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A Leaf-Closing Substance of *Albizzia julibrissin* Durazz

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Abstract—Potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**) was isolated as a leaf-closing substance of a nyctinastic plant, *Albizzia julibrissin* Durazz. Compound **1** was quite effective for the leaf-closing of *A. julibrissin* at 2×10^{-5} M. And **1** was also effective for leaf closure of *Albizzia lebbeck*, which belongs to the same plant genus as *A. julibrissin*, at 4×10^{-4} M. On the other hand, HPLC and FAB MS analysis revealed that **1** was contained in *A. lebbeck*. Thus, leaf-closing substance of *A. lebbeck* would be a chemical substance the same as or structurally related to **1**.

Most Leguminosae plants close their leaves in the evening, as if to sleep, and open them in the morning.[1] This rhythmic movement of the leaves is called nyctinasty, which has been known to be controlled by an internal biological clock.[2] Among them, the nyctinastic leaf-movement of *Albizzia julibrissin* Durazz (nemu-no-ki in Japanese) is the most well known.

Recently, we have identified several bioactive substances that regulate this leaf-movement,[3–16] and revealed that the nyctinastic movement of plants is controlled by interaction between leaf-closing and -opening substances.[11–14] From *A. julibrissin*, we have already isolated a leaf-opening substance, *cis-p*-coumaroylagmatine (**2**). However, the counterpart of **2**, the leaf-closing substance, remained unidentified. We have now isolated potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**) as the leaf-closing substance of *A. julibrissin*.

Isolation of **2**, the leaf-opening substance of *A. julibrissin*, was carried out based on a bioassay using a leaf of *A. julibrissin*. The status of the leaf of *A. julibrissin* is easily affected by the prolonged radiation of sunlight. The sunlight prevented the leaf-closure of *A. julibrissin*. Thus, our bioassay previously established for other nyctinastic plants could not be used in the case of the leaf-closing substance of *A. julibrissin*. We, therefore, developed a new method that can be carried out in a short time. The most important problem encountered in the method was the difficulty for the leaf to pump up the sample solution through the vessel. To circumvent this difficulty, we developed the suction method: the leaf immersed in the sample solution is put in a bell funnel, and decompressed using an aspirator. The sample solution is then pumped up through the vessel to the stomata by compulsion. We were able to distinguish the bioactive fraction from others because the bioactive fraction made the leaf closed within a few minutes in this method.

We carried out the isolation of the leaf-opening and -closing substances of *A. julibrissin*. The entire process is demonstrated in Scheme 1. The fresh whole plant of *A. julibrissin* (2.6 kg) was extracted with MeOH–H_2O (8:2) for two weeks and concentrated in vacuo. The concentrated extract was partitioned with ethyl acetate, then with *n*-BuOH. From the result of the partition with organic solvents, it was found that both leaf-opening and -closing substances were water-soluble. Comparatively hydrophobic leaf-opening substance (**2**) was dispersed in both aqueous and *n*-BuOH layers. Thus, after partition, the aqueous layer showed weak leaf-closing activity because both leaf-closing and -opening substance contained in the same aqueous layer cancel each other. Then, we removed the leaf-opening substance mostly by repetitive partition with *n*-BuOH. However, it was impossible to remove it entirely; thus, we attempted to apply polymer gel chromatography for the separation of the two inversely bioactive substances. The bioactive aqueous layer was carefully separated by Amberlite XAD-7 column chromatography eluted with MeOH–H_2O, and the 30% MeOH aq. fraction showed leaf-closing activity. The 30% MeOH aq. fraction was further purified by gel filtration column chromatography using Toyopearl HW-40S with 30% MeOH aq., and then HPLC using preparative Cosmosil 5C18AR column with 20% MeOH aq repeatedly to give potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**, 1.8 mg). As previously reported, the leaf-opening substance **2** was also isolated from the *n*-BuOH layer.[9]

Keywords: *Albizzia julibrissin* Durazz; Leguminosae; leaf-closing substance.

