# Studies on Antitumor Screening of Medicinal Plants and Their BIOASSAY-GUIDANCED ISOLATION: CYTOTOXIC LIMONOIDS FROM *MELIA AZEDARACH* [1-3]

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**Abstract**

*Melia azedarach* L. (Meliaceae) is a widely distributed tree, whose bark, fruit and leaves have been traditionally used as anthelmintics in China and to cure malaria, fevers and venereal diseases in Africa. During our preliminary screening test for antitumor agents from higher plants, the ethanolic extract of the root bark of *M. azedarach* exhibited significant cytotoxic activity against P388 cells *in vitro*. An aqueous solution of the EtOH extract was partitioned with $CH_2Cl_2$ and *n*-BuOH, successively. When each extract was subjected to the cytotoxic bio-assay, the activity was found to be concentrated in the $CH_2Cl_2$ extract. Systematic cytotoxic bioassay-guidanced fractionation of the $CH_2Cl_2$ extract by means of various chromatography using silica gel or ODS column led to the isolation of seven azadirachtin-type limonoids and twelve tetranortriterpenoids. These structures were confirmed by various spectroscopic methods. Also they were demonstrated for cytotoxic activity against P388 cells.

**Key Word Index:** *Melia azedarach*, cytotoxity, limonoid, tetranortriterpenoid, azadirachtin, sendanin, trichilin, Meliaceae

## Introduction

Limonoids have atracted much attention because of the marked insect antifeedant[4] and growth regulating property[5], cytotoxic[6] and antiviral antivities[7]. *Melia azedarach* L. (Meliaceae), which is well known to contain limonoids as the chemical constituents, is a widely distributed tree, whose barks, fruit and leaves have been traditionally used as anthelmintics in China[8] and to cure malaria, fevers and venereal diseases in Africa.[9] During our preliminary screening test for antitumour agents from plants, the ethanolic extract of the root bark of *M. azedarach* exhibited significant cytotoxic activity against P388 cells *in vitro*. In this paper, we describe the isolation, structrural elucidation and cytotoxic activity of the effective principles from this plant.

## Material and Methods

**General Procedure** Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. $[\alpha]_D$: JASCO DIP-4. MS: VG AutoSpec. IR: Perkin Elmer 1710. $^1$H- and $^{13}$C-NMR: Bruker AM 400 and 500 MHz at 303 K. NOESY experiments were carried out with a 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 Merck Kieselgel 60 (70-230 mesh) using amounts equivalent to 100 times the sample. MPLC was performed on a column (22 mm i.d. x 300 mm) packed with 20 μm silica gel or 20 μm ODS. Final purification was made by HPLC using a Hibar RT RP-18 column (20 mm i.d. x 250 mm) packed with 7 μm ODS. The NMR coupling constants ($J$) are given in Hz.

**Plant Material** Fresh root bark of *M. azedarach* L. was collected at Jiangsu, China in 1993. The species was identified by Professor Z.-Y. 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 three times with 24 L 70% EtOH at 70 °C. The concentrated extract (241 g) was partitioned between $CH_2Cl_2$ and $H_2O$, then between *n*-Butanol and $H_2O$. The $CH_2Cl_2$ soluble fraction (56 g) was subjected to silica gel column chromatography using *n*-hexane - EtOAc (1:0 - 0:1) as an eluting system to give fourteen fractions (frs.A - N). Fraction K (1.9 g) was further chromatographied on silica gel column eluted with n-hexane - acetone (8 : 2) and purified by ODS MPLC and ODS HPLC with MeOH / $H_2O$ or $CH_3CN$ / $H_2O$ eluting systems to give compounds **1** (30 mg), **2** (6 mg), **4** (2 mg) and **18** (1.7 mg). Fraction L (15 g) , one of the most active fractions, was further chromatographed on a silica gel column and eluted with $CH_2Cl_2$ - MeOH (60:1 - 10:1). Then the fractions eluted by $CH_2Cl_2$ - MeOH, (60:1) and (30:1), were further subjected to ODS MPLC and purified using ODS HPLC with MeOH / $H_2O$ or MeCN / $H_2O$ solvent systems to give compounds **3** (4 mg), **5** (8 mg) **6** (54 mg), **7** (33 mg), **8** (18 mg), **9** (400 mg), **10** (300 mg), **11** (17 mg), **12** (4.5 mg), **13** (3.8 mg), **14** (2.5 mg), **15** (3 mg), **16** (9 mg), **17** (45.4 mg), **18** (1.7 mg) and **19** (12.6 mg).

