Flavonoids and Isoflavonoids from the Fresh Immature Seeds of Sophora tomentosa L.

Flavonoids and Isoflavonoids from the Fresh Immature Seeds of *Sophora tomentosa* L.

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Abstract

Three compounds were isolated from immature seeds of *Sophora tomentosa* L. using solvent extraction and chromatographic separation. Their structures were identified to be 7,4'-dihydroxy-3'-methoxyisoflavone, calycosin (7,3'-dihydroxy-4'-methoxyisoflavone), and farnisin (7,3'-dihydroxy-4'-methoxyflavone) by spectroscopic methods.

Introduction

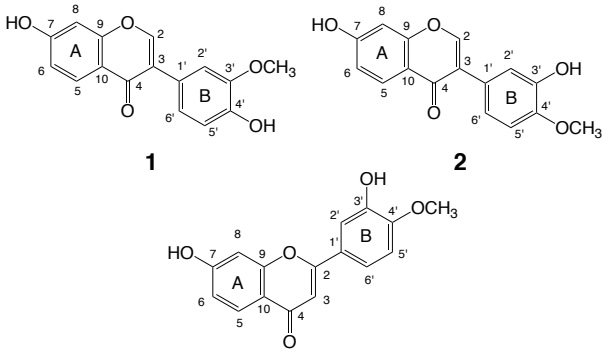
Sophora tomentosa L. (Leguminosae) is a small tree with yellow flower commonly found at the coastal area in Okinawa Islands. Flavonoids, isoflavonoids, alkaloids and benzofurane derivatives have been isolated from the aerial parts, leaves, roots and stems of *S. tomentosa*.[1-8] Some flavonoids have potent antimicrobial activities against methicillin resistant *Staphylococcus aureus*, and some isoflavonoids show estrogenic activities.[9-10] The flavonoids from *Sophora* species have antitumor, antimicrobial, anti-HIV, and enzyme inhibitory activities.[11]

Six compounds useful for human health and medicinal chemistry have been isolated from the mature seeds of *S. tomentosa* L[12] However, immature legumes of *S. tomentosa* L have been rarely investigated to find bioactive compounds.

In the present study, compounds in the immature seeds of *S. tomentosa* L were investigated. Three compounds, two isoflavonoids and one flavonoid, were isolated and structurally characterized.

Results and Discussion

The chloroform-soluble fraction from a methanol extract of the fresh immature seeds of *Sophora tomentosa* L. was subjected to several chromatographic separation and purification to give two isoflavonoids (**1** and **2**) and one flavonoid (**3**).



1 **2**

3

Structures

Compound **1** was obtained as white needles and has a molecular formula of $C_{16}H_{12}O_5$ by observation of a quasi-molecular ion peak at *m*/z 285.0727 $[M+H]^+$ (calcd for $C_{16}H_{13}O_5$: 285.0763) in its high resolution-electron spray ionization-mass spectrum (HR-ESI-MS). Its ultra violet (UV) spectrum showed absorption maxima at 249 (log ε = 4.49), 259 (4.47), 292 (4.29) and 309 nm (4.13) characteristic of isoflavone, indicating that **1** was an isoflavone derivative. Its IR spectrum showed a band due to OH at 3238 cm^{-1}, indicating that **1** was an isoflavone possessing hydroxy group(s). Its ^1H and ^{13}C NMR spectra showed a singlet at δ_H 8.05 due to an oxygenated olefinic proton (H-2) characteristic of isoflavone and a signal at δ_C 152.5 due to oxygenated olefinic carbon (C-2) of isoflavone, supporting that **1** was the isoflavone

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Table 1. ^{1}H and ^{13}C NMR spectral data of **1**–**3**