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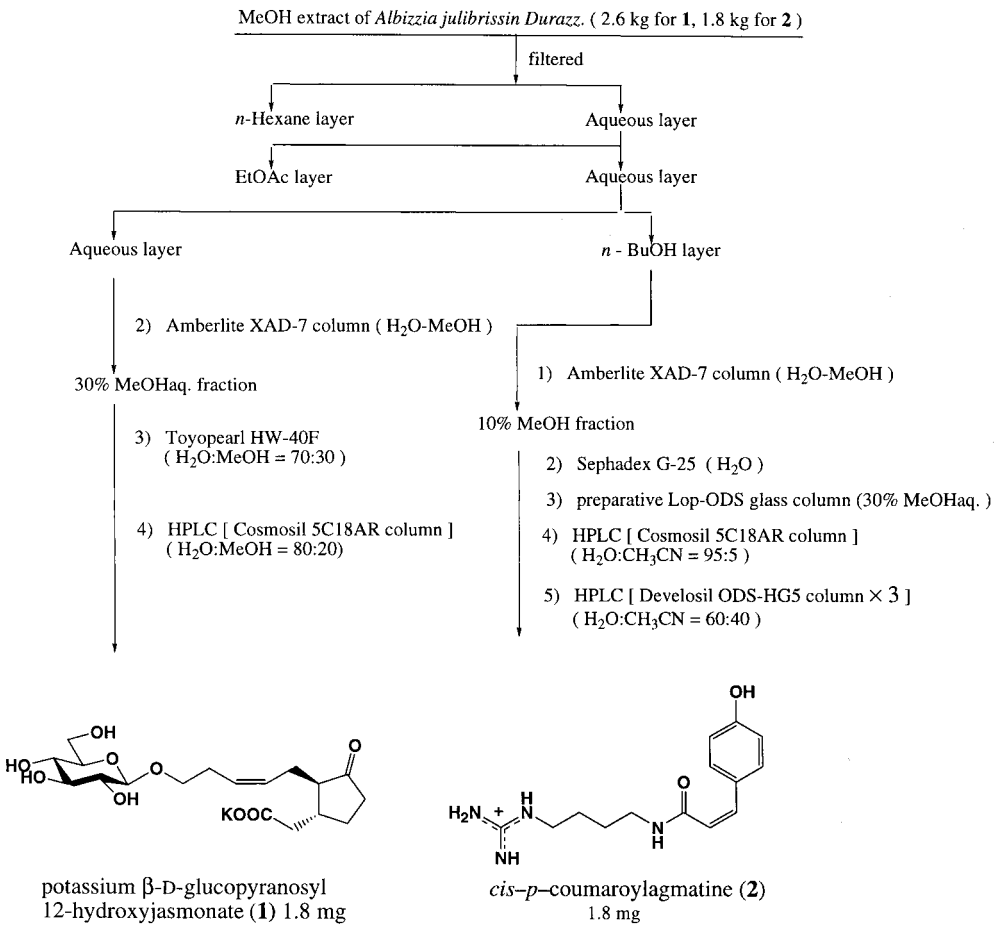

Scheme 1. Isolation of leaf-opening and -closing substances from *A. julibrissin*.

The structural determination of **1** was carried out by means of NMR and HR FAB MS (negative) experiments.[17] Negative-ion mode FAB MS experiment gave a strong quasi-molecular ion peak at *m/z* 387, which corresponded to $C_{16}H_{27}O_8$ in the negative HR FAB MS experiment. On the other hand, a strong molecular ion corresponding to **1** was observed at *m/z* 411 in the positive-ion mode FAB mass spectrum, suggesting **1** exists as a potassium salt. HMQC, HMBC, and HOHAHA experiments gave the planar structure of **1** (Fig. 1).[17] The correlations in HMBC between the distinctive *cis*-olefin and the surrounding parts revealed the structure of the side chain containing C_7 to C_{12}. The anomeric proton of β-glucopyranoside gave the correlation with C_{12} (70.7 ppm in the ^{13}C NMR spectrum) in the HMBC spectrum. And also, NOE correlation (5.6%) was observed between $H_{1'}$ and H_{12}. Thus, the hydroxyl group on C_{12} was determined to be glycosilated with β-glucopyranoside. The correlation between carbonyl group (C_6) and H_8 in HMBC and correlation between H_8 and H_7 in HOHAHA spectrum revealed that this side chain was connected with C_7. The specific carboxylate (C_1) showed correlations between two H_2 signals in HMBC. Moreover, H_2 signals gave correlations with C_7 and C_4 in HMBC. With correlation between H_4 and C_3 in HMBC, the carboxy methyl group was connected with C_3. Finally, the correlation between H_5 and C_6 revealed that C_5 should be located between the carbonyl (C_6) and C_4.

