In Vitro Leishmanicidal Constituents of *Millettia pendula*

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The *in vitro* leishmanicidal constituents of *Millettia pendula* were examined. Two new compounds, 1 (millettilone A) and 2 (millettilone B), were isolated from the methanol extract of *M. pendula*, together with six known compounds: 3*R*-claussequinone (3), pendulone (4), secundiflorol I (5), 3,8-dihydroxy-9-methoxypterocarpan (6), 3,10-dihydroxy-7,9-dimethoxypterocarpan (7), and formononetin (8). Among these, pendulone showed the most potent leishmanicidal activity. Compound 2 was found to be a purple pigment in this heartwood. Their chemical structures were elucidated using spectral methods.

Key words leishmaniasis; *Millettia pendula*; Leguminosae; Myanmar; pigment

Due to tropical monsoons and a subtropical climate, Myanmar abounds in natural plant resources, especially timber such as teakwood. However, the medicinal properties of these plant materials have not yet been thoroughly investigated.

In general, woody plants biosynthesize defensive compounds within the heartwood to protect the tissue against attack by pathogens, such as fungi and bacteria, and from oxidants. Several pharmacologic properties of heartwood constituents (for example, antibacterial, antifungal, and antioxidant activities) that play a role in the defense of heartwood tissue have been reported.[1—3)]

Millettia pendula BENTH. timber is produced in Myanmar and Thailand and is called *Thinwin* in Myanmar. It has a brownish purple color and the presumed chemical structure of the purple pigment has been previously reported, although it has not been isolated.[4)] This timber is mainly used for heavy construction, as in bridges and residential buildings and is also an excellent material for agricultural implements, joinery work, ornamental flooring, and cabinet making. However, its pharmaceutical applications have not yet been investigated.

Leishmaniasis is endemic in tropical regions, currently affecting 12 million people in 88 countries.[5)] This disease is transmitted by small biting sandflies (*Phlebotomus* sp.). The first-line drugs for the treatment of leishmaniasis are pentavalent antimonials such as *N*-methylglucamine antimonate (Glucantime) and sodium stilbogluconate (Pentostam). However, these drugs are toxic and generally expensive. Although some quinone derivatives are known to have antiprotozoal properties,[6,7)] only a few with antiprotozoal activity have been extracted from heartwood (a rich source of quinone compounds). Therefore we focused our screening on the antileishmanial activity of heartwood constituents and previously reported the screening results of 75 timber extracts.[8)] In this paper, we describe the leishmanicidal constituents of *M. pendula*, which has previously only been used as building timber.

Results and Discussion

In our previous paper,[8)] we examined 75 types of timber belonging to 27 families with regard to their leishmanicidal activities, and *M. pendula* showed the most potent activity, presumably due to its isoflavan skeleton compound pendulone. Further purification of the extract led us to isolate two new compounds, millettilone A (**1**) and millettilone B (**2**), together with six known compounds. Compound **2** is thought to be the genuine purple pigment of this heartwood.

Millettilone A (**1**), a yellow amorphous powder, has the formula $C_{17}H_{16}O_6$ based on high-resolution time-of-flight (HR-TOF) MS. The IR spectrum suggested the presence of a hydroxyl group (3434 cm^{-1}), conjugated carbonyl groups (1647 cm^{-1}), and an aromatic ring (1604, 1509 cm^{-1}). Since the UV spectrum of **1** was similar to that of 3*R*-claussequinone (**3**), **1** was assumed to be an isoflavan. In the ^{13}C-NMR spectrum, the chemical shifts of the A and B rings were in good agreement with those of 3*R*-claussequinone, and therefore **1** was thought to have the same substituent system as **3** on the A and B rings. Considering the presence of an additional signal (δ_C=56.7; δ_H=3.42) and the above data, **1** was thought to have a methoxyl group on ring C. The plane structure of **1** was established by a combination of 2D-NMR (COSY, HMQC, and HMBC) spectra (Figs. 1, 2). Since a small coupling constant (<1.0 Hz) was observed between H-