*1-Tigloyl-3,20-diacetyl-11-methoxylmeliacarpinin* (**1**) Colourless powder, mp 150 - 152°C (from $CHCl_3$); $[\alpha]_D$ -3.57° ($CHCl_3$; c 0.6); IR $\nu_{max}$ cm$^{-1}$ ($CHCl_3$): 3460,1735, 1717 (sh), 1700, 1600, 1373, 1054; EI-MS *m/z*: 732 [M$^+$], 700, 673, 640, 518.

*3-Tigloyl-1,20-diacetyl-11-methoxylmeliacarpinin* (**2**) Colourless powder, mp 214 - 216°C (from $CHCl_3$); $[\alpha]_D$ +7.94° ($CHCl_3$; c 0.6); IR $\nu_{max}$ cm$^{-1}$ ($CHCl_3$): 3470, 1735, 1720 (sh), 1700, 1650, 1615, 1600, 1373, 1060; EI-MS *m/z*: 732 [M$^+$], 700, 673, 640, 549, 518.

*1-Cinnamoyl-3-hydroxyl-11-methoxymeliacarpinin* (**3**) Colourless powder, mp 124 - 126°C (from $CHCl_3$); $[\alpha]_D$ -2.39° ($CHCl_3$; c 0.2); IR $\nu_{max}$ cm$^{-1}$ ($CHCl_3$): 3400, 1740, 1710 (sh), 1630, 1600; EI-MS *m/z*: 637 [M-COOMe]$^+$.

*1-Deoxy-3-methacrylyl-11-methoxymeliacarpinin* (**4**) Colourless powder, mp 274 - 276°C (from $CHCl_3$); $[\alpha]_D$ -16.3° ($CHCl_3$; c 0.2); IR $\nu_{max}$ $cm^{-1}$ ($CHCl_3$): 3400, 1735, 1700, 1596; EI-MS *m/z*: 618 [$M^+$], 559, 447, 373.

*1-Cinnamoyl-3-acetyl-11-methoxymeliacarpinine* (**5**) is a known limonoid.[10]

*1-Tigloyl-3-acetyl-11-methoxymeliacarpinin* (**6**) Colourless crystals, mp 165 - 167°C (from acetone), $[\alpha]_D$ -12.6° ($CHCl_3$; *c* 0.5); IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 3240, 1742, 1707, 1624; EI-MS *m/z*: 690 [$M^+$], 658, 631, 575, 519; HRMS *m/z*: found 690.2865, required for $C_{35}H_{46}O_{14}$ 690.2887.

*1-Acetyl-3-tigloyl-11-methoxymeliacarpinin* (**7**) Colourless crystals, mp 149 - 151°C (from acetone), $[\alpha]_D$ +5.8° ($CHCl_3$; *c* 0.2); IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 3450, 1742, 1705, 1650, 1630; EI-MS *m/z*: 690 [$M^+$], 658, 631, 575, 519, 477.

The structures of 29-isobutylsendanin (**8**),[11] 12-hydroxyamoorastin (**9**) and 29-deacetyl-sendanin (**10**)[12-14] were confirmed by comparing their physical and spectral data with that in the literature.

**Acetylation of 9** 12-Hydroxyamoorastin (**9**, 50 mg) was acetylated with 2 ml $Ac_2O$ - pyridine (1:1) for 16 h at room temperature. Then toluene was added and the reaction mixture concentrated under reduced pressure. The residual material was subjected to ODS HPLC using a MeCN - $H_2O$ (1:1) solvent system to give four acetylated derivatives, sendanin (**9a**, 13.5 mg), 1-acetylsendanin (**9b**, 17.5 mg), 7-acetylsendanin (**9c**, 14.5 mg) and 1,7-diacetylsendanin (**9d**, 5 mg). The structure of sendanin (**9a**) was elucidated by comparing the physical and spectral data with that in the literature.[15]

