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Lignans from the Leaves of *Meliosma lepidota* ssp. *squamulata* and their Biological Activity toward *Leishmania major*

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Research Article

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ABSTRACT

From the leaves of *Meliosma lepidota* ssp. *squamulata*, one new sesamin-type lignan was isolated. Its structure was first elucidated by spectroscopic investigation and then finally determined by X-ray crystallographic analysis. The anti-*Leishmania* activity of the new lignan and related ones, (+)-aptosimon, (+)-sesamin and (+)-paulownin, isolated from this plant was evaluated.

INTRODUCTION

Meliosma lepidota Blume ssp. *squamulata* (Hance) Beusekom (Syn. *M. squamulata* Hance) is a tall evergreen tree belonging to the family Sabiaceae. However, in the International Plant Names Index, *Meliosma* species are currently classified in the Meliosmaceae, whereas the latest taxonomic publication observes the classical taxon, Sabiaceae [1]. The Sabiaceae comprises about one hundred species in five major genera, and *M. lepidota* ssp. *squamulata* grows wild in the Amami Islands, the Okinawa Islands, Taiwan and Southern China [2]. This plant is also known as a feeding plant for the larvae of a butterfly, *Dichorragia nesimachus*. Leishmaniasis is a parasitic disease that is found in parts of the tropics and subtropics. It is classified as a neglected tropical disease. Leishmaniasis is caused by infection with the *Leishmania* parasite, which is spread by the bite of phlebotomine sand flies. The most common forms are cutaneous Leishmaniasis, which involves skin sores, and visceral Leishmaniasis. There has been no report on the constituents of the title plant and its medicinal use is also uncertain. This paper deals with the isolation work on the plant and the biological assaying of lignans, isolated.

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RESULTS AND DISCUSSION

From the EtOAc-soluble fraction of a MeOH extract of the leaves of *M. lepidota* ssp. *squamulata*, one new sesamine-type ligna, meliosmin **(1)** was isolated along with three known lignans, (+)-aptosimon **(2)** [3,4], (+)-sesamin **(3)** [5,6], and (+)-paulownin **(4)** [7], one known diterpenoid, ribenol **(5)** [8], and four known triterpenes, alphitolic acid **(6)** [9], virgatic acid **(7)** [10], 1-oxo-erythrodiol **(8)** [11], and maslinic acid (**9**) [7] **(Figure 1)**.

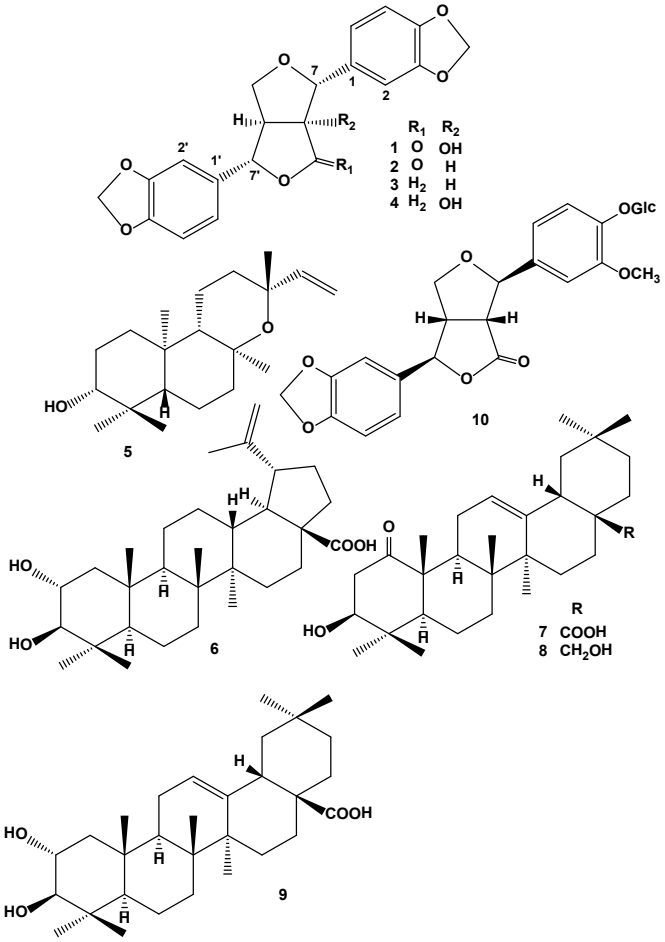

Figure 1. Structures of compounds isolated.