Table 1. ^{13}C-NMR Data for Compounds **1**—**3**

	1[a)]	**1**[b)]	**3**[a)]		**2**[a)]
2	64.8	63.8	69.1	2	147.9
3	35.7	34.5	32.0	3	116.4
4	75.1	74.4	29.9	3a	122.7
4a	112.5	111.6	112.6	4	124.0
5	133.2	132.2	131.0	5	114.6
6	109.6	109.3	109.4	6	159.5
7	159.8	157.7	157.8	7	98.2
8	103.4	103.2	103.7	7a	157.5
8a	156.0	154.9	155.7	1′	134.6
1′	147.4	146.6	149.7	2′	186.2
2′	187.3	186.7	187.4	3′	108.7
3′	108.5	107.7	108.6	4′	160.1
4′	159.6	158.5	159.6	5′	181.8
5′	182.4	182.2	182.4	6′	125.1
6′	132.1	131.6	131.5	4′-OCH_3	56.8
4-OCH_3	56.0	56.1			
4′-OCH_3	56.7	56.2	56.7		

a) In acetone-d_6; *b*) in $CDCl_3$.

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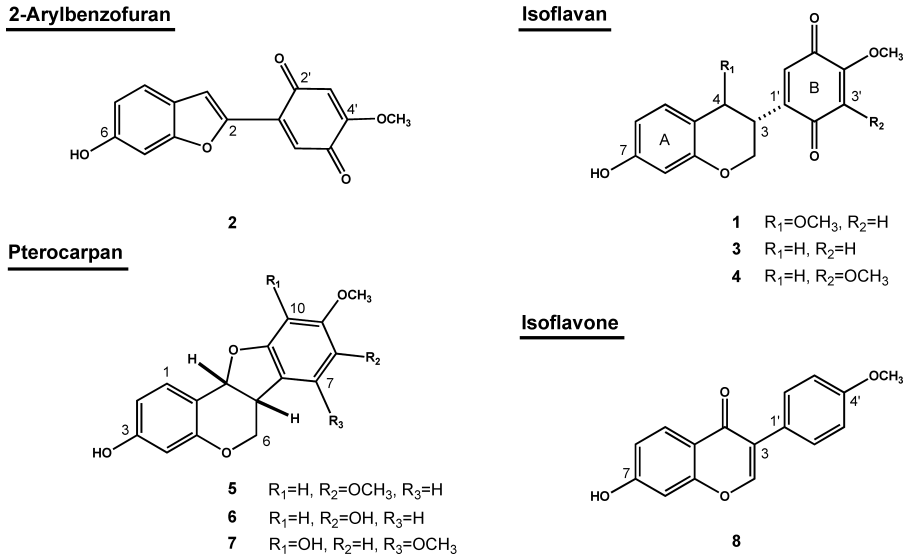

Fig. 1. Structures of Compounds **1**—**8**

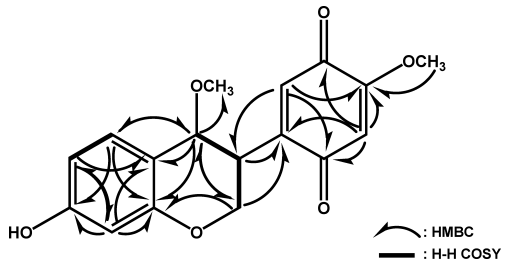

Fig. 2. HMBC and H–H COSY Correlations of **1**

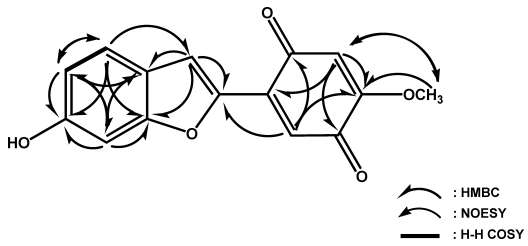

Fig. 3. HMBC, NOESY and H–H COSY Correlations of **2**

3 and H-4, the relative configuration between the methoxyl group at C-4 and the benzoquinone ring was determined to be *cis*. However, the absolute configuration of C-3 has not been determined because of the degradation after measurement of other spectral data. Thus, the chemical structure of **1** was established to be as shown in Fig. 1.