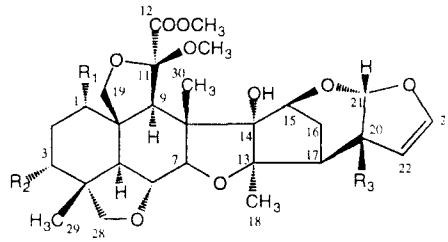
*1-Acetylsendanin* (**9b**) Colourless crystals, mp 158 - 160°C (from acetone), $[\alpha]_D$ -8.6° ($CHCl_3$, *c* 0.1); IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 1740, 1720 (sh), 1600; HRMS *m/z*: found 658.2608, required for $C_{34}H_{42}O_{13}$ 658.2625.

*7-Acetylsendanin* (**9c**) Colourless crystals, mp 150 - 152°C (from acetone), $[\alpha]_D$ -15.6° ($CHCl_3$, *c* 0.1); IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 1740, 1600; HRMS *m/z*: found 658.2629, required for $C_{34}H_{42}O_{13}$ 658.2625.

*1,7-Diacetylsendanin* (**9d**) Colourless crystals, mp 253 - 255°C (from acetone), $[\alpha]_D$ -13.0° ($CHCl_3$, *c* 0.9); IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 1740, 1720 (sh), 1600; EI-MS *m/z*: 700 [$M^+$].

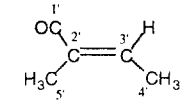
*12-Deacetyltrichilin I* (**11**) Colourless powder, mp 175 - 176 °C (from MeOH), $[\alpha]_D$ -32.4° (MeOH, c 0.12), IR $\nu_{max}cm^{-1}$ (KBr): 3450 (br), 1710, 1455, 1375, 1062; EI-MS *m/z*: 614 [$M^+$ - 18], 596, 555, 531[$M^+$ - 101], 512, 494, 470, 452,435.

*1-Acetyltrichilin H* (**12**) Colourless powder, mp 170 - 172 °C (from $CHCl_3$), $[\alpha]_D$ -19.7° ($CHCl_3$, c 0.6), IR $\nu_{max}cm^{-1}$ ($CHCl_3$): 3730, 1742, 1720 (sh), 1700 (sh), 1602, 1373, 1095; EI-MS *m/z*: 657 [$M^+$ - 87], 614, 554, 494, 452, 374.

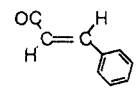


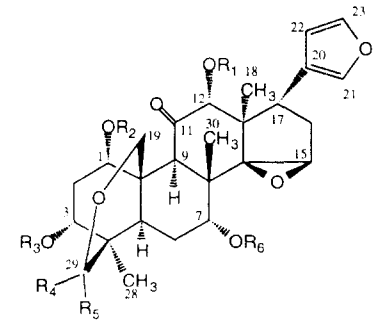
(1) $R_1$=OTig, $R_2$=$R_3$=$OCOCH_3$
(2) $R_1$=$R_3$=$OCOCH_3$, $R_2$=OTig
(3) $R_1$=OCin, $R_2$=R3=OH
(4) $R_1$=H, $R_2$=$OCOC(CH_3)$=$CH_2$, $R_3$=OH
(5) $R_1$=OCin, $R_2$=$OCOCH_3$, $R_3$=OH
(6) $R_1$=OTig, $R_2$=$OCOCH_3$, $R_3$=OH
(7) $R_1$=$OCOCH_3$, $R_2$=OTig, $R_3$=OH

Tig=Tigloyl



Cin=Cinnamoyl





(8) $R_1$=$R_3$=$COCH_3$, $R_2$=$R_5$=$R_6$=H, $R_4$=Isobutyryl
(9) $R_1$=$R_2$=$R_6$=H, $R_3$=$COCH_3$, $R_4$=OH, $R_5$=H or $R_4$=H, $R_5$=OH
(10) $R_2$=$R_6$=H, $R_1$=$R_3$=$COCH_3$, $R_4$=OH, $R_5$=H or $R_4$=H, $R_5$=OH
(9a) $R_2$=$R_5$=$R_6$=H, $R_1$=$R_3$=$COCH_3$, $R_4$=$OCOCH_3$
(9b) $R_5$=$R_6$=H, $R_1$=$R_2$=$R_3$=$COCH_3$, $R_4$=$OCOCH_3$
(9c) $R_2$=$R_5$=H, $R_1$=$R_3$=$R_6$=$COCH_3$, $R_4$=$OCOCH_3$
(9d) $R_5$=H, $R_1$=$R_2$=$R_3$=$R_6$=$COCH_3$, $R_4$=$OCOCH_3$