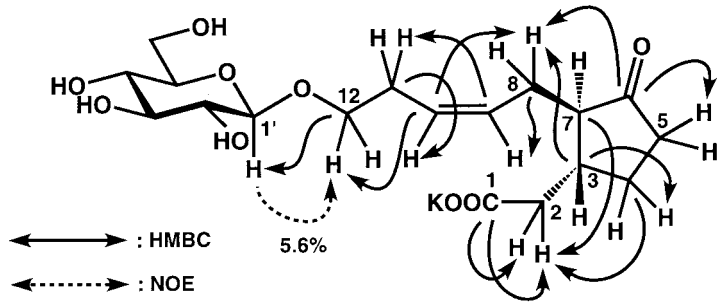

Figure 1. HMBC and NOE correlations in potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**).

Table 1. Bioactivity of the MeOH extracts collected during the daytime and at night (++ completely open; + nearly open; + − at random; − nearly closed; −− completely closed)

Time of collection	Concentration (g/L)	Movement of the leaves	
		Daytime	Night
Daytime	10	++	++
	1	++	+
	1×10^{-1}	++	+
Night	10	++	++
	1	−	+−
	1×10^{-1}	−	−−

The stereochemical relationship between H_3 and H_7 was supposed to be *anti* from the coupling constant (J=10 Hz); also, no NOE was observed between H_3 and H_7. As a glucoside of jasmonic acid, β-D-glucopyranosyl tuberonic acid (**3**), in which the stereochemical relationship between H_3 and H_7 is *syn*, was previously isolated as a potato-tuber inducing stimulus.[18] However, the ^{1}H NMR spectrum of the potassium salt of authentic **3** was slightly different from that of **1** on the signals assigned to the five-membered ring. From these results, the relative stereochemistry of **1** was determined as shown in Fig. 1.

Yoshihara et al. reported that, in the process of isolation, β-D-glucopyranosyl tuberonic acid (**3**) readily changed to another biologically inactive compound, β-D-glucopyranosyl 12-hydroxyjasmonate (**1**), through epimerization at C_7.[18] However, in our case, it is interesting that the thermodynamically stable *trans*-form is the bioactive compound.

Potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**) was effective for the leaf-closing of *A. julibrissin* at 2×10^{-5} M, and not effective for the leaf of other nyctinastic plants, such as *Phyllanthus urinaria* L., *Mimosa pudica* L., and *Aeshynomene indica* L. even at 1×10^{-4} M. Interestingly, **1** was also effective for *Albizzia lebbeck*, which belongs to the same *Albizzia* genus as *A. julibrissin*, at 4×10^{-4} M. The leaf-closing activity of **1** was one-tenth weaker for *A. lebbeck* than for *A. julibrissin*. This result showed that the chemical structure of bioactive substances controlling the plant leaf-movement would be the same or closely related among the plant genus. Then, we carried out the HPLC analysis of **1** in the methanol extract of *A. lebbeck*. We separated the methanol extract of *A. lebbeck* on the Amberlite XAD-7 column chromatography, and then each fraction was analyzed on HPLC. The identification of **1** was based on the comparison of retention time and UV spectrum obtained on the photodiode array detector with that of authentic **1**. And we can detect **1** in the 30% MeOH aq. fraction from the Amberlite column. Moreover, negative mode FAB MS analysis of the 30% MeOH aq. fraction gave the molecular ion of **1** at *m/z* 387. From this result, it was suggested that the leaf-closing substance of *A. lebbeck* would be **1**. The diversity of bioactive substance between nyctinastic plants is very interesting and presents an important problem from the viewpoint of the evolutional aspect of a plant genus. We are now working on the identification of the leaf-movement factors of *A. lebbeck* based on the bioassay using the leaf of *A. lebbeck*, and more detailed study using the plants which belong to the same *Albizzia* genus should be carried out.