Millettilone B (**2**), a dark purple amorphous powder, was determined to have a molecular formula of $C_{15}H_{10}O_5$ based on HR-TOF-MS. Its methanol solution exhibited a deep purple color, which was due to a maximum absorption at 513 nm in the UV/VIS spectrum. Its ^{13}C-NMR spectrum showed no aliphatic signals except for a methoxyl group (δ_C=55.9; δ_H=3.88). The presence of a benzoquinone ring bearing a methoxyl group, for which the chemical shifts were in good agreement with those of **1**, was deduced from the ^{13}C-NMR spectrum. Since the coupling patterns of the aromatic ring proton signals indicated a 1,2,4-trisubstituted benzene ring, **2** was thought to have the same ring systems as **1** and **3**. Considering the unsaturation number along with the above data and the lack of a carbon atom in the ^{13}C-NMR spectrum compared with the isoflavan skeleton, **2** was considered to be a 2-arylbenzofurane. Finally, the chemical structure of **2** was established by a combination of 2D-NMR (COSY, HMQC, HMBC) spectra, as shown in Figs. 1 and 3. The coloration mechanism of the purple pigment of *M. pendula* has been reported by Mitsunaga *et al.*,[4] who described the deduced structure of the pigment as an isoflavan skeleton. Compound **2** is thought to be one of the purple pigments contained in heartwood of *M. pendula*.

The leishmanicidal activities of the compounds isolated from *M. pendula* heartwood were examined. Among them, pendulone exhibited the most potent activity (IC_{50} 0.07 μg/ml). In general, isoflavan compounds bearing a *p*-benzoquinone moiety showed moderate activity (3*R*-clausse-quinone, IC_{50} 1.2 μg/ml; **1**, IC_{50} 9.3 μg/ml). While pterocarpane bearing a hydroxyl group at C-8 showed moderate activity (3,8-dihydroxy-9-methoxypterocarpan, IC_{50} 2.9 μg/ml), two other pterocarpanes that have no hydroxyl group at C-8 showed weak activity (secundiflorol I, IC_{50} 86 μg/ml; 3,10-dihydroxy-7,9-dimethoxypterocarpan, IC_{50} 77 μg/ml). These results indicate that the *p*-benzoquinone moiety is important for potent leishmanicidal activity, and that the hydroxyl group at C-8 of pterocarpan is involved in this activity. The leishmanicidal activity of compound **2** was not examined because of a shortage of sample available for assay.

In conclusion, we found that the leishmanicidal activity of *M. pendula* heartwood extract was due to isoflavans with a 1,4-benzoquinone moiety and, among them, pendulone showed the most potent activity. Through this investigation, two new compounds, millettilone A and B, were isolated, the latter of which was a purple pigment in this heartwood.

Experimental

Materials *M. pendula* timber samples were from "Myanmar Timber Samples" produced by the Forest Research Institute, Forest Department, Yangon, and were kindly donated by the Ministry of Forestry of Myanmar.

Chemicals TetraColor ONE was purchased from Seikagaku Kogyo Co. Ltd. Medium 199 and ES Cell Qualified Fetal Bovine Serum were from In-

vitrogen Co. Ltd.

The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL Alpha 500 spectrometer. UV spectra were recorded on a Hitachi U-2000 spectrometer and IR spectra on a JASCO FTIR-5300 spectrometer. Mass spectra were measured with an Applied Biosystems QSTAR XL spectrometer (TOF-MS). HPLC was run on a Shimadzu LC-10A apparatus with a UV detector (Shimadzu SPD-M10A).

Cultivation of Leishmania Promastigotes Medium 199 was used for the cultivation of promastigotes of *Leishmania major* (MHOM/SU/73/5ASKH). Promastigotes were cultured in the medium [supplemented with heat-inactivated (56 °C for 30 min) fetal bovine serum (10%)] at 27 °C, in a 5% CO_2 atmosphere in an incubator.[8]

Leishmanicidal Activity Assay The leishmanicidal effects of timber extracts were assessed using the improved 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) method as follows. Cultured promastigotes were seeded at 4×10^5/50 μl of the medium per well in 96-well microplates, and then 50 μl of different concentrations of test compounds dissolved in a mixture of DMSO and the medium were added to each well. Each concentration was tested in triplicate. The microplate was incubated at 27 °C in 5% CO_2 for 48 h. TetraColor ONE (10 μl) a mixture of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt and 1-methoxy-5-methylphenazinium methosulfate was added to each well and the plates were incubated at 27 °C for 6 h. Optical density values (test wavelength 450 nm; reference wavelength 630 nm) were measured using a microplate reader (Thermo BioAnalysis Japan Co., Ltd., Kanagawa, Japan). Leishmanicidal activity was expressed as the Minimum lethal concentration (MLC) and Minimum inhibitory concentration (MIC). The inhibitory concentration of 50% (IC_{50}) values of compounds were estimated from the dose–responce curve.