*3-Deacetyltrichilin H* (**13**) Colourless powder, mp 162 - 164 °C (from $CHCl_3$), $[\alpha]_D$ -4.0° ($CHCl_3$, c 0.2), IR $\nu_{max}cm^{-1}$ ($CHCl_3$): 3600, 1735, 1715 (sh), 1598; EI-MS *m/z*: 573 [$M^+$ - 87], 614, 554, 494, 452, 374.

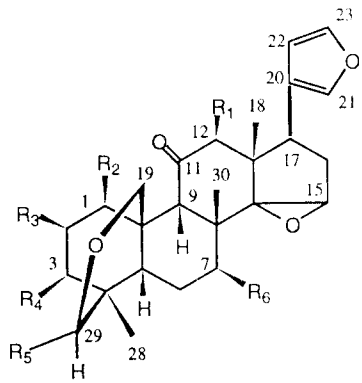
*1-Acetyl-3-deacetyltrichilin H* (**14**) Colourless powder, mp 162 - 164 °C (from $CHCl_3$), $[\alpha]_D$ -11.7° ($CHCl_3$, c 0.2), IR $\nu_{max}cm^{-1}$ ($CHCl_3$): 3600, 1745, 1596; HRMS *m/z*: found 702.288345, required for molecular of $C_{36}H_{46}O_{14}$ 702.288757. EI-MS *m/z*: 702 [$M^+$], 684, 642, 624, 615, 572, 554, 512, 452.

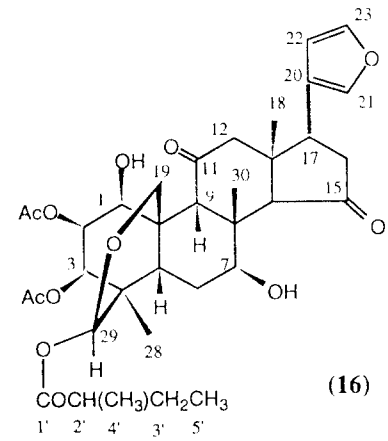
*1-Acetyl-2-deacetyltrichilin H* (**15**) Colourless powder, mp 218 - 220 °C (from $CHCl_3$), EI-MS *m/z*: 615 [$M^+$ - 87], 573, 375.

The compouds **16 - 19** were known ones which were confirmed to be meliatoxin $B_1$ (**16**), trichilin H (**17**), trichilin D (**18**) and 1,12-diacetyltrichilin B (**19**) by comparing the spectral and physical data with those in the literature.[16,17]

**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.[22] 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. Mouse P388 leukemia cells (2 x $10^4$ cells/ml) were inoculated in each well with 100 µl/ml 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 transplantation. At day 3, 20 μl MTT solution (5 mg/ml) per well was added to each cultured medium. After a further 4 h of incubation, 100 μl 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-A4*i*) at two wavelengths (550 and 700 nm). In all these experiments, 3 replicate wells were used to determine each data point.





