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Leaf-opening Substance in the Nyctinastic Plant, *Albizzia julibrissin* Durazz

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***cis-p*-Coumaroylagmatine (1) was isolated from *Albizzia julibrissin* Durazz, a nyctinastic plant, as a leaf-opening substance. The compound was quite effective for opening the plant leaves at 5×10^{-6} M at night, but was not effective for other nyctinastic plants. The bioactive fraction with leaf-closing activity was also separated from the plant extract. Although the leaf-opening activity of the plant extract changed between the day and night, the content of 1 was almost constant through a 24-h day. These results suggest that the change in content of an unknown leaf-closing factor induced balance between the two leaf-movement factors through a 24-h day.**

Key words: *Albizzia julibrissin* Durazz; nyctinasty; leaf-opening substance; *cis-p*-coumaroylagmatine; biological clock

Nyctinasty is a well-known leaf-movement phenomenon which can be observed in almost all leguminosae plants.[1] This movement is controlled by a chemical substance whose concentration in a plant changes according to the circadian rhythm. Hitherto, we have isolated several chemical substances that induce the leaf-closing movement of nyctinastic plants.[2–5] These results strongly suggested that different leaf-closing substances exist in each nyctinastic plant. Furthermore, the identification of potassium lespedezate (**2**) and potassium isolespedezate (a geometrical isomer of **2**) in *Lespedeza cuneata* G. Don,[6] and of calcium 4-*O*-*β*-D-glucopyranosyl-*p*-coumarate (**3**) in *Cassia mimosoides* L. as leaf-opening substances[7] implies that the nyctinastic movement would be controlled not only by a change in the concentration of the leaf-closing factor,[8] but also by competitive interaction between leaf-closing and leaf-opening substances (Fig. 1). Nyctinastic leaf movement is known to be induced by a flow of K^+ ions into and out of the motor cell. Thus, the existence of a leaf-opening substance together with a leaf-closing substance can be reasonably understood in view of the recent finding that the K^+ ion enters and leaves a plant cell by different ion channels which are differently regulated.[9]

Nyctinasty of the silk tree, *Albizzia julibrissin* Durazz (Nemu-no-ki in Japanese), is a well-known plant movement, together with the rapid movement of *Mimosa pudica* L. We identified *cis-p*-coumaroylagmatine (**1**) as a leaf-opening factor in *A. julibrissin* by a bioassay that used a leaf of the original plant,[10] and a quantitative HPLC analysis of **1** was performed to measure the internal change in the concentration of **1** through a daily cycle.

Materials and Methods

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. A Jasco PU-980 pump equipped with an MD-910 pho-

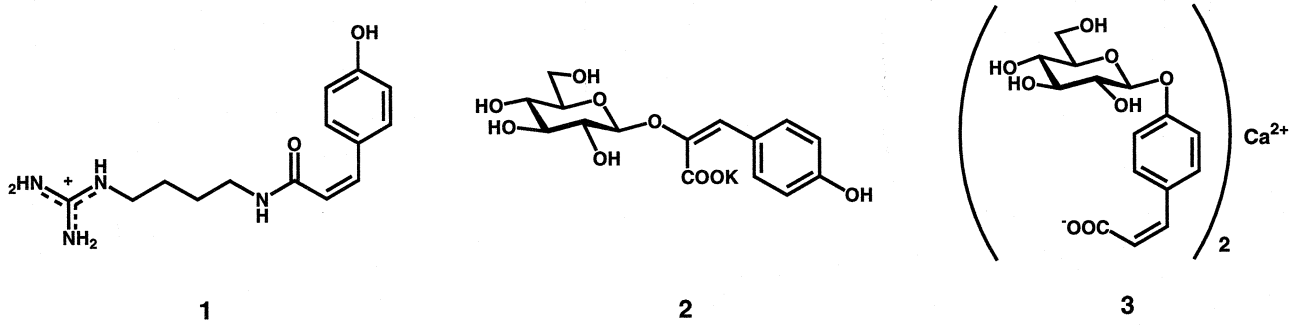

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Fig. 1. Leaf-opening Substances from Several Nyctinastic Plants.