Meliosmin **1**, $[\alpha]_D^{25}$ -21.2, was isolated as colorless needles and high-resolution (HR)-electrospray ionization (ESI)-mass spectral data, *m/z*: 407.0739, revealed its elemental composition to be $C_{20}H_{16}O_8$. The IR spectrum showed absorption maxima for hydroxyl groups (3463 cm^{-1}), a ketone carbonyl group (1776 cm^{-1}), and aromatic rings (1609 and 1502 cm^{-1}), and UV absorption bands at 237 and 281 nm were indicative of the presence of the aromatic rings. In the ^{1}H-NMR spectrum, two sets of three aromatic protons coupled in an ABX system, two singlet protons each equivalent to two protons, and five aliphatic methylene or methine protons were observed **(Table 1).** The ^{13}C-NMR spectral data revealed two dioxymethylene carbons signals and 12 resonances for two aromatic rings, the remaining six signals comprising those of one oxymethylene, two oxymethines, one methine, one oxygenated tertiary and one carbonyl carbon signals **(Table 1).** From the above evidence, compound **1** was assumed to possess a sesamin-type lignan framework. The ^{1}H-^{1}H correlation spectrum revealed that four protons in a sequence, i.e., -OCH-CH-CH_2O-, and one proton resonating at δ_H 5.07 (s) must be at the isolated position. Therefore, the planar structure of meliosmin was expected to be as shown in **Figure 1**. Confirmation of the planar structure and the relative configuration of the substituents in the dioxabicyclooctane core was performed by X-ray crystallographic analysis, and an ORTEP drawing shows the relative stereoscopic structure of **1 (Figure 2).** The absolute configuration of **1** was assigned as the same as that of the related compounds, (+)-aptosimon [Δε (nm) +2.06 (241) and +1.45 (289)] **(2)**, (+)-sesamin Δε(nm) +1.63 (224) and +1.04 (287)] **(3),** and (+)-paulownin [Δε (nm) +2.15 (239) and +2.14 (285)] **(4)**, which were isolated from this plant, showing positive Cotton effects at around 285 nm and 230 nm in the CD spectrum. An antipodically substituted compound, styraxlignolide B **(10)**, consequently showed negative Cotton effects at due wavelengths ($[\theta]_{235}$ -459.5 and $[\theta]_{280}$ -255.9) [12]. Therefore, the structure of **1** was elucidated to be as shown in **Figure 1** and termed meliosmin. Four lignans and other isolates were evaluated their anti-*Leishmania* and cytotoxicic activities.

CONCLUSION

From the leaves of *M. lepidota* ssp. *squamulata*, a sesamin-type lignan **(1)** was isolated along with three known lignans,

(+)-aptosimon **(2)**, (+)-sesamin **(3)**, and (+)-paulownin **(4)**. Structure **1** was first spectroscopically elucidated, and then confirmed by X-ray crystallographic and CD spectral analyses. A closely related compound, vitelignin A, was isolated from *Vitex negundo* [13]. New lignan **(1)** possessed a hydroxy group at the 8-position, whereas vitelignin A was positional isomer of the hydroxy group at the 8'-position.

Table 1. ^{13}C- and ^{1}H-NMR spectroscopic data for compound **1** (100 MHz and 400 MHz, respectively, DMSO-d_6, δ ppm and J in Hz).

Compound 1		
	^{13}C	**^{1}H**
1	129.8	-
1'	132.4	-
2	107.4	6.85 d 2
2'	106.8	7.05 d 2
3	147.7	-
3'	147.8	-
4	146.6	-
4'	146.7	-
5	108.1	6.84 d 8
5'	108.2	6.93 d 8
6	120.7	6.76 dd 8, 2
6'	120.8	6.96 dd 8, 2
7	84.2	5.07 s
7'	81.5	5.38 d 8
8	84.1	-
8'	58.1	3.18 ddd 8, 7, 3
9	174.7	-
9'	69.4	4.01 dd 9, 3
		4.27 dd 9, 7
-OCH_2O-	100.8	5.98 2H, s
-OCH_2O-	101.3	6.03 2H, s