**(16)**

**(11)** $R_1$=$R_2$=$R_3$=$R_6$=OH, $R_4$=OAc, $R_5$=$OCOCH(CH_3)CH_2CH_3$

**(12)** $R_1$=$R_2$=$R_3$=$R_4$=OAc, $R_6$=OH, $R_5$=$OCOCH(CH_3)_2$

**(13)** $R_1$=$R_3$=OAc, $R_2$=$R_4$=$R_6$=OH, $R_5$=$OCOCH(CH_3)_2$

**(14)** $R_1$=$R_2$=$R_3$=OAc, $R_4$=$R_6$=OH, $R_5$=$OCOCH(CH_3)_2$

**(15)** $R_1$=$R_2$=$R_4$=OAc, $R_3$=$R_6$=OH, $R_5$=$OCOCH(CH_3)_2$

**(17)** $R_1$=$R_3$=$R_4$=OAc, $R_2$=$R_6$=OH, $R_5$=$OCOCH(CH_3)_2$

**(18)** $R_1$=H, $R_2$=$R_6$=OH, $R_3$=$R_4$=OAc, $R_5$=$OCOCH(CH_3)CH_2CH_3$

**(19)** $R_1$=$R_2$=$R_3$=$R_4$=OAc, $R_6$=OH, $R_5$=$OCOCH(CH_3)CH_2CH_3$

## Results and Discussion

*1-Tigloyl-3,20-diacetyl-11-methoxylmeliacarpinin* (**1**) obtained as colourless powder was assigned with a molecular formula $C_{37}H_{48}O_{15}$ from the $M^+$ ion at *m/z* 732 in the EI-MS. The NMR spectra indicated the presence of meliacarpinin skeleton with three methyl (δ 1.49, s, 3H; 1.35, s, 3H; 0.98, s, 3H) , two methoxyl (δ 3.33, s, 3H; 3.67, s, 3H ), two acetyl (δ 1.91, s, 3H; 2.09, s, 3H), one tigloyl (δ 6.90, qq, 1H; 1.76, dd, 3H; 1.81, d, 3H ).and one hydroxyl (δ 4.14, s, 1H) groups and were similar to those of 1-tigloyl-3-acetyl-11-methoxymeliacarpinin.[1] However, instead of one acetyl group in 1-tigloyl-3-acetyl-11-methoxymeliacarpinin, there are two acetyl groups in the compound **1**. One acetyl group

was assigned in C-3 from the cross peak of $CH_3\underline{C}OO$ / H-3 in the HMBC spectra of **1**. Another acetyl group in **1** was assigned in C-20, since the signal of $C_{20}$-OH (δ 6.09, s, 1H) in 1-tigloyl-3-acetyl-11-methoxymeliacarpinin was disappeared in **1**. That suggestion was also supported by downfield moving in the chemical shifts of H-17 (0.8 ppm) and H-22 (0.5 ppm) as well as upfield moving in those of C-17 (2.6 ppm), C-21 (3.3 ppm) and C-22 (2.5 ppm) in **1** compared with those of 1-tigloyl-3-acetyl-11-methoxymeliacarpinin. The 1-O-tigloyl group was proved from the cross peak of C-1' / H-1 in the HMBC spectra of **1**. The stereochemistry of **1** was elucidated by NOESY of Me-18 / H-9, Me-18 / H-17, Me-30 / H-7, Me-30 / H-15, Me-30 / H19a, Me-29 / H-3, Me-29 / H-28b, Me-29 / H-6, H-5 / H-9, and H-7 / H-21.

*3-Tigloyl-1,20-acetyl-11-methoxymeliacarpinin* (**2**) obtained as colourless powder had a same molecular formula of **1** from the $M^+$ ion at *m/z* 732 in the EI-MS. The NMR data are quite similar to those of 3-tigloyl-1-acetyl-11-methoymeliacarpinin.[1)] The assignment of 20-acetyl group was done as same in **1**. The structural difference between the compounds **1** and **2** was assumed that 1α-O-tigloyl and 3α-O-acetyl groups in **1** were interchanged each other in **2**. That was deduced by comparing the chemical shift of 1β-H in **2** with that in 3-tigloyl-1-acetyl-11-methoymeliacarpinin.[1)] When the 1α-O-acetyl group was changed into the 1α-O-tigloyl group, the chemical shift of 1β-H moved to downfield about 0.16 ppm.[1)]

*1-Cinnamoyl-3-hydroxyl-11-methoxymeliacarpinin* (**3**) obtained as colourless powder showed a molecular formula of $C_{37}H_{44}O_{13}$ from the $[M\text{-}COOMe]^+$ ion at *m/z* 637 in the EI-MS. The NMR spectra are similar to those of **5**[10)] with three methyl (δ 1.47, s, 3H; 1.33, s, 3H; 0.98, s, 3H) , two methoxyl (δ 3.37, s, 3H; 3.73, s, 3H ) , and one cinnamoyl (δ 7.40-7.45, m, 5H; 7.72, d, 1H; 6.43, d, 1H) groups. The presnece of 1α-O-cinnamoyl and 3α-hydroxyl groups was deduced from the NOE of H-7' / Me-18, H-8' / Me-18, and H-3 / Me-29. That was also supported from the downfield moving in the chemical shifts of 28a-H (0.5 ppm), C-4 (1.5 ppm) and C-2 (2.8 ppm) of **3**, compared with those of **5**, because of the anisotropic effect of 3α-hydroxyl group.