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todiode-array detector (Jasco) and using BORWIN software for data processing was employed for the quantitative analysis. 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. A. julibrissin that was used for isolating the leaf-opening factor was collected on the campus of Keio University in August 1996. *A. julibrissin* that was used for the bioassay was grown in a greenhouse at Keio University at 25–33°C for a few weeks.

Bioassays. Young leaves separated from the stems of *A. julibrissin* plants with a sharp razor blade were used for the bioassay. Two leaves were immersed in distilled water (*ca.* 1.0 ml) in a 20-ml glass tube in a greenhouse and allowed to stand overnight. Leaves which opened again the next morning (at around 10 a.m.) were used for the bioassay. Each test solution dissolved in water was poured into test tubes by a microsyringe at around 11 a.m., and the threshold value for the leaf-opening activity was judged by leaf opening until 9:00 p.m. In the bioassay for the leaf-closing factor, the reaction time depended on the concentration of the bioactive substance, the fraction with leaf-closing activity making the leaves closed or half-closed within a few hours. All bioassays were repeated several times to check their reproducibility.

Isolation of cis-p-coumaroylagmatine (*1*) *from A. julibrissin.* Fresh leaves of *A. julibrissin* (1.8 kg) were extracted with methanol (13 l) for two weeks. The extract was filtered and evaporated to 700 ml under reduced pressure. The aqueous residue (pH 6) was centrifuged at 2800 rpm for 20 min, and partitioned with *n*-hexane (200 ml × 3), EtOAc (200 ml × 3) and water-saturated *n*-BuOH (200 ml × 3). The bioactive butanol layer was further partitioned with water. The resulting bioactive aqueous layer was evaporated to dryness under reduced pressure. The residue was chromatographed in an Amberlite XAD-7 column (ϕ 5.4 × 54 cm, Organo Co.), eluting with MeOH–H_2O (0:10, 1:9, 3:7, 5:5, and 10:0; two liters each). The bioactive 10% MeOH eluate was then chromatographed repeatedly in a Sephadex G-25 Fine column (ϕ 3.8 × 47 cm, Pharmacia Biotech Co.) with H_2O as the eluent. The bioactive fraction was separated by MPLC in a preparative Lop-ODS column (ϕ 24 × 360 mm, Nomura Chemical Co.; mobile phase, 30% MeOH aq.; flow rate, 4.0 ml·min^{-1}; detection at 260 nm; Rt=24–26 min), and then by HPLC in a preparative Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co.; mobile phase, 30% MeOH aq.; flow rate, 3.5 ml·min^{-1}; detection at 260 nm; Rt=17–18 min). The active fraction was finally purified by HPLC with a combination of three Develosil ODS HG-5 columns (ϕ 4.6 × 250 mm; mobile phase, 40% CH_3CNaq.; flow rate, 0.5 ml·min^{-1}; temperature, 40°C; detection at 260 nm; Rt=9.6 min) to give *cis-p*-coumaroylagmatine (**1**) as a colorless syrup (1.8 mg). **1**: ^{1}H-NMR (400 MHz, D_2O) δ: 7.27 (2 H, d, J=7 Hz, H_3), 6.85 (2 H, d, J=7 Hz, H_2), 6.81 (1 H, d, J=12 Hz, H_α), 5.96 (1 H, d, J=12 Hz, H_β), 3.19 (2 H, t, J=6.6 Hz, $H_{4'}$), 3.09 (2 H, t, J=7.0 Hz, $H_{1'}$), 1.48–1.45 (2 H, m, $H_{3'}$), 1.38–1.34 (2 H, m, $H_{2'}$); ^{13}C-NMR (100 MHz, D_2O, 35°C) δ: 172.5 (carbonyl), 157.6 (C_4), 157.5 (guanidine), 138.0 (C_α), 131.9 (C_3), 129.2 (C_1), 123.4 (C_β), 116.8 (C_2), 42.3 ($C_{1'}$), 40.3 ($C_{4'}$), 26.7 ($C_{2',3'}$); FAB-MS (positive) m/z: 277 (M^+); ν_{max} (film) cm^{-1}: 1651; UV λ_{max} (H_2O) nm (ε): 278 (2,000).