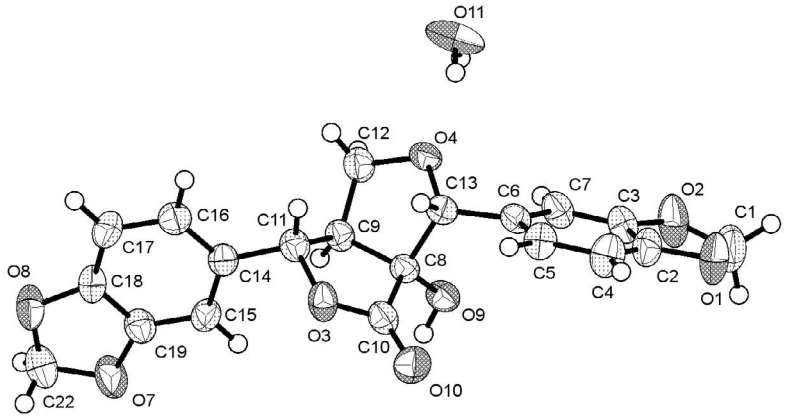

Figure 2. An ORTEP drawing of compound 1. The molecule has X-ray crystallographic numbering.

Four lignans and other five known isolates were evaluated as to anti-*Leishmania* activity and their cytotoxicity was also evaluated as to A549 human lung adenocarcinoma epithelial cells **(Table 2)**. Although new lignan **(1)** did not show any activity, (+)-paulownin **(4)** showed moderate anti-*Leishmania* activity at IC_{50}=23.7 ± 7.4 μM, however, it did not show any cytotoxicity at 100 μM **(Table 2)**. Thus, (+)-paulownin **(4)** is expected to be a promising selective anti-*Leishmania* agent and it is implied that molecular action point is not at the common event of cell cycle, but, at some stage of life cycle of the protozoa. To show some activity, one electronegative substituent on the dioxabicyclo[3.3.0]octane ring was required, but two of those one the ring decreased the activity. Known compounds **(6–9)** were generally inactive toward *Leishmania*, but compound **5** showed slight anti-*Leishmania* activity at IC_{50}=64.1 μM.

EXPERIMENTAL

Plant Material

Leaves of *M. lepidota* ssp. *squamulata* were collected in Kunigami-gun, Okinawa, Japan, in July 2006, and a voucher specimen was deposited in the Herbarium of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Hiroshima University (06-MLS-Okinawa-0703).

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Table 2. Anti-*Leishmania* activity and cytotoxicity toward A549 (IC_{50}: μM)

	Anti-Leishmania	Cytotoxicity
1	>100	>100
2	60.7 ± 5.7	>100
3	>100	>87.0 ± 8.5
4	23.7 ± 7.4	>100
Am	0.39 ± 0.1	Nt
Dox	nt	0.50 ± 0.2

Note: Am and Dox: amphotericin B and doxorubicin as positive controls, respectively. nt: Not Tested.

General Experimental Procedures

Melting point was measured with a Yanagimoto micro melting point apparatus and is uncorrected. Optical rotation was measured on a JASCO P-1030 polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/Vis spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL α-400 spectrometer at 400 MHz and 100 MHz, respectively, with tetramethylsilane as an internal standard. A CD spectrum was obtained on a JASCO J-720 spectropolarimeter. Positive-ion HR-MS were performed with an Applied Biosystem QSTAR XL system ESI (Nano Spray)-MS.

Silica gel CC and reversed-phase [octadecylsilanized silica gel (ODS)] open CC were performed on silica gel 60 (Merck, Darmstadt, Germany) and Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan), respectively. HPLC was performed on an ODS column (Inertsil; GL Science, Tokyo, Japan), and the eluate was monitored with a UV detector at 254 nm and a refractive index monitor.

Extraction and Isolation

Leaves of *M. lepidota* spp. *squamulata* (8.80 kg) were extracted three times with MeOH (4.5 L × 3) at room temperature for one week and then concentrated to 3 L *in vacuo*. The concentrated extract was washed with *n*-hexane (3 L, 41.0 g), and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water (3 L) and then extracted with EtOAc (3 L) to give 104.0 g of an EtOAc-soluble fraction. The aqueous layer was extracted with 1-BuOH (3 L) to give a 1-BuOH-soluble fraction (125.0 g), and the remaining water-layer was concentrated to furnish 198.0 g of a water-soluble fraction.