*1-Deoxy-3-methacrylic acid-11-methoxymeliacarpinin* (**4**) obtained as colourless powder exhibited a molecular formula of $C_{32}H_{42}O_{12}$ from the $M^+$ ion at *m/z* 618 in the EI-MS. The NMR spectra were quite similar to those of 1-deoxy-3-tigloyl-11-methoxymeliacarpinin[18)] except for the change of 3α-O-tigloyl to 3α-O-methacrylate (δ 5.61, t, 1H; 6.13, t, 1H; 1.96, t, 3H; δ 166.2, s, C-1'; 136.2, s, C-2'; 126.1, t, C-3', 18.3, q, C-4') in **4**.

*1-Tigloyl-3-acetyl-11-methoxymeliacarpinin* (**6**) obtained as colourless crystals, mp 165 - 167° and $[\alpha]_D$ -12.6°, had the molecular formula $C_{35}H_{46}O_{14}$ from HR-EIMS. NMR indicated the presence of two methoxyl (δ 3.35 and 3.70, each 3H, *s*), one acetyl (δ 1.94, 3H, *s*) and one tigloyl (δ 6.90, 1H, *qq*; δ 1.78, 3H, *dd*; δ 1.83, 3H, *d*) group similar to those of 1-tigloyl-3-acetyl-11-methoxyazadirachtin (**6a**).[10,19)] However, instead of the three

methoxyl, five methyl and four carbonyl (δ 170.1, 173.3, 169.7 and 166.8) groups in **6a**, two methoxyl, six methyl and three carbonyl (δ 170.1, 169.0 and 166.6) groups were observed in compound **6**. This suggested that one of the methoxycarbonyl groups in **6a** was replaced by the methyl group in **6**. The deduction that a methyl group (δ 0.98, 3H, *s*) was attached to the position C-4 in **6** was also supported by the movement upfield (0.5 ppm) of the chemical shifts due to the 3b, 6b and 28b positional protons in comparison with **6a**. This was also supported by the cross-peak between the 3b-proton and the 29-methyl protons in the NOESY spectrum. The position of the acetyl and tigloyl groups was confirmed by the $^{1}H$-$^{13}C$ long-range correlation of the HMBC spectrum (Fig. 1). From above results, compound **6** was determined to be 1-tigloyl-3-acetyl-11-methoxymeliacarpinin.

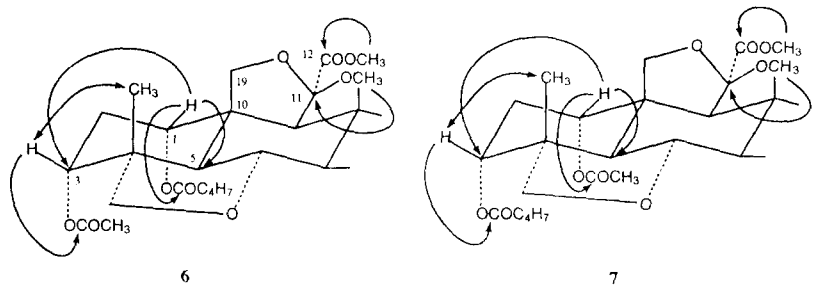



Fig. 1 HMBC (⟶) and NOESY (⟷) correlations in partial structures of **6** and **7**

*1-Acetyl-3-tigloyl-11-methoxymeliacarpinin* (**7**), colourless crystals, mp 149 - 151°, $[\alpha]_D$ +5.8°, had the same molecular formula $C_{35}H_{46}O_{14}$ as **6** from EI-MS and $^{13}C$-NMR spectral data suggesting the presence of two methoxyl, one acetyl and one tigloyl group to be similar to those of **6**. The structural difference between compounds **6** and **7** was assumed that the 1α-O-tigloyl and 3α-O-acetyl groups in **6** were interchanged in **7.** This assumption was confirmed by the $^{1}H$-$^{13}C$ long-range correlation of the HMBC spectrum (Fig. 1). Therefore, compound **7** was established to be 1-acetyl-3-tigloyl-11-methoxymeliacarpinin.

