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The Chemistry of Leaf-movement in *Mimosa pudica* L.

Minoru Ueda and Shosuke Yamamura*

Department of Chemistry, Faculty of Science and Technology,

Keio University, Hiyoshi, Yokohama 223, Japan.

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Abstract : Two well-known movements are observed in *Mimosa pudica* L. (Ojigi–so in Japanese): one is the very rapid movement of the leaves when it is stimulated by touch, heating, etc., and the other is the very slow, periodical movement of the leaves called nyctinastic movement which is controlled by a biological clock. We have isolated chemical substances controlling these two leaf-movements. These movements were regulated by the different chemical substances. We have succeeded in the identification of the chemical substances related to the two different kinds of mimosa leaf-movement, which have been discussed for over 80 years. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: plants; natural products; biologically active compounds.

INTRODUCTION

The sensitive plant, *Mimosa pudica* L. (Ojigi–so in Japanese) is known by the very rapid movement of the leaves when it is stimulated by touch, heating, etc. This remarkable movement has been known from the pre-Christian era. The first record on this leaf-movement of mimosa goes back to the era of Alexander the Great. Also, *Mimosa* shows very slow, periodical movement of the leaves called nyctinastic movement which is controlled by a biological clock: the leaves open in the daytime and close at night. Historically, the nyctinastic movement of mimosa was the beginning for the discovery of a biological clock. As early as the 18th century, a French scientist discovered that nyctinastic movement continued even in complete darkness.[1] This inexplicable phenomenon prompted both biologists and chemists to make great efforts to understand the mechanism of the thigmonastic and nyctinastic movements of this plant. For example, C. Darwin published voluminous records on observations of plant leaf-movement, including that of mimosa.[2]

Generally, leaf-movement could be explained by the movement of water into or out of motor cells, which is driven by fluxes of ions, especially K^+ ions.[3] One of the most interesting problems is how the stimulus is transported from the stimulated point to other parts of the plant. From the viewpoint of electrophysiology, different rates of stimulus conduction were observed by Houwick[4] and Shibaoka[5] in the rapid movement of mimosa. Regardless of the mode of action, some stimulants may interact directly or indirectly with a motor cell. On the other hand, with regard to the nyctinastic movement, the signals by which a biological clock controls the rhythms, such as the K^+ channel state, have long remained unknown.

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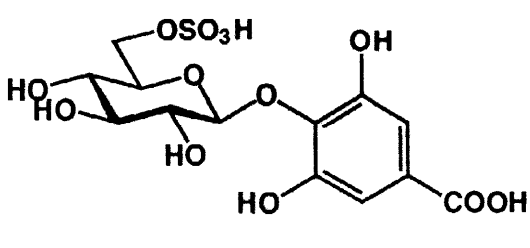
In 1916, Ricca strongly suggested that some stimulants related to the rapid leaf-movement are present in the *Mimosa* plant.[6] Since then, many scientists have attempted to isolate the true excitatory substance of *M. pudica*, and a wide variety of bioactive compounds have been isolated from the *Mimosa* plant.[7-10]

Especially, in 1983, Schildknecht reported on the successful isolation and structural determination of four leaf movement factors from *M. pudica*, *Acacia karroo*, *Oxalis strica* and others after extensive efforts in the search for the true leaf-movement factors.[11] Through these experiments, finally, he proposed that the leaf-movement factors, collectively known as turgorins, are a new class of phytohormones that control both thigmonastic and nyctinastic movements. However, in 1984, Umrath *et al.* questioned whether K-PLMF 1 (**1**), a representative turgorin, was the true excitatory substance in *M. pudica*.[12] Additionally, they obtained two bioactive fractions (substances E and G)[12] from *M. pudica* by extraction with boiling water followed by purification with Sephadex LH 20. The percentage activities were 80% for E and 20% for G, of which the main leaf-closing substance E has been assigned to be the excitatory substance in *M. pudica*. And β-glucosidase was not effective for the former, while substance G lost its activity on the treatment with β-glucosidase. In addition, it should be noted that substance E completely lost its leaf-closing activity on further purification. They reported that substance E would be an unstable aliphatic oxyacid which lost its bioactivity on further purification. Therefore, it was apparently almost impossible to isolate such an unstable substance from *M. pudica*.

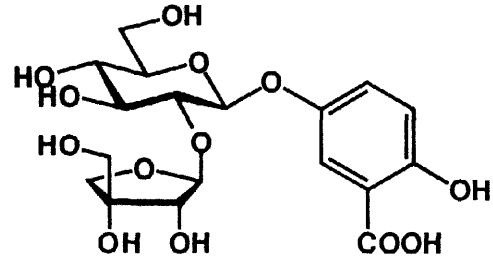
On the other hand, the other leaf-movement of mimosa, a slow nyctinastic movement, had been practically neglected so far, because this movement was thought to be caused by the same chemical substance as that in the rapid movement.[11] For this reason, the two leaf–movements of mimosa have often been confused.

This paper discloses the true character of the leaf-movement factors of