The EtOAc-soluble fraction (104.0 g) was subjected to silica gel (300 g) CC with solvent systems of *n*-hexane (C_6H_{14}) and EtOAc [C_6H_{14} (3 L), and C_6H_{14}-EtOAc (20:1, 3 L), (10:1, 3 L), (5:1, 3 L), (2:1, 3 L), (1:1, 3 L), and (1:2, 3 L)], EtOAc (3 L), EtOAc-acetone (1:1 3 L), acetone (3 L), and MeOH (3 L), 3-L fractions being collected. The residue (3.07 g) in fraction 4 was separated by ODS CC (*Φ*=3.5 cm, *L*=20 cm) with H_2O-MeOH [(9:1, 1 L), (17:3, 1 L), (4:1. 1 L), (3:1. 1 L), (7:3, 1 L), (3:2, 1 L), (1:1, 1 L), (2:3, 1 L), (3:7, 1 L), (1:4, 1 L), and (1:9, 1 L)], 1-L fractions being collected. From fraction 10, 30.1 mg of **5** was obtained as crystals. The residue (13.6 g) in fraction 5 was separated by ODS CC (*Φ*=3.5 cm, *L*=20 cm) with H_2O-MeOH [(9:1, 1 L), (17:3, 1 L), (4:1. 1 L), (3:1. 1 L), (7:3, 1 L), (3:2, 1 L), (1:1, 1 L), (2:3, 1 L), (3:7, 1 L), (1:4, 1 L), and (1:9, 1 L)], 1-L fractions being collected. The residue (120 mg) in fraction 7 was purified by HPLC (*Φ*=10 mm, *L*=25 cm; H_2O-acetone, 8:17; flow rate: 2.8 mL/min) to give 10.6 mg of **4** from the peak at 10.0 min. The residue (190. mg) in fraction 8 was purified by HPLC (*Φ*=10 mm, *L*=25 cm; H_2O-acetone, 17:33; flow rate: 2.8 mL/min) to give 17.4 mg of **1**, 19.1 mg of **2** and 27.6 mg of **3** from the peaks at 15.0 min, 20.0 min and 24.0 min, respectively.

The residue (14.1 g) in fraction 6 was separated by ODS CC (*Φ*=3.5 cm, *L*=20 cm) with H_2O-MeOH [(9:1, 1 L), (17:3, 1 L), (4:1. 1 L), (3:1. 1 L), (7:3, 1 L), (3:2, 1 L), (1:1, 1 L), (2:3, 1 L), (3:7, 1 L), (1:4, 1 L), and (1:9, 1 L)], 1-L fractions being collected. The residue (0.12 g) in fraction 10 was purified by HPLC (*Φ*=10 mm, *L*=25 cm; H_2O-acetone, 34:66; flow rate: 2.8 mL/min) to give 10.4 mg of **6**, 45.0 mg of **7** and 43.7 mg of **8** from peaks at 21 min, 32 min and 37 min, respectively.

The residue (12.6 g) in fraction 7 was separated by ODS CC (*Φ*=3.5 cm, *L*=20 cm) with H_2O-MeOH [(9:1, 1 L), (17:3, 1 L), (4:1. 1 L), (3:1. 1 L), (7:3, 1 L), (3:2, 1 L), (1:1, 1 L), (2:3, 1 L), (3:7, 1 L), (1:4, 1 L), and (1:9, 1 L)], 1-L fractions being collected. The residue (4.21 g) in fraction 10 was purified by HPLC (*Φ*=10 mm, *L*=25 cm; H_2O-acetone, 32:68; flow rate: 2.8 mL/min) to give 13.0 mg of **9** from a peak at 22 min.