	1 in acetone-d6		**2** in acetone-d_6		**3** in CD_3OD	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	8.05 s	152.5	8.17 s	153,4		164,3
3		124.0		125.1	6.62 s	104.3
4		174.8		175.4		178.8
5	7.94 d (8.8)	127.6	8.08 d (8.8)	128.5	7.93 d (8.7)	126.4
6	6.87 dd (8.8, 2.0)	114.7	7.01 dd (8.8, 2.2)	115.4	6.89 dd (8.7, 2.0)	115.0
7		162.6		163.0		163.7
8	6.78 d (2.0)	102.3	6.92 d (2.2)	103.2	6.92 d (2.0)	102.1
9		157.9		158.8		158.3
10		117.6		118.7		115.8
1'		124.3		126.3		124.0
2'	7.16 d (1.9)	112.8	7.18 d (2.1)	116.9	7.37 d (1.7)	112.5
3'		147.0		147.1		146.8
4'		146.6		148.3		150.1
5'	6.74 d (8.2)	114.7	7.00 d (8.3)	112.2	7.04 d (8.5)	111.3
6'	6.95 dd (8.2, 1.9)	121.7	7.08 dd (8.3, 2.1)	121.1	7.47 dd (8.5, 1.7)	118.5
OCH_3-3'	3.75 s	55.4				
OCH_3-4'			3.89	56.3	3.90 s	55.1

* Coupling constants (*J*) are expressed in Hz.

derivative.

The ^{1}H and ^{13}C NMR spectra of **1** showed a carbonyl carbon signal at δ_C 174.8 due to a chromone skeleton, AMX pattern signals at δ_H 7.94 (1H, d, *J*=8.8 Hz), 6.87 (1H, dd, *J*=8.8, 2.0 Hz), and 6.78 (1H, d, *J*=2.0 Hz) due to aromatic protons, another AMX pattern signals at δ_H 7.16 (1H, d, *J*=1.9 Hz), 6.95 (1H, dd, *J*=8.2, 1.9 Hz), and 6.74 (1H, d, *J*=8.2 Hz) due to aromatic protons, and signals at δ_C 162.6, 152.5, 147.0 and 146.6 due to oxygenated aromatic carbons and a singlet at δ_H 3.75 due to a methoxy group. These data indicated that **1** was an isoflavone derivative possessing one methoxy and two hydroxy groups and that ring A was C-6, C-9, C-10-trisubstituted (pattern X) or C-7, C-9, C-10-trisubstituted (pattern Y) aromatic ring and ring B was C-1', C-2', C-5'-trisubstituted (pattern A), C-1', C-2', C-4'-trisubstituted (pattern B) or C-1', C-3', C-4'-trisubstituted (pattern C) aromatic ring. Full assignments of ^{1}H and ^{13}C NMR signals were performed by correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple band coherence (HMBC) NMR analyses and analyses of coupling patterns (Table 1). The AMX pattern signals including the doublet (1H, *J*=8.8 Hz) at δ_H 7.94 due to H-5 were identified as aromatic protons of A ring, suggesting no substitution at C-6 position, because a correlation between the doublet at δ_H 7.94 due to H-5 and a signal at δ_C 174.8 due to the carbonyl carbon was observed in the HMBC spectrum

(Fig. 1). Therefore, ring A was C-7, C-9, C-10-trisubstituted (pattern Y) aromatic ring.

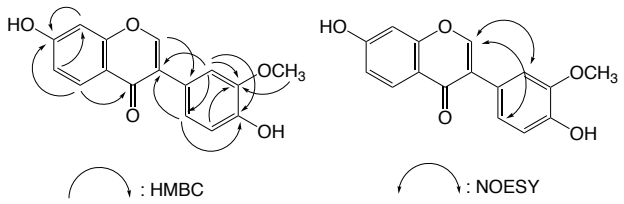

Fig. 1. Selected HMBC and NOESY correlations of **1**.

