Degraded and Oxetane-Bearing Limonoids from the Roots of *Melia azedarach*

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Brine shrimp lethality test (BST)-guided fractionation of a methanol extract of the roots of *Melia azedarach* resulted in the isolation of two new limonoids, 9α-hydroxy-12α-acetoxyfraxinellone (1) and 7,14-epoxy-azedarachin B (2), together with the known compounds, 12α-hydroxyfraxinellone (4), 9α-hydroxyfraxinellone (5), azedarachin B (6), and neoazedarachin B (7). The structures of 1 and 2 were elucidated by analysis of spectroscopic data and comparison of their NMR data with those of the known compounds. Compounds 1, 2 and 4—7 exhibited significant activity in the BST, in particular, azedarachin B (6) showed remarkable BST activity with an LC_{50} value of 0.0098 μM.

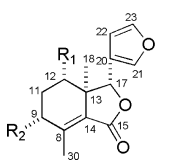
Key words *Melia azedarach*; limonoid; fraxinellone; azedarachin; brine shrimp lethality; cytotoxicity

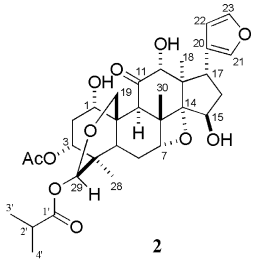
In the pursuit of biologically active natural products by using the brine shrimp (*Artemia salina*) lethality test (BST),[1—3] methanol extract of the roots of *Melia azedarach* was found to show significant lethal activity at 200 μg/ml.[4] Hence we investigated chemical components of the active MeOH extract of *M. azedarach* collected in Tokushima, Japan, using the BST, thereby resulting in the isolation of two new limonoids named 9α-hydroxy-12α-acetoxyfraxinellone (**1**) and 7,14-epoxy-azedarachin B (**2**) together with the previously known compounds 12α-hydroxyfraxinellone (**4**),[5] 9α-hydroxyfraxinellone (**5**),[6] azedarachin B (**6**),[7] and neoazedarachin B (**7**).[8] In this paper, we report the structure elucidation of the two new compounds **1**, **2** and their BST activity.

Results and Discussion

Since methanol extract of the roots of *M. azedarach* showed strong BST lethal activity at 200 μg/ml, it was fractionated into six fractions by silica gel column chromatography eluting with a CH_2Cl_2–EtOAc gradient. The active fractions C and E were purified by a combination of silica gel, reversed-phase ODS (octadecylsilyl) column chromatography, and preparative HPLC to give two new limonoids **1** and **2**.

Compound **1** had a $[M]^+$ peak at *m*/*z* 306.1109 in high resolution (HR) EI-MS, corresponding to the molecular formula $C_{16}H_{18}O_6$. Consequently, **1** has an unsaturation index of 8. Its IR spectrum displayed absorptions attributable to the presence of a hydroxy group (3447 cm^{-1}), an ester (1743 cm^{-1}) group, a γ-lactone (1755 cm^{-1}) moiety, and a furan ring (2880, 1506 cm^{-1}). The NMR spectra of **1** contained signals due to two tertiary methyls at δ_H 1.04 and 2.28 (each s), a β-furan at δ_H 6.33 (dd, J=1.2, 0.7 Hz), 7.42 (dd, J=1.2, 0.7 Hz), and 7.45 (t, J=1.2 Hz), a conjugated ester carbonyl at δ_C 168.4, an acetyl group at δ_H 1.98, δ_C 21.2, and δ_C 170.3, and a tetrasubstituted double bond at δ 128.6 and 146.7. The ^{13}C-NMR data suggested the presence of three oxy-methins at δ 70.6 (C-12), 69.8 (C-9), and 81.9 (C-17) and one sp^3 quaternary carbon at δ 47.1 (C-13). These spectroscopic data suggested that **1** was closely related to degraded limonoids such as 12α-hydroxyfraxinellone (**4**) and 9α-hydroxyfraxinellone (**5**).[5,6] In addition, there was a correlation spectroscopy (COSY) spin system, H-9/H_2-11/H-12, among which the H-12 signal at δ 5.37 showed a heteronuclear multiple bond connectivity (HMBC) correlation with the carbonyl signal of the sole acetyl group, indicating that the acetyloxy group was located at C-12. The remaining hydroxy group was placed at the C-9 position on the basis of its chemical shift (δ 69.8) as well as of the HMBC correlation





