:2) and purified with ODS HPLC with CH_3CN:H_2O (6:1) to give compound **8** (t_R = 7.75 min) 1.7 mg.

12-Deacetyltrichilin I (1). Colorless powder, mp 175–176 °C (from MeOH), $[\alpha]_D$ −32.4° (0.12, *c* MeOH); IR ν_{max} cm^{-1} (KBr): 3450 (br), 1710, 1455, 1375, 1062; EIMS *m/z*: 614 [M^+-18], 596, 555, 531[M^+-101], 512, 494, 470, 452, 435; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

1-Acetyltrichilin H (2). Colorless powder, mp 170–172 °C (from $CHCl_3$), $[\alpha]_D$ −19.7° (0.6, *c* $CHCl_3$), IR ν_{max} cm^{-1} ($CHCl_3$): 3730, 1742, 1720 (sh), 1700 (sh), 1602, 1373, 1095; EIMS *m/z*: 657 [M^+-87], 614, 554, 494, 452, 374; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

3-Deacetyltrichilin H (3). Colorless powder, mp 162–164 °C (from $CHCl_3$), $[\alpha]_D$ −4.0° (0.2, *c* $CHCl_3$), IR ν_{max} cm^{-1} ($CHCl_3$): 3600, 1735, 1715 (sh), 1598; EIMS *m/z*: 573 [M^+ - 87], 614, 554, 494, 452, 374; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

1-Acetyl-3-deacetyltrichilin H (4). Colorless powder, mp 162–164 °C (from $CHCl_3$), $[\alpha]_D$ −11.7° (0.2, *c* $CHCl_3$), IR ν_{max} cm^{-1} ($CHCl_3$): 3600, 1745, 1596; HRMS *m/z*: found 702.288345, required for molecular of $C_{36}H_{46}O_{14}$ 702.288757, EIMS *m/z*: 702 [M^+], 684, 642, 624, 615, 572, 554, 512, 452; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

1-Acetyl-2-deacetyltrichilin H (5). Colorless powder, mp 218–220 °C (from $CHCl_3$), EIMS *m/z*: 615 [M^+-87], 573, 375; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

Bioassay of cytotoxic activity against P388 cells

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate.[13] The cytotoxic activity of the complexes along with free ligands was examined against P388 lymphocyctic leukemia cells. The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. Mouse P388 leukemia cells (2×10^4 cells/mL) were inoculated in each well with 100 μL/mL of RPMI-1640 medium (Nissui Pharm. Co., Ltd) supplemented with 5% fetal calf serum (Mitsubishi Chemical Industry Co., Ltd) and kanamycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. Various drug concentrations (10 μL) were added to the cultures at day 1 after the transplantation. At day 3, 20 μL of MTT solution (5 mg/mL) per well was added to each cultured medium. After a further 4 h of incubation, 100 μL of 10% SDS–0.01 N HCl solution was added to each well and the formazan crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a microplate reader (Tohso MPR-A4i) with a two wavelength system (550 and 700 nm). In all these experiments, three replicate wells were used to determine each point. The results (IC_{50} values) shown in Table 3 are expressed by the concentration of each compound, which achieved 50% reduction of growth in sample-treated cells with respect to the control.

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