The nyctinastic leaf-movement of *A. julibrissin* is strongly affected by light exposure; when the plant is put under the exposure of constant light, the leaf keeps open until late at night compared with the natural leaf. Tanada reported that delay in nyctinastic closure of *A. julibrissin* pinnules was introduced by a combined red and far-red irradiation.[19,20] Interestingly, the leaf closed with **1** opens again within a few hours on exposure to sunlight. This result suggests that some mechanism that decomposes **1** would operate in the sunlight exposure. *A. julibrissin* would have a highly organized mechanism to adjust the biorhythm of leaf-movement in accordance with the surrounding light condition. We are now trying to analyze the change of content of **1** in the leaf of *A. julibrissin* under continuous light condition.

It has already been revealed that a change in the balance of concentration between leaf-closing and -opening factors induced the leaf-movement of *L. cuneata*.[5,15] On this plant, extracts collected during the day and night exhibited opposite bioactivity to each other. The same result was obtained for the case of *A. julibrissin*, the extract collected in the daytime exhibited leaf-opening activity, and kept the leaves open even at night, while the extract collected at night exhibited weak leaf-closing activity, and kept the leaves closed even in daytime (Table 1). In the case of *L. cuneata*, the change in the balance between two leaf-movement factors was attributed to the change in content of the leaf-opening factor through a day.[15] We are now investigating an analytical condition for a quantitative analysis of the contents of **1** and **2** in *A. julibrissin*.

Experimental

General procedures

UV–VIS spectra were obtained in an aqueous solution by a Jasco UVIDEC-610A spectrophotometer at room temperature. 2D NMR, ^{1}H NMR (400 MHz), and ^{13}C NMR spectra (100 MHz) were recorded by a JEOL JNM-A400 spectrometer in D_2O, using *t*-BuOH as an internal standard [^{1}H NMR (δ 1.23) and ^{13}C NMR (δ 31.2)] at various temperatures. The positive-mode FAB-MS spectrum was measured by a JEOL JMS-700 spectrometer, using thioglycerol as a matrix. The HPLC analysis was carried out with a Jasco PU-960 pump equipped with a UV-970 detector. All solvents used for HPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 μm pore size, 47 mm. dia.) before use.

Plant materials

The used plant, *Albizzia julibrissin* Durazz was collected in the campus of Keio University, Japan, in August, 1998. The plant used for bioassay of leaf–closing was grown in the greenhouse of Keio University at 23–33°C for several weeks. *A. lebbeck* used in the bioassay was grown from seeds which were collected in Okinawa prefecture, Japan, in August 1999. The methanol extract of *A. lebbeck* was

prepared from the fresh leaves of *A. lebbeck* (8.41 kg) collected in Okinawa prefecture, Japan, in August, 1999.

HPLC

A Jasco PU-980 pump equipped with a Jasco MD-910 photodiode-array detector and using BORWIN software for data processing was employed for the quantitative analysis. HPLC experiments were carried out on a Jasco 880-PU and 875-UV system equipped with Cosmosil 5C18AR column and a Jasco 980-PU and 970-UV system, respectively, monitoring UV absorbance at 220 nm. Analyses were carried out at room temperature by using the following columns: Cosmosil 5C18AR (Nacalai Tesque Co., 5 μm particle size, 250 mm×20 mm ID), Develosil ODS HG-5 (Nomura Chemicals Co., 5 μm particle size, 250 mm×4.6 mm ID). The flow rate of the mobile phase was 4.0 mL/min for 20 mm ID columns, and 1.0 mL/min for 4.6 mm ID

The HPLC analysis of the methanol extract of *A. lebbeck* was carried out with a Jasco MD-910 photodiode array detector [column: Cadenza CD-C18 (Imtact Co., 250 mm× 4.6 mm ID), temp: 20°C, detection: 200–700 nm, mobile phase: 12% aq.CH_3CN containing 1.0% AcOH].

Bioassay

The young leaves detached from the stem of the plant *A. julibrissin* or *A. lebbeck* with a sharp razor blade were used for the bioassay. One leaf was immersed in distilled water (ca. 1.0 mL) in a 20-mL glass tube in the greenhouse and allowed to stand overnight. The leaves which open again the next morning (around 10:00 a.m.) are used for the bioassay. Each test solution (ca. 100 μL) was carefully poured into the test tubes with a microsyringe around 11:00 a.m. The leaf-opening activity was judged by leaf-opening until 9:00 p.m. On the other hand, the bioassay of the leaf-closing activity was carried out using a bell funnel in vacuo. The leaf immersed in the sample solution is put in a bell funnel, and decompressed using an aspirator. The bioactive fraction made the leaf close within a few minutes in this bioassay.