1: R_1 = OAc, R_2 = OH
3: R_1 = R_2 = H
4: R_1= OH, R_2 = H
5: R_1 = H, R_2 = OH

2

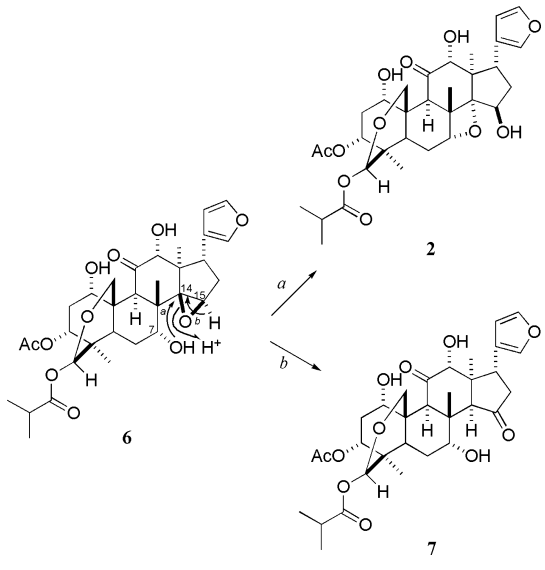


Chart 1. Plausible Biogenetic Route from **6** to **2** and **7**

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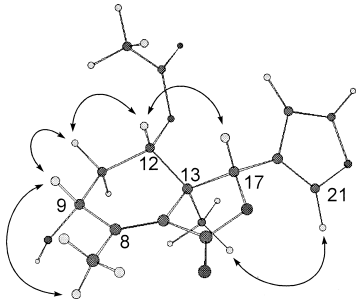


Fig. 1. NOESY Correlations and Relative Stereochemistry for **1**

between the methyl (δ 2.28, H_3-30) and C-9 signals. The above analyses of the NMR data provided 9-hydroxy-12-acetoxyfraxinellone for the plane structure of **1**. The relative stereochemistry of **1** was elucidated based on the nuclear Overhauser effect spectroscopy (NOESY) correlations as shown in Fig. 1 and the large $J_{12\text{-}11\beta}$ value (10.8 Hz), which were identical to those of **4** and **5**. Thus compound **1** was assigned as 9α-hydroxy-12α-acetoxyfraxinellone.

Compound **2** showed a quasi-molecular ion peak at *m/z* 625.2653 $[M+Na]^+$ in its positive FAB mass spectrum corresponding to the molecular formula $C_{32}H_{42}O_{11}$, indicating 12 degrees of unsaturation. The IR spectrum displayed hydroxyl (3445 cm^{-1}) and carbonyl (1738, 1733, 1715 cm^{-1}) absorptions. The ^{1}H- and ^{13}C-NMR data (Table 1) of **2** showed signals due to a β-furan ring at δ 7.23 (d, J=1.1 Hz, H-21), 6.46 (d, J=1.6 Hz, H-22), and 7.43 (dd, J=1.6, 1.1 Hz, H-23), an isopropionyl group at δ 1.17 (3H, q, J=1.4 Hz), 1.21 (3H, q, J=1.6 Hz), 2.62 (1H, qq, J=1.6, 1.4 Hz), and 175.7, and three tertiary methyl groups at δ 0.95 (s, H_3-18), 0.76 (s, H_3-28), and 1.35 (s, H_3-30). Also detected were an acetyl group at δ 2.08, 21.4, and 169.9, one ketone at δ 209.5 (C-11), four methylenes including one oxymethylene at δ 3.90 and 4.11 (d, J=12.4 Hz, H_2-19), eight methins including six oxygen-bearing methins, an acetal ring at δ 5.74 (s, H-29) and 93.2 (C-29), which was found to bear the isopropionyl group by the HMBC correlation between H-29 and the carbonyl (δ 175.7) of the isopropionyl group, five quaternary carbons at δ 39.7 (C-4), 39.6 (C-10), 43.7 (C-8), 50.0 (C-13), and 97.2 (C-14). In addition, there were three COSY spin systems, H-1/H_2-2/H-3, H-5/H_2-6/H-7, and H-15/H_2-16/H-17. These NMR data suggested that compound **2** resembled a tetranortriterpenoid, azedarachin B (**6**).[7] In comparison with the ^{13}C-NMR data[7] for C-7 (δ 70.1), C-14 (δ 72.9), and C-15 (δ 59.2) in **6**, in the case of **2**, the corresponding carbons shifted downfield at δ 82.0, 97.2, and 76.5, respectively. Considering an unsaturation index of 12, the unusual downfield shifts for C-7, C-14, and C-15 in **2** reflected that the epoxide ring existing at the C-14 and C-15 positions in **6** should be opened, resulting in the formation of an oxetane ring between C-7 and C-14. According to the literature,[9—12] the ^{13}C-NMR assigned for oxetane rings has appeared at lower field. This was substantiated by the observation of the HMBC between H-7 and C-14. Additionally, the C-7 and C-14 signals gave HMBC correlations with the H_3-30 singlet signal at δ 1.35. The other HMBC correlations as shown in Fig. 2a indicated that **2** has the same functional groups at the same positions as azedarachin B (**6**). From small J values for H-1, H-3, and H-7, the functional groups at C-1, C-3, and C-7 were in axial and α configurations. In NOESY experiments as summarized in Fig. 2b, the H-7 signal showed a cross peak to the H-15 and H-30 signal, and H-15 signal showed a NOE correlation with H-21 of the β-furan ring, indicating that the oxetane ring and C-15 hydroxy group took a α- and β-configuration, respectively. The other NOEs indicated that the relative stereochemistry of **2** was the same as that of **6**. Thus the structure of **2** was assigned as 7,14-epoxy-azedarachin B. Herein, it is worthy of note that **2** is the first limonoid discovered bearing an oxetane ring.