Purification of the leaf-closing substance from A. julibrissin. The substance was purified from leaves collected at night (7:00 p.m.) according to the procedure described for the isolation of **1**. The aqueous layer obtained after partitioning between water and water-saturated *n*-BuOH was used for the purification. After it had been concentrated *in vacuo*, the residue was applied to an Amberlite XAD-7 column (ϕ 5.4 × 54 cm, Organo Co.) and eluted with MeOH–H_2O (0:10, 1:9, 3:7, 5:5, and 10:0; two liters each). The bioactive H_2O eluate was then analyzed by HPLC in a Develosil ODS HG-5 column (ϕ 4.6 × 250 mm) with 26% MeOH aq. containing 0.1% TFA as the mobile phase (flow rate, 0.6 ml/min; detection at 270 nm) to confirm no co-existence with **1**.

Chemical synthesis of cis-p-coumaroylagmatine (*1*). A methanolic solution of *trans-p*-coumaric acid (**4**; Sigma Chemical Co., 100 mg) in an argon-flushed 10-ml flask was irradiated with UV light (Ushio Co.; 254 nm) at 4°C for 5 h. After the resulting mixture had been concentrated *in vacuo*, the residue was separated by HPLC in a preparative Cosmosil 5C18AR column (ϕ 20 × 250 mm; Nacalai Tesque Co.; mobile phase, 50% MeOH aq.; flow rate, 5.0 ml/min; detection at 260 nm) to afford **5** (32 mg, 32%).

cis-p-Coumaroylagmatine (**1**) was prepared according to the method of Negrel and Smith.[11] Synthetic **5** (1.64 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) were dissolved in pyridine (20 ml), and 2.26 g (11 mmol) of DCC was added. The reaction mixture was allowed to stand under ice-cooling for 24 hrs, and then evaporated to dryness. The residue was dissolved in EtOAc and filtered through a Hyflo super-cell (Wako Pure Chemical Industries). The filtrate was washed with sat. $NaHCO_3$ aq., and then evaporated to give *cis-p*-coumaroylsuccinimide (**6**) as a yellow powder (1.95 g, 7.47 mmol, 74.7%).

This compound (300 mg, 1.15 mmol) was then dissolved in acetone (20 ml). To this solution was added agmatine sulfate (207 mg, 0.9 mmol), and then sat. $NaHCO_3$ aq. was added to adjust the pH of this solution to 8. The resulting solution was allowed to stand at room temperature and then evaporated to dryness. The residue was purified by HPLC in a Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co., mobile phase, 30% MeOH aq.; flow rate, 5.0 ml·min^{-1}; detection at 260 nm) to give **1** (18.6 mg, 0.0655 mmol, 5.7%) as a clear syrup. All physical data completely corresponded with isolated natural **1**.

trans-p-Coumaroylagmatine (**8**) was also prepared ac-

cording to the method reported by Negrel and Smith.[11]

Extraction of plants collected during a 24-h day. Fresh leaves of *A. julibrissin* (30 g) were collected every three hours throughout the day (1:00 a.m., 4:00 a.m., 7:00 a.m., 10:00 a.m., 1:00 p.m., 4:00 p.m., 7:00 p.m., and 10:00 p.m.), and were then extracted with methanol (1 l) for one week. Each of these extracts was filtered, evaporated to 100 ml under reduced pressure, partitioned between *n*-hexane (150 ml × 3) and ethyl acetate (100 ml × 4), and evaporated to 50 ml under reduced pressure. These residues were used for a quantitative analysis of *p*-coumaroylagmatines.

Quantitative analysis of p-coumaroylagmatines. The amount of **1** in each aqueous layer was determined by an HPLC analysis using a Develosil ODS HG-5 column (ϕ 4.6 × 250 mm) with 20% MeOH aq. containing 0.1% TFA as the mobile phase (flow rate, 0.8 ml/min; detection at 270 nm). Water content of the plant material was estimated to be 80% of the total weight in a calculation of the concentration. The identification of each peak was established by its retention time and confirmed by co-injecting an authentic sample. The content of **8** was analyzed by means of the NMR spectrum of the separated fractions corresponding to **1** and **8** that had been prepared according to the isolation procedure already described.