Meliosmin

Colorless needles; mp. 105-108°C; $[\alpha]_D^{25}$-21.2 (*c* 0.34, $CHCl_3$); IR ν_{max} (film) cm^{-1}: 3463, 2892, 2781, 1776, 1609, 1502, 1448, 1249, 1038, 930, 757; UV λ_{max} (MeOH) nm (log ε): 281 (2.73), 237 (2.87), 208 (3.04); ^{1}H-NMR (400 MHz, DMSO-d_6) and ^{13}C-NMR (100 MHz, DMSO-d_6): **Table 1**; CD (MeOH) $\Delta\varepsilon$ (nm): +2.15 (239), +0.36 (285) (*c* 2.37 × 10^{-5} M); HR-ESI-MS (positive-ion mode) *m/z*: 407.0739 $[M+Na]^+$ (Calcd for $C_{20}H_{16}O_8Na$: 407.0737).

X-ray analysis of meliosmin (1)

A suitable crystal (0.27 mm × 0.22 mm × 0.10 mm) was used for analysis. Data were obtained using a Bruker SMART APEX II Ultra CCD diffractometer, using graphite-monochromated Mo*K*α radiation (λ=0.71073 Å). The structure was solved by a direct method using the program SHELXTL-97 [14]. The refinement and all further calculations were carried out using SHELXTL-97. Absorption correction was carried out utilizing the SADABS routine [15]. The H atoms were included at calculated positions and treated as riding atoms using the SHELXTL default parameters. Crystal data: $C_{20}H_{18}O_9$, *M*=402.34, monoclinic, *P*2(1), *a*=10.1257(17)

Å, *b*=7.6121(13) Å, *c*=12.426(2) Å, *β*=107.0868(18)°, *V*=915.4(3) Å^3, *T*=296 K, *Z*=2, $D_{c=}$1.460 g cm^{-3}, μ(Mo*K*α)=0.106 mm^{-1}, *F*(000)=420, 5638 reflections were measured in the range of 2*θ*<56.74°, 3381 being unique and used in all calculations. The final goodness-of-fit on F^2 was 1.067, and the final *R* indices were R_1=0.0338 and wR_2=0.0895 based on *I*>2*σ*(*I*), and R_1=0.0365 and wR_2=0.0915 with all data. The largest differences of the peak and the hole were 0.280 and −0.179 eÅ^{-3}, respectively. The CCDC deposit (No. 1061064) contains supplementary crystallographic data.

Anti-Leishmania Activity

The anti-*Leishmania major* activity toward promastigotes was determined by colorimetric cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16]. Promastigotes at the logarithmic growth phase were cultured in M199 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. One μL aliquots of sample solutions (DMSO) and *L. major* cells (1 × 10^5 cells/well) in 100 μL medium were added to each well of a 96-well plate, and then the plate was incubated at 27°C under an ambient atmosphere for 48 h. A solution of MTT (100 μL) was then added to each well and the incubation was continued for a further 24 h. The formazan product on MTT reduction was then dissolved in DMSO and the absorbance was measured using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and amphotericin B as a positive control. The experiment was performed in triplicate. The anti-*Leishmania major* activity was determined as the percentage of the control absorbance of reduced dye at 540 nm. The inhibitory activity was calculated as:

$$\% \text{ inhibition}=[1-(A_{test}-A_{blank})/(A_{control}-A_{blank})]\times 100$$

Where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} the absorbance of the test wells, and A_{blank} the absorbance of the cell-free wells.

Cytotoxic Activity toward A549 Lung Adenocarcinoma

Cytotoxic activity toward lung adenocarcinoma cells was determined by colorimetric cell viability assaying using MTT. A549 lung adenocarcinoma was purchased from the Japanese Collection of Research Bioresorces Cell Bank, Japan. A549 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FCS, and kanamycin (100 μg/mL) and amphotericin B (5.6 μg/mL).

One μL aliquots of sample solutions (DMSO) and the cancer cells (5 10^3 cells/well) in 100 μL medium were added to each well of a 96-well plate, which was then incubated at 37°C under a 5% CO_2 atmosphere for 72 h. A solution of MTT (100 μL) was then added to each well and the incubation was continued for a further 1 h. The absorbance of each well was measured at 540 nm using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and doxorubicin as a positive control. The cytotoxic activity was calculated as:

$$\% \text{ inhibition}=[1-(A_{test}-A_{blank})/(A_{control}-A_{blank})]\times 100$$

Where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} the absorbance of the test wells, and A_{blank} the absorbance of the cell-free wells.

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