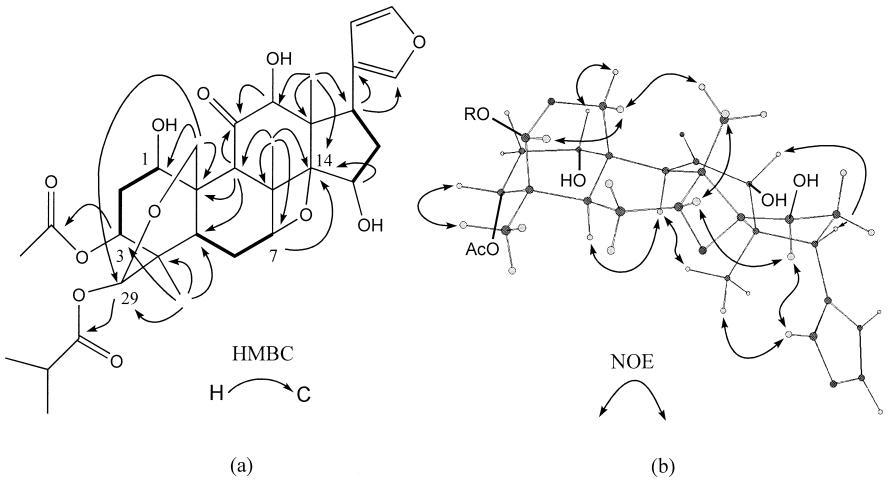
Table 1. ^{13}C-NMR (150 MHz) and ^{1}H-NMR (600 MHz) Spectral Data of Compound **2** in $CDCl_3$[a)]

Position	δ_C	δ_H
1	69.6	4.87 t (4.8)
2	36.1	1.89 dt (16.4, 4.8)
		2.87 dt (16.4, 4.8)
3	73.4	5.36 t (4.8)
4	39.7	
5	32.0	2.69 dd (12.6, 5.9)
6	22.6	1.81 ddd (12.6, 12.6, 4.3)
		1.95 ddd (12.6, 5.9, 1.2)
7	82.0	5.00 dd (4.3, 1.2)
8	43.7	
9	51.5	4.51 dd (4.3, 1.2)
10	39.6	
11	209.5	
12	80.0	3.93 s
13	50.0	
14	97.2	
15	76.5	4.95 dd (3.2, 2.0)
16	38.1	2.00 m (2H)
17	42.8	3.57 dd (10.1, 6.2)
18	14.2	0.95 s
19	64.5	3.90 dd (12.4, 1.6)
		4.11 d (12.4)
20	124.8	
21	140.1	7.23 d (1.1)
22	112.2	6.46 d (1.6)
23	142.4	7.43 dd (1.6, 1.1)
28	17.4	0.76 s
29	93.2	5.74 s
30	18.2	1.35 s
3-OAc	169.9	
	21.4	2.08 s
1′	175.7	
2′	34.0	2.62 qq (1.4. 1.6)
3′, 4′	18.6	1.21 q (1.6)
	18.9	1.17 q (1.4)