Results and Discussion

Several compounds have been isolated as leaf-movement factors of *A. julibrissin*,[8,12] but these previous studies were based on a bioassay using the leaf of *Mimosa*. As already mentioned, however, most of the nyctinastic plants have their individual leaf-movement factors. Therefore, the previously isolated leaf-movement factors of this plant might not be valid in respect of *A. julibrissin*. We therefore needed to develop a new bioassay that used the leaf of the original plant to identify the actual leaf-movement factors. However, it is difficult for a leaf to pump the sample solution up through the vessel, because of the stiffness of the leaf stem of a grown plant (or small tree). We used the young first or second leaf of a plant which had been cultivated from seed to circumvent this difficulty. Fortunately, the soft stem of the young leaf pumped up the sample very well, and was satisfactory for the bioassay.

The bioactive substance was purified while monitoring the leaf-opening activity with a leaf of *A. julibrissin*. After partitioning with organic solvents, the residual aqueous layer showed weak leaf-closing activity, whereas strong leaf-opening activity was observed in the water-saturated *n*-butanol layer. We could separate the two leaf-movement activities by this partition. After repeated chromatography of the *n*-butanol layer, leaf-opening substance **1** (1.2 mg) was isolated as a colorless syrup. No other leaf-opening substance was observed in the water-saturated *n*-butanol layer. The leaf-closing activity was concentrated in the H_2O-eluate by Amberlite XAD-7 column chromatography, which contained no **1** by an HPLC analysis; thus, the leaf-closing substance would have been a highly polar substance. However, the activity of the bioactive fraction was still weak because of the low content or instability of the leaf-closing substance. This fraction could close leaves at 50 g/l, and its purification is now in progress.

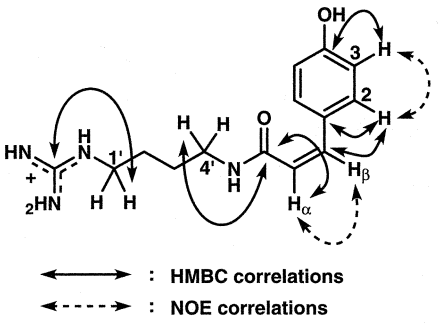
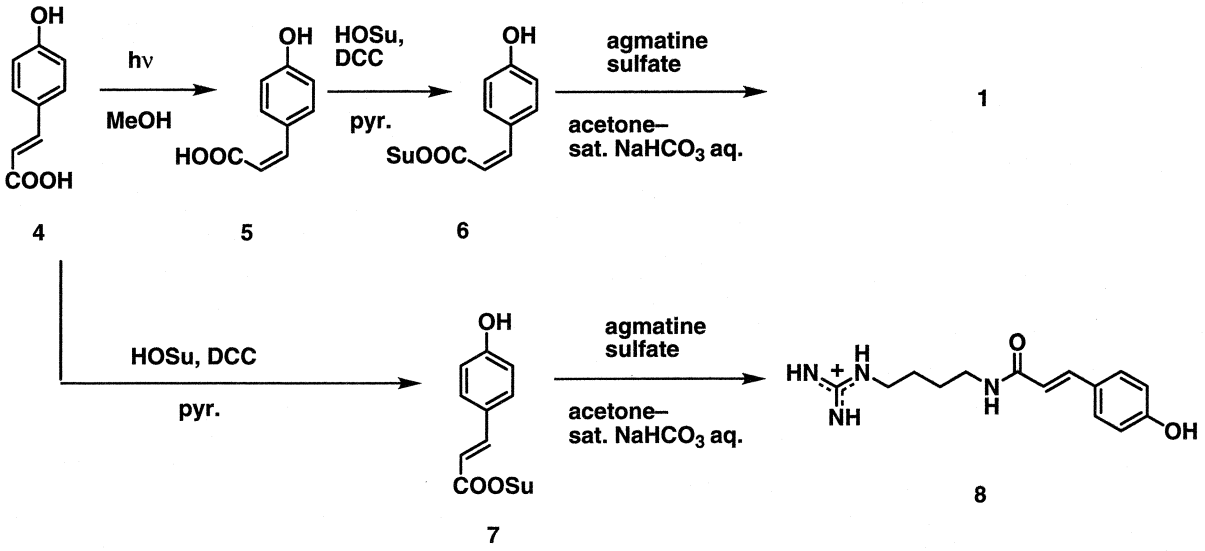