In the nuclear Overhauser effect spectroscopy (NOESY) spectrum, correlations were observed between a singlet due to H-2 at δ_H 8.05 and both a doublet due to H-2' at δ_H 7.16 and doublet of doublets due to H-6' at δ_H 6.95. The other AMX pattern signals including doublet of doublet (1H, *J*=8.2, 1.9 Hz) at δ_H 6.95 were assigned as aromatic protons of B ring, indicating no substitution at C-5' position, because correlation between the doublet of doublets due to H-6' at δ_H 6.95 and the signal due to C-3 carbon at δ_C 124.0 was observed in the HMBC spectrum (Fig. 1). These facts indicated that ring B was C-1', C-3', C-4'-trisubstituted (pattern C) aromatic ring.

Moreover, observation of the correlation between a singlet due to the methoxy protons and a signal due to C-3' aromatic carbon at δ_C 147.0 revealed that the methoxy group was bonded to C-3'. In the NOESY spectrum, a correlation was observed between the signal due to the methyl protons of the methoxy group at δ_H 3.75 and that due to H-2' at δ_H 7.16, supported that the methoxy group was bonded to C-3'. The bond positions of two hydroxy groups were assigned to be C-7 and C-4'. The positions of one methoxy and two hydroxy groups also confirmed by band-shift experimental by means of shift reagents in UV spectrum as follows. In comparison of the bands in UV spectrum to which added with $AlCl_3$ with those in original UV spectrum in MeOH, no band shift was observed for Band I (309 nm) and II (259 nm) at both spectra. This fact was shown that position of two hydroxy groups were not 3-hydroxy derivatives and 5-hydroxy derivatives[13]. Comparing of the UV spectrum to which added with NaOMe with original UV spectrum in MeOH, a bathochromic shift (309 → 337 nm, Δ 28 nm) of band I was observed, suggesting that a hydroxy group exists at the C-4' position of the B ring[13]. Comparing the spectrum to which NaOAc was added with the original UV spectrum in MeOH, the bathochromic shift (309 → 336 nm, Δ 27 nm) of Band I was observed, suggesting that a hydroxy group exists at the C-7 position[13].

Thus, **1** was identified with 7, 4'-dihydroxy-3'-methoxyisoflavone (**1**). The physical and spectral data of **1** were in agreement with those in reference.[14] Although it has been reported that **1** was isolated from the fresh mature seeds,[12] the structural elucidation was not sufficient due to the low yield and the inclusion of a lot of noise in the spectrum. This time, **1** could be completely identified because the yield could have been increased and spectra with less noise and additional spectra could have been measured.

Compound **2** was obtained as pale yellow oil. Its ^{1}H and ^{13}C NMR spectra showed a singlet at δ_H 8.17 due to an oxygenated olefinic proton (H-2) characteristic of isoflavone and a signal at δ_C 153.4 due to oxygenated olefinic carbon (C-2) of isoflavone, suggesting that **2** was an isoflavone derivative.

The ^{1}H and ^{13}C NMR spectral data of **2** coincided with those of **1**, suggested that **2** was regio-isomer of **1** (Table 1). The ^{1}H NMR spectrum showed a carbonyl carbon signal at δ_C 175.4 due to a chromone skeleton, AMX pattern signals at δ_H 8.08 (1H, d, *J*=8.8 Hz), 7.01 (1H, dd, *J*=8.8, 2.2 Hz), and 6.92 (1H, d, *J*=2.2 Hz) due to aromatic protons, ABX pattern signals at δ_H 7.18 (1H, d, *J*=2.1 Hz), 7.08 (1H, dd, *J*=8.3, 2.1 Hz), and 7.00 (1H, d, *J*=8.3 Hz) due to aromatic protons, signals at δ_C 163.0, 158.8, 148.3 and 147.1 due to oxygenated aromatic carbons and a singlet at δ_H 3.89 due to a methoxy group. These data indicated that **2** was an isoflavone derivative possessing one methoxy and two hydroxy groups and that both ring A