a) All assignments were made by extensive analyses of 1D and 2D NMR (COSY, DEPT, HMQC, and HMBC).

As depicted in Chart 1, biogenetic formation of compound **2** can be presumably explained by an intramolecular nucleophilic attack of the hydroxyl group on the C-14 position of the epoxide ring according to route *a*. This process *a* results in the formation of a highly strained oxetane ring, which is likely to be unfavorable due to an increase of high ring strain. In contrast, a preferable alternative route *b* leads to neoazedarchin B (**7**) with a 1,2-hydrogen shift.[13,14] It is, however, often observed that nature produces compounds with high ring strain. It should be commented that we failed to convert **6** to **2** by acidic treatment of **6** a though the formation of **7** was observed.

As the results of BST are listed in Table 2, all limonoids **1**—**7** showed 100% lethality in BST at 100 μM except for



(a) (b)

Fig. 2. (a) HMBC Correlations for **2**; (b) NOESY Correlations and Relative Stereochemistry for **2**

Table 2. Brine Shrimp Lethality Test (BST) Results for Compounds **1**—**7**

Compound	LC_{50} (μM)
1	6.5
2	20.5
3	>10 0
4	50.2
5	32.0
6	0.0098
7	58.5
Podophyllotoxin	5.8
Berberin	66.9

fraxinellone (**3**). In particular, azedarachin B (**6**) exhibited highly potent lethality in BST with an LC_{50} value of 0.0098 μM. It is well known that BST activity often has a positive correlation to cytotoxicity and pesticidal activity. The compounds were also evaluated against the KB cell line. Compound **6** exhibited significant cytotoxic activity at IC_{50} 0.0049 μM, whereas the degraded limonoids **1**—**5** showed no cytotoxicty at 50 μM. Compound **6** was also reported to show potent insect antifeedent activity.[14] Most tetracyclic sendanin-[15,16] and trichilin-type[17,18] limonoids with a 14,15-epoxide ring and a C-19/C-29 bridged acetal ring were reported to show strong cytotoxicity against P388 cells. The aforementioned structural features are probably responsible for the cytotoxic and BST activities of **6**.

Experimental

General Experimental Procedures Optical rotations were measured by JASCO DIP-1000 digital polarimeter. IR spectra were measured by JASCO FT-IR 5300 infrared spectrophotometer. 1D- and 2D-NMR spectra were recorded on a Varian Unity 600 in $CDCl_3$. Chemical shifts are given as δ (ppm) with TMS as internal standard. MS were recorded on a JEOL AX-500 instrument. Column chromatography was carried out on Kieselgel 60 (70—230 mesh and 230—400 mesh).

Plant Material The roots of *Melia azedarach* Linn. var. *japonica* Makino were collected in Tokushima, Japan and a voucher specimen (1428RT) has been deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Isolation MeOH extract (91 g) of the dried powdered root of *M. azedarach* was chromatographed on a silica gel column eluted with a step gradient of CH_2Cl_2 (100%), CH_2Cl_2–EtOAc (9 : 1), CH_2Cl_2–EtOAc (2 : 3), CH_2Cl_2–EtOAc (3 : 2), EtOAc (100%), and EtOAc–MeOH (9 : 1) to give six fractions (A—F).