Fig. 2. Structural Determination of *cis-p*-Coumaroylagmatine.

The structural determination of **1** was carried out by means of 2D-NMR experiments (Fig. 2). Aromatic and aliphatic moieties were contained in **1** from the ^{1}H-NMR spectrum. ^{1}H–^{1}H COSY and HMQC experiments gave the connectivity related to the *p*-disubstituted benzene moiety, olefinic moiety, and $C_{1'}$–$C_{4'}$ moiety of agmatine. The structure of the aromatic moiety was determined to involve a *p*-coumaroyl group by the correlation between δ 6.85 (H-2) and δ 123.4 (C-β), δ 6.81 (H-α) and δ 172.5 (*C*-carbonyl), and δ 7.27 (H-3) and δ 157.6 (C-4) that were observed in an HMBC experiment. The existence of a guanidine function in the aliphatic moiety was presumed by the chemical shift of the very broad ^{13}C signal at 157.5 ppm, which was correlated with δ 3.09 (H-1′) in the HMBC data. The signal at 157.5 ppm was observed only as a cross peak in the HMBC experiment due to its breadth. Together with the chemical shift of H-4′ (δ 3.19), the structure of the aliphatic moiety was determined to be that of agmatine. These two partial structures were connected by the correlation between δ 3.19 (H-4′) and δ 172.5 (C-carbonyl) that was observed in the HMBC experiment to give the structure of *p*-coumaroylagmatine. Finally, the structure was confirmed by the molecular ion observed at *m/z* 277 in the FAB-MS experiment. From the coupling constant (J=12 Hz) and observed NOE correlation between the olefinic protons, the configuration of the olefinic proton was determined to be *cis*. Thus, the structure was determined to be *cis-p*-coumaroylagmatine (**1**). The *trans*-isomer (**8**) had been reported to have been isolated from nature,[13] but the *cis*-isomer has not been previously reported. It is interesting that *A. julibrissin* contained the thermodynamically unstable *cis*-isomer solely, with no *trans*-isomer present. Although synthetic **8**, which is depicted later on, had almost the same retention time as that of **1**, no *E*-isomer was isolated from the fraction containing **1**.

We synthesized **1** and its *trans*-isomer (**8**) to confirm the structure and investigate the structure-activity relationship. *cis-p*-Coumaroylagmatine (**1**) was synthesized from agmatine and *cis-p*-coumaric acid (**5**) (Scheme 1) which had been prepared by photoisomeriza-



Scheme 1. Chemical Synthesis of *p*-Coumaroylagmatines.

tion of the commercially available *trans*-isomer (**4**). This was converted to the hydroxysuccineimide ester (**6**), and then coupled with agmatine by using DCC to give **1**. Synthetic **1** had absolutely the same physical properties as those of natural **1**, including ^{1}H- and ^{13}C-NMR spectra.

cis-p-Coumaroylagmatine (**1**) was quite effective for the leaf-opening of *A. julibrissin* at 5×10^{-6} M at night, but was not effective for other nyctinastic plants such as *Aeschynomene indica, Phyllanthus urinaria* L. and *Mimosa pudica* L., even at 1×10^{-2} M. Synthetic **1** was as effective as isolated natural **1**, but synthetic **8** was effective only at 1×10^{-3} M toward the leaf of *A. julibrissin*. Thus, the *cis*-configuration of **1** is presumed to be very important for the leaf-opening activity.