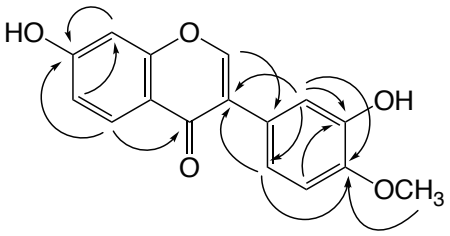

Fig. 2. Selected HMBC correlations of **2**.

and B were 1, 2, 4-trisubstitution aromatic rings, same as **1**. Full assignments of ^{1}H and ^{13}C NMR signals were performed by COSY, HSQC, and HMBC NMR analyses and analyses of coupling patterns (Table 1). The AMX pattern signals including the doublet (1H, *J*=8.8 Hz) at δ_H 8.08 due to H-5 were identified as aromatic protons of A ring, suggesting no substitution at C-6 position same as **1**, because a correlation between the doublet at δ_H 8.08 due to H-5 and a signal at δ_C 175.4 due to the carbonyl carbon was observed in the HMBC spectrum (Fig. 2). The ABX pattern signals including doublet of doublets (1H, *J*=8.3, 2.1 Hz) at δ_H 7.08 were assigned as aromatic protons of B ring, indicating no substitution at C-5' position, because correlation the doublet of doublets at δ_H 7.08 and the signal due to C-3 carbon at δ_C 125.1 was observed in the HMBC spectrum (Fig. 2). Moreover, the correlation observed between a singlet due to the methoxy protons and a signal due to C-3' aromatic carbon at δ_C 147.1 in the HMBC spectrum of **1** was not observed in the spectrum of **2** and the correlation between a singlet due to the methoxy protons and a signal due to C-4' aromatic carbon at δ_C 148.3 in the HMBC spectrum of **2** was newly observed. The fact revealed that the methoxy group was bonded to C-4'. The bond positions of two hydroxy groups were assigned to be C-7 and C-3'.

Thus, **2** was identified with 7, 3'-dihydroxy-4'-methoxyisoflavone (**2**, calycosin). The physical and spectral data of **2** were in agreement with those in reference. [15]

Compound **3** was obtained as pale yellow amorphous and had a molecular formula $C_{16}H_{12}O_5$ by observation of a quasi-molecular ion peak at *m*/z 285.0741 $[M+H]^+$ (calcd for $C_{16}H_{13}O_5$: 285.0763) in the HR-ESI-MS. Its UV spectrum showed absorption maxima at 240 (log ε 3.99) and 341 nm (4.08) characteristic of flavone, indicating that **3** was a flavone derivative. Its IR spectrum showed a band due to OH at 3364 cm^{-1}, indicating that **3** was a flavone possessing hydroxy group(s).

The ^{1}H and ^{13}C NMR spectra of **3** showed a carbonyl carbon signal at δ_C 178.8 due to a chromone skeleton, ABX pattern signals at δ_H 7.93 (1H, d, *J*=8.7 Hz), 6.89 (1H, dd, *J*=8.7, 2.0 Hz) and 6.92 (1H, d, *J*=2.0 Hz) due to aromatic protons, AMX pattern signals at δ_H 7.47 (1H, dd, *J*=8.5, 1.7 Hz), 7.37 (1H, d, *J*=1.7 Hz) and 7.04 (1H, d, *J*=8.5 Hz) due to aromatic protons, and signals at δ_C 163.7, 158.3, 150.1 and 146.8 due to oxygenated aromatic carbons and a singlet due to a methoxy group at δ_H 3.91. These data indicated that **3** was a flavone derivative possessing one methoxy and two hydroxy groups and that ring A was C-6, C-9, C-10-trisubstituted (pattern X) or C-7, C-9, C-10-trisubstituted (pattern Y) aromatic ring and ring B was C-1', C-2', C-5'-trisubstituted (pattern L), C-1', C-2', C-4'-trisubstituted (pattern M) or C-1', C-3', C-4'-trisubstituted (pattern N) aromatic ring. Complete assignments of ^{1}H and ^{13}C NMR signals were performed by COSY, HSQC and HMBC NMR analyses and analyses of sprit patterns of signals (Table 1). The ABX pattern signals including