Isolation Procedure Powdered *M. pendula* Benth. timber (68 g) was extracted with hot methanol (2 l×2) and the extract was then filtered. The filtrate was concentrated *in vacuo* and the residue (4.8 g) was purified on column chromatography over silica gel using chloroform–methanol to obtain 20 fractions. Fractions eluted with 5—15% methanol/chloroform were combined and purified by silica gel column chromatography using chloroform/ethylacetate to give 100 fractions. Among them, fractions eluted with 10—20%, 25—30%, and 30—40% ethylacetate/chloroform were combined respectively as fr. A, fr. B, and fr. C. Fr. A was rechromatographed on silica gel (hexane/chloroform) and then on Sephadex LH-20 column chromatography (methanol) to give pendulone (**4**) (85 mg) and a dark purple residue. This residue was rechromatographed on reversed-phase HPLC using 40% CH_3CN/H_2O to give millettilone B (**2**) (1 mg) and secundiflorol I (**5**) (1 mg). Fr. B was rechromatographed on Sephadex LH-20 using methanol as an eluent to give dark brown and pale brown residues. Each residue was purified on reversed-phase HPLC using 38 and 35% CH_3CN/H_2O, respectively, to obtain millettilone A (**1**) (9 mg) and 3,8-dihydroxy-9-methoxypterocarpan (**6**) (7 mg) from the former residue, and 3*R*-claussequinone (**3**) (16 mg) and formononetin (**8**) (3 mg) from the latter. Fr. C was rechromatographed on Sephadex LH-20 using methanol as an eluent to give 3,10-dihydroxy-7,9-dimethoxypterocarpan (**7**) (20 mg).

Compound 1 (millettilone A) A yellow amorphous powder, $[\alpha]_D^{24}$ −35.9° (c=0.1, MeOH). HR-TOF-MS m/z: 317.1040 ($[M+H]^-$) (Calcd for $C_{17}H_{17}O_6$: 317.1025). UV λ_{max} (MeOH) nm (ε): 203.0 (36000), 264.5 (7800). IR (KBr) cm^{-1}: 3434 (OH), 1647 (C=O), 1604, 1509 (Ar). ^{1}H-NMR (acetone-d_6) δ: 8.59 (1H, br s, –OH), 7.10 (1H, d, J=8.3 Hz, H-5), 6.44 (1H, dd, J=8.3, 2.3 Hz, H-6), 6.39 (1H, s, H-6′), 6.30 (1H, br s, H-8), 6.06 (1H, s, H-3′), 4.36 (1H, dd, J=10.5, 3.1 Hz, H-2), 4.26 (1H, d, J=10.5 Hz, H-2), 4.20 (1H, s, H-4), 3.81 (3H, s, $-OCH_3$), 3.48 (1H, m, H-3), 3.42 (3H, s, $-OCH_3$). ^{13}C-NMR (acetone-d_6, $CDCl_3$): Table 1.

Compound 2 (millettilone B) A dark purple amorphous powder. HR-TOF-MS m/z: 271.0626 ($[M+H]^+$ (Calcd for $C_{15}H_{11}O_5$: 271.0607). UV λ_{max} (MeOH) nm (ε): 204.0 (22000), 265.0 (11000), 292.0 (12000), 345.0 (4100), 513.0 (4700). IR (KBr) cm^{-1}: 3454 (OH), 1656 (C=O), 1626 (C=O), 1579 (C=C). ^{1}H-NMR (acetone-d_6) δ: 7.80 (1H, s, H-3), 7.57 (1H, d, J=8.3 Hz, H-4), 7.00 (1H, br s, H-7), 6.96 (1H, s, H-6′), 6.88 (1H, dd, J=8.3, 1.8 Hz, H-5), 6.08 (1H, s, H-3′), 3.88 (3H, s, $-OCH_3$). ^{13}C-NMR (acetone-d_6): Table 1.

Compounds **3**, **4**,[9] **5**—**7**,[10—12] **8**[13,14] were characterized by comparison of their spectroscopic properties with the values reported in the literature.

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