The BST active (100% death at 100 μg/ml) fraction C (3.7 g) was first subjected to reversed-phase Cosmosil 75 C_{18}-OPN chromatography eluting with MeOH–H_2O (4 : 1) to give fractions 1—8. Fraction 2 (12 mg) was separated by reversed-phase HPLC (Cosmosil 5C_{18}-AR-II, 10×250 mm, particle size 5 μm) with MeOH–H_2O (9 : 1) to give compounds **1** (3.0 mg), **4** (1.2 mg), and **5** (3.1 mg). The BST active (100% death at 100 μg/ml) fraction E (7.7 g) was chromatographed on a silica gel column eluting with CH_2Cl_2–MeOH (4 : 1) to give fractions 9—19. The fraction 10 (20 mg) was separated by reversed-phase HPLC (Cosmosil 5C_{18}-AR-II, 10×250 mm, particle size 5 μm) using MeOH–MeCN–H_2O (37 : 55 : 8, 2 ml/min) to give compound **2** (2.6 mg). Fraction 23 (46 mg) was separated by reversed-phase HPLC (Cosmosil 5C_{18}-AR-II, 10×250 mm, particle size 5 μm) using MeOH–MeCN–H_2O (3 : 3 : 4) to give azedarachin B (**6**) (8.7 mg) and neoazedarachin B (**7**) (8.6 mg).

9α-Hydroxy-12α-acetoxyfraxinellone (**1**): Colorless amorphous solid, $[\alpha]_D^{21}$ −25.8° (c=0.38, $CHCl_3$). IR (film) ν_{max}: 3447 (OH), 3180, 2880 (furan), 1755 (γ-lactone), 1743 (C=O), 1676 (C=C), 1506 (furan) cm^{-1}. EI-MS *m/z*: 306 (M^+, 7), 105 (100). ^{1}H-NMR ($CDCl_3$, 600 Mz) δ 1.04 (3H, s, H_3-18), 1.98 (3H, s, OAc), 2.05 (1H, ddd, J=10.8, 10.8, 5.5 Hz, H-11α), 2.07 (1H, ddd, J=10.8, 5.5, 2.0 Hz, H-11β), 2.28 (3H, s, H_3-30), 4.34 (1H, br s, H-9), 5.13 (1H, s, H-17), 5.37 (1H, dd, J=10.8, 5.5 Hz, H-12), 6.33 (1H, dd, J=1.2, 0.7 Hz, H-22), 7.42 (1H, dd, J=1.2, 0.7 Hz, H-21), 7.45 (1H, t, J=1.2 Hz, H-23). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 15.0 (C-18), 15.3 (C-30), 21.2 ($\underline{CH_3}CO$), 33.8 (C-11), 47.1 (C-13), 69.8 (C-9), 70.6 (C-12), 81.9 (C-17), 109.3 (C-22), 120.0 (C-20), 128.6 (C-14), 141.1 (C-21), 143.2 (C-23), 146.7 (C-8), 168.4 (C-15), 170.3 ($CH_3\underline{CO}$); HR-EI-MS *m/z*: 306.1109, Calcd for $C_{16}H_{18}O_6$: 306.1104.

7,14-Epoxy-azedarachin B (**2**): Colorless amorphous solid, $[\alpha]_D^{21}$ −21.7° (c=0.50, EtOH). IR (film) ν_{max}: 3445 (OH), 1738 (C=O), 1733 (C=O), 1715 (C=O) cm^{-1}. FAB-MS *m/z*: 625 $[M+Na]^+$, 43 (100). HR-FAB-MS *m/z*: 625.2653 $[M+Na]^+$, Calcd for $C_{32}H_{42}O_{11}Na$: 625.2624. ^{1}H- and ^{13}C-NMR, see Table 1.

Brine Shrimp Bioassay The bioassay was carried out essentially according to the procedure reported in the literature.[1] A half spoon of brine shrimp eggs (Nihon Animal Pharmaceutical Inc., Tokyo, Japan) were hatched in a container filled with air-bubbled artificial sea water that was prepared with 10 g of commercial salt mixture (GEX Inc., Osaka, Japan) and 500 ml of distilled water. After 48 h, the phototropic shrimps were collected by pipette for bioassay. Ten shrimp were transferred to each vial, and artificial water was added to make 5 ml. To this vial was added each sample dissolved in 0.025 ml of dimethyl sulfoxide (DMSO), whereas the vial containing 0.025 ml of DMSO was regarded as control. The surviving shrimps were counted in the stem of the pipette against a lightened background after 24 h. The percent death at each dose was counted as the following formula: % deaths=(the number of dead shrimps+the number of dying shrimps×0.7)/10×100. The assay results are summarized in Table 2.

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