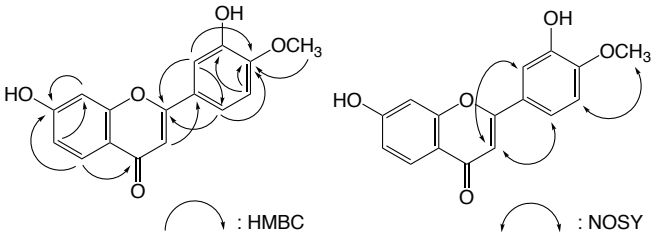

Fig. 3. Selected HMBC and NOESY correlations of **3**.

doublet (1H, *J*=8.7 Hz) at δ_H 7.93 due to H-5 were identified as aromatic protons at A ring and suggested no substitution at C-6 position, because correlation between the doublet at δ_H 7.93 due to H-5 and a signal due to the carbonyl carbon at δ_C 178.8 was observed in the HMBC spectrum (Fig. 3). Therefore, ring A was C-7, C-9, C-10-trisubstituted (pattern Y) aromatic ring.

In the NOESY spectrum, correlations were observed between a singlet due to H-3 at δ_H 6.62 and both doublet due to H-2' at δ_H 7.37 and doublet of doublets due to H-6' at δ_H 7.47. The AMX pattern signals including the doublet of doublets (1H, *J*=8.5 1.7 Hz) due to H-6' at δ_H 7.47 were assigned as aromatic protons at B ring and suggested no substitution at C-5' position, because both doublet (1H, *J*=1.7 Hz) at δ_H 7.37 due to H-2' and the doublet of doublets at δ_H 7.47 due to H-6' showed correlation to the signal at δ_C 164.3 due to C-2 oxygenated olefinic carbon in the HMBC spectrum (Fig. 3). These facts indicated that ring B was C-1', C-3', C-4'-trisubstituted (pattern N) aromatic ring. Furthermore,

observation of the correlation between a singlet at δ_H 3.90 due to the methoxy proton and signal at δ_C 150.1 due to C-4' aromatic carbon revealed that the methoxy group was bonded to C-4'. This was supported by the observation of correlation between the signal due to the methoxy protons at δ_H 3.90 and that due to H-5' at δ_H 7.04 in the NOESY spectrum. Therefore, the bond positions of two hydroxy groups were assigned to be C-7 and C-3'.

Thus, **3** was identified as 7, 3'-dihydroxy-4'-methoxyflavone (**3**, farnisin). The physical and spectral data of **3** were in agreement with those in reference. [16)]

A number of flavonoids and isoflavonoids were isolated from aerial parts, roots, stems and stem barks of *S. tomentosa* and all of them were 5-hydroxy derivatives. Those isolated from fresh immature and mature[12)] seeds in the investigation were no substitution skeletons at C-5 position. Equol derived from isoflavone such as daidzein has estrogenic effect to human and animals.[17)] Daidzein possesses the structure similar to that (no substitution at C-5) isolated from not only mature seeds but also immature seeds of *S. tomentosa*. The estrogenic effect was expected in the isoflavones from the immature seeds of *S. tomentosa*.

Experimental

Analytical and preparative TLCs were carried out on Merck 60 F_{254} silica gel plate (thickness: 0.25 mm) and on the plates (thickness: 0.5 and 2.0 mm), respectively. Column chromatography (CC) and flash-column chromatography (FCC) were carried out with Kieselgel 60 F_{254} (Merck). ^{1}H-, ^{13}C-, and two-dimensional (2D) NMR spectra were acquired on a Bruker AVANCE III 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) or 400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) spectrometers in CD_3OD for **3** and in acetone-d_6 for **1** and **2**. The symbols s, d, m, t, q and dd denote singlet, doublet, multiplet, triplet, quartet and doublet of doublets, respectively. HR-ESI-MS was obtained on a JEOL JMS-T100LP mass spectrometer.