To investigate the relationship between structures and activities, various acetylated derivatives of **9** were prepared. Acetylation of **9** was carried out in the usual way using acetic anhydride and pyridine to give sendanin (**9a**), 1-acetylsendanin (**9b**), 7-acetylsendanin (**9c**) and 1,7-diacetylsendanin (**9d**). The structure of **9a** was confirmed to be sendanin by comparing its physical and spectral data with that in the literature.[15] Both **9b** and **9c** had the same molecular formula $C_{34}H_{42}O_{13}$ from HRMS and their NMR spectra were similar to those of **9a** except for one additional acetyl group. The 1β- and 7β-positional proton chemical shifts, δ 4.28 and 3.67 in **9a**, were shifted downfield to δ 5.16 in **9b** and to δ 4.78 in **9c**, respectively. Consequently, **9b** and **9c** were shown to be 1-

acetylsendanin and 7-acetylsendanin, respectively; **9d** was also established as 1,4-diacetylsendanin.

Compounds **9** and **10** are present as a mixture of two 29-positional epimers in solution. Normally, the chemical shift of 3β-H appears more downfield in the *exo*-configuration of 29-OR than in the *endo*-configuration, since the 29-positional alcoholic oxygen located in a quasi-1,3-diaxial direction exerts a marked deshielding effect on 3β-H.[20] In 29-deacetylsendanin (**10**), the chemical shifts of the 3β-H in the *exo*- and *endo*-configurations of 29-OH are observed at δ 5.34 and 4.88 ($CDCl_3$) respectively, while in **9** they are at δ 5.55 and 4.91 ($CDCl_3$ & 20% pyridine-$d_5$). In sendanin with an *exo*-configuration of 29-OAc, it appears at δ 5.25 ($CDCl_3$).[20] Comparing the chemical shifts of 3b-H in **9a**, **9b**, **9c** and **9d** with the above data, the four acetylated derivatives of **9** should be due to the *exo*-configuration of the 29-acetyl group. This assumption was confirmed by the NOESY experiment on **9c**, which showed a clear correlation between 29-H and 6β-H as well as 19β-H. The fact that only the *exo*-configuration existed after acetylation suggested that the 29-OH in the *exo*-configuration could be more easily acetylated than one in the *endo*-configuration, by converting the *endo*-type gradually to the *exo*-type during the acetylation process.

*12-Deacetyltrichilin I* (**11**) was obtained as colourless 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 EI-MS. The NMR data indicated the presence of one 2-methylbutyryl and one acetyl groups 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 15a-H, 59.2 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 (δ 213.4 s C-11), and a furyl moiety (δ 7.22 1H, δ 6.52 1H and δ 7.32 1H ) like trichilins.[10,15] It was also suggested that **11** 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.[23] The fact that the acetyle group was connected to C-3 in **11** was deduced from the $^1$H-NMR signal of H-3 (δ 5.45 d, J=4.7Hz) . 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 **11** was done from the chemical shift (δ 3.00) of 17β-H, which was shifted downfield to δ 3.39 in trichilin A (12β-OH).[24] The 29-*exo*-configuration of **11** 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.[17]

*1-Acetyltrichilin H* (**12**) was obtained as colourless 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 EI-MS. The NMR data is quite similar to those of 1,12-diacetyltrichilin B (**19**),[17] except for the changing of the ester moiety of 2-methylbutyryl group at C-29 to 2-methylpropionyl group (δ 2.66 hept, 1.20 d, 1.21 d) in **12**.

*3-Deacetyltrichilin H* (**13**) obtained as colourless powder was 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 EI-MS. The structure of **13** was elucidated by comparison of the NMR data with those of trichilin H (**17**).[17] It was identical to trichilin H except for the signal due to 3-acetyl group in **17**.