Isolation of potassium β-D-glucopyranosyl 12-hydroxy-jasmonate (1)

The leaf-closing substance was isolated from leaves collected at night (7:00 p.m.). The fresh whole plant of *A. julibrissin* (2.6 kg) was extracted with MeOH–H_2O (8:2) (22.5 L) for two weeks, filtered, and evaporated to 500 mL under reduced pressure. The aqueous residue was centrifuged at 2800 rpm for 20 min, and partitioned with *n*-hexane (200 mL×5), EtOAc (200 mL×5) and water-saturated *n*-BuOH (200 mL×5). The bioactive aqueous layer was carefully separated by Amberlite XAD–7 column chromatography (5.4×54 cm ID, Organo Co.) eluted with MeOH–H_2O (0:100, 1:9, 3:7, 5:5, and 100:0). The weakly bioactive 30% MeOH aq. fraction was further purified by Toyopearl HW-40S column chromatography (3.8×47 cm ID, Tosoh Co.) with 30% MeOH aq., and then repeated HPLC purification using a preparative Cosmosil 5C18AR column (20×250 mm ID, Nacalai Tesque Co.) with 20% MeOH aq. to give potassium β-D-glucopyranosyl 12-hydroxy-jasmonate (**1**, 1.8 mg). **1** ^{1}H NMR (400 MHz, D_2O, rt.): 5.67 (1H, td, J=7, 11 Hz, H_{10}), 5.61 (1H, td, J=7, 11 Hz, H_9), 4.47 (1H, d, J=8 Hz, $H_{1'}$), 4.02 (1H, d, J=10 Hz, H_{12a}), 3.91 (1H, dd, J=2, 12 Hz, $H_{6'a}$), 3.82 (1H, td, J=7, 10 Hz, H_{12b}), 3.72 (1H, dd, J=6, 12 Hz, $H_{6'b}$), 3.49 (1H, t, J=9 Hz, $H_{3'}$), 3.45 (1H, dd, J=2, 6 Hz, $H_{5'}$), 3.38 (1H, t, J=9 Hz, $H_{4'}$), 3.26 (1H, dd, J=8, 9 Hz, $H_{2'}$), 2.68 (1H, dd, J=5, 14 Hz, H_{2a}), 2.56 (2H, q, J=7 Hz, H_8), 2.52 (2H, q, J=7 Hz, H_{11}), 2.43–2.41 (1H, m, H_3), 2.32 (1H, td, J=9, 18 Hz, H_{4a}), 2.30 (1H, dd, J=9, 14 Hz, H_{2b}), 2.22 (1H, td, J=7, 10 Hz, H_7), 1.52 (1H, m, H_{4b}) ppm.; ^{13}C NMR (100 MHz, D_2O, 35°C): 228.7 (C_6), 184.0 (C_1), 129.43 (C_{10}), 128.5 (C_9), 103.2 ($C_{1'}$), 76.8 ($C_{3'}$), 76.7 ($C_{5'}$), 74.1 ($C_{2'}$), 70.6 ($C_{4'}$), 70.7 (C_{12}), 62.0 ($C_{6'}$), 55.0 (C_7), 39.4 (C_3), 39.0 (C_5), 28.2 (C_8), 27.7 (C_4), 25.8 (C_{11}) ppm.; HR FAB-MS (negative): $[M-K]^-$ Found m/z387.1687, $C_{18}H_{27}O_9$ requires m/z 387.1655.

HPLC analysis of the MeOH extract of *A. lebbeck*

The fresh leaves of *A. lebbeck* (8.41 kg) was extracted with MeOH (36 L) for a week. The extract was concentrated in vacuo, and the extract was partitioned with *n*-hexane (200 mL×5), ethyl acetate (200 mL×5), and *n*-butanol (200 mL×5). The aqueous layer was separated on the Amberlite XAD-7 column (5.4×54 cm ID, Organo Co.) eluted with MeOH–H_2O (0:100, 10:90, 30:70, 50:50, and 100:0). Each fractions were analyzed on a HPLC equipped with photodiode array detector. The retention time of **1** was confirmed by the comparison with authentic **1**.

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