Extraction and isolation. Fresh immature seeds (weight: 5.424 kg) of *Sophora tomentosa* collected at the campus of University of the Ryukyus, Okinawa-prefecture in April were ground in a blender and immersed in methanol (MeOH) for ca. 2 weeks. After filtration, the residue was re-extracted with MeOH at room temperature 10 times and at 60 °C for 8 hours 8 times. The MeOH soln combined were concentrated *in vacuo* into dryness to give tarry matters (428.03 g). The tarry matters obtained was successively extracted with hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), 1-butanol (*n*-BuOH) and MeOH to give hexane- (50.6 g), $CHCl_3$- (23.8 g), EtOAc- (1.6 g), *n*-BuOH- (46.8g) and MeOH- soluble fractions (160.6 g), respectively. The portion (9.75 g) of $CHCl_3$-soluble fraction was subjected to FCC on silica gel (Si-gel) with solvent system of $CHCl_3$ increasing MeOH and H_2O as solvent ratio of 10:0:0, 9:1:0.1, 7:3:0.5, EtOAc-MeOH-H_2O (7:3:0.5) and EtOAc to give fractions A–E. As fraction B showed several absorption spots on analytical TLC under UV light at 254 nm and luminescence spots on analytical TLC under UV light at 365 nm, this fraction was subjected to FCC on Si-gel with solvent system of $CHCl_3$-MeOH-H_2O (9:1:0.1) to give fractions B1-B5. The fraction B4 was re-chromatographed on Si-gel with $CHCl_3$-MeOH (12:1) to give fractions B451-B456. Fraction B454 was subjected to CC on Si-gel to give fractions B4541-B4543. The fraction B4542 was subject to preparative TLC on si-gel with $CHCl_3$-acetone (8:1) to give **1** (4 mg), **2** (1 mg), and **3** (5 mg).

***7, 4'-dihydroxy-3'-methoxyisoflavone* (1).** White needles, mp 240 - 244℃(MeOH). HR-ESI-MS: *m*/z 285.0727 $[M+H]^+$ (Calcd for $C_{16}H_{13}O_5$: 285.0763. UV: λmax (log ε) nm: 249 (4.49), 259 (4.47, band II), 292 (4.29) and 309 (4.13, band I). UV (MeOH+NaOMe) λ_{max} nm : 257, 304, 337. UV (MeOH+$AlCl_3$) λ_{max} nm :249, 264, 292, 308. UV (MeOH+NaOAc) λ_{max} nm : 259, 290, 336. IR ν cm^{-1}: 3238 (OH); ^{1}H (acetone-d_6, 500 MHz): Table 1; ^{13}C NMR (acetone-d_6, 125 MHz): Table 1. These spectral data coincided with those in reference. [14)]

***7, 3'-dihydroxy-4'-methoxyisoflavone* (2, *calycosin*).** Pale yellow oil. ^{1}H (acetone-d_6, 400 MHz): Table 1; ^{13}C NMR (acetone-d_6, 100 MHz): Table 1. These spectral data coincided with those in reference. [15)]

***7, 3'-dihydroxy-4'-methoxyflavone* (3, *farnisin*).** Pale yellow amorphous. HR-ESI-MS: *m*/z 285.0741 $[M+H]^+$

(Calcd for $C_{16}H_{13}O_5$: 285.0763). UV: λmax (log ε) nm: 240 (3.99), 290 (3.99, sh) and 341 (4.08). IR ν cm^{-1}: 3364 (OH); ^{1}H (CD_3OD, 400 MHz): Table 1; ^{13}C NMR (CD_3OD, 100 MHz): Table 1. These physical and spectral data coincided with those in reference.[16)]

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