*1-Acetyl-3-deacetyltrichilin H* (**14**) and *1-acetyl-2-deacetyltrichilin H* (**15**) obtained as colourless powder had both the same molecular formulas $C_{36}H_{46}O_{14}$ as **17** from the $[M^+]$ ion at m/z 702 and $[M - OCOCH(CH_3)_2]^+$ ion at m/z 615 in the EI-MS respectively. The structures of **14** and **15** were also elucidated by comparison of the NMR data with those of **17**, that is, the $^1$H-NMR spectrum of **14** was identical to that of **17** except for the fact that the 1-hydroxyl-3-acetoxyl moiety in **17** was changed to the 1-acetoxyl-3-hydroxyl in **14**. The assignment of 1-acetoxyl in **14** was also supported by the upfield moving of H-9 (δ 4.13 s) in **14**, compared with that (δ 4.64 s, H-9) in **17**. The assignment of 1-acetoxyl in **15** was done as the same manner in **14**. The another acetoxyl group in **15** was deduced at C-3 from the doublet signals with J=4.5 Hz of H-3 (δ 5.47).

The cytotoxic activities ($IC_{50}$ values, μg/ml) of compounds (**1 - 5**) against P388 lymphocytic leukemia cells were 100 (**1**), 48.0 (**2**), 1.5 (**3**), 47.0 (**4**), and 10.5 (**5**), respectively. Compound **3** showed a significant cytotoxic activity, however the activity of **5**, which has one more acetyl group than **3** at C-3, decreased. The similar phenomena were observed in the compounds **1** and **2**, that is, the cytoxic activities ($IC_{50}$ values) of 1-tigloyl-3-acetyl-11-methoxymeliacarpinin (**6**) and 1-acetyl-3-tigloyl-11-methoxymeliacarpinin (**7**) were 3.2 and 3.3 μg/ml, but the cytotoxic activities of **1** and **2**, which were additional acetylated compounds of $C_{20}$-OH of above two substances were almost lost. The compound **4** with a 1-deoxy structrure showed very weak activity.

The cytotoxic activity of compounds **6 - 10** and **9a - 9d** against P388 lymphocytic leukemia cells are showed in Table 1. Three sendanin-type limonoids, **8**, **9** and **10**, isolated from fr. L exhibited very strong cytotoxic activity against P388 cells *in vitro*. It was assumed that the cytotoxic activity of fr. L was due to synergism among their sendanin-type limonoids. Also, acetylation of the 1α- or 7α-OH of compound **9** decreased the cytotoxic activity. In particular, when both the 1α- and 7α-OH of **9** were acetylated, the cytotoxity was almost lost. In addition, azadirachtin-type limonoids, **6** and **7**, also exhibited significant cytotoxic activity, but to a lesser degree than the sendanin-type limonoids except for **9d**. The azadirachtin-type compounds are interest because of their remarkable inhibition of insect feeding and ecdysis inhibiting activity,[4, 21] but their cytotoxic activity has not been reported until now.

The cytotoxic activities of compounds **11 - 19** against P388 lymphocytic leukemia cells *in vitro* were examined and their $IC_{50}$ values were determined as shown in Table 1. As can be seen from Table 1, 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 **11**, **13** and **18**, which have one or two acetyl groups in the structures, exhibited strong activities. The activities of compounds **12**, **14**, **15**, **17** and **19**, which have more than three acetyl groups in the structures, decreased slightly. When the 14,15-epoxide structure was broken , like a compound **16**, the cytotoxic activity decreased greatly.

Table 1 Cytotoxic Activities against P388 Cells *In Vitro*

| compounds | yield (mg) | $IC_{50}$ (μg/ml) |
|---|---|---|
| **1** | 30 | 100 |
| **2** | 6 | 48.0 |
| **3** | 4 | 1.5 |
| **4** | 2 | 47.0 |
| **5** | 8 | 10.5 |
| **6** | 54 | 3.2 |
| **7** | 33 | 3.3 |
| **8** | 18 | 0.034 |
| **9** | 400 | 0.090 |
| **10** | 300 | 0.026 |
| **11** | 17 | 0.011 |
| **12** | 4.5 | 0.47 |
| **13** | 3.8 | 0.045 |
| **14** | 2.5 | 0.40 |
| **15** | 3 | 0.66 |
| **16** | 9 | 5.4 |
| **17** | 45.4 | 0.16 |
| **18** | 1.7 | 0.055 |
| **19** | 12.6 | 0.46 |
| **9a** | | 0.078 |
| **9b** | | 0.44 |
| **9c** | | 0.55 |
| **9d** | | > 10 |

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