Indole-3-acetic acid (IAA) has also been reported to influence the leaf-opening of *Mimosa*.[14] The leaf-opening activity of **1** was much stronger than that of IAA which was also effective for the leaf-opening of *A. julibrissin* at 5×10^{-4} M. Interestingly, **1** was also effective on the leaf of *C. mimosoides* L. at a much higher concentration of 1×10^{-2} M. The leaf-opening substance of *C. mimosoides* L, **4**,[7] also has a *cis-p*-coumaroyl moiety, so this structure would be important for the leaf-opening activity of **1**. However, the leaf-opening activity of potassium *cis-p*-coumarate was very weak for the leaf of these plants; thus, such attached highly polar moieties as β-glucoside and the *N*-acylagmatine moiety are presumed to serve as some carrier for the *p*-coumaroyl moiety.

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] 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 during the daytime

Table 1. Bioactivity of the MeOH Extracts Collected during the Daytime and at Night

Time of collection	Concentration [g·l^{-1}]	Movement of the Leaves	
		Daytime	Night
Daytime	10	++	++
	1	++	+
	1×10^{-1}	++	+
Night	10	++	++
	1	−	+−
	1×10^{-1}	−	−−

++ completely open; + nearly open; +− at random; − nearly closed; −− completely closed.

Table 2. Quantitative HPLC Analysis of the Concentration of **1** in *A. julibrissin* Collected through a Day

Time of collection	Concentration of **1** [mol·l^{-1}]
1:00 AM	8.7×10^{-4}
4:00 AM	10.2×10^{-4}
7:00 AM	11.0×10^{-4}
10:00 AM	11.8×10^{-4}
1:00 PM	9.4×10^{-4}
4:00 PM	10.9×10^{-4}
7:00 PM	11.6×10^{-4}
10:00 PM	9.6×10^{-4}

Analytical conditions: mobile phase, 26% MeOH aq. containing 0.1% TFA; flow rate, 0.6 mL/min; detection, 270 nm.

(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] Thus, we carried out a quantitative analysis of **1** throughout a 24-h day, using the plant extracts prepared every three hours during a day, with HPLC equipped with a photodiode array detector (Fig. 3). The result shows that the content of **1** was almost constant throughout a day (Table 2).[16] Even the extract

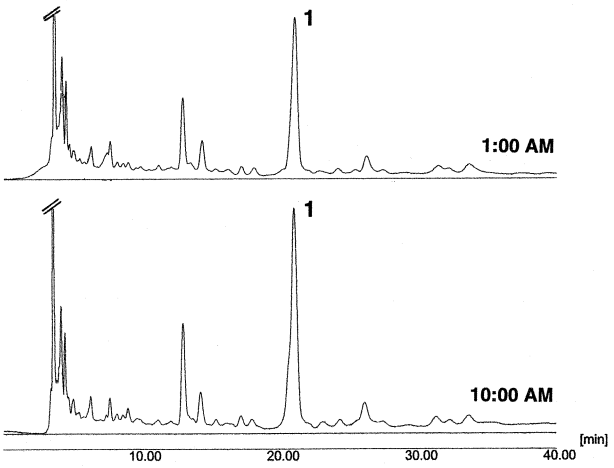


Fig. 3. Chromatogram of the extract containing **1** that had been prepared from the plant collected at 10:00 a.m. and 1:00 a.m., respectively.
Column: Develosil ODS HG-5 ϕ 4.6 × 250 mm; mobile phase: 26% MeOH aq.-0.1% TFA; flow rate: 0.6 ml/min; detection: 270 nm.

collected at 10:00 a.m. contained about 1.35-fold as much **1** as the one collected at 1:00 a.m, whereas, the leaves kept open from 4:00 a.m. until 6:00 p.m. were closed during the rest of the day. Thus, the difference in bioactivity between the two plant extracts seems to have been attributable to the difference in the content of the leaf-closing factor. We are further working on the isolation of the leaf-closing substance in *A. julibrissin* to carry out its quantitative analysis.

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16) There is another possibility that **1** was deactivated into **8** at night, because **1** and **8** were not separable by an HPLC analysis. NMR measurements of the HPLC peak corresponding to **1** disclosed that no **8** was contained in either of the extracts collected during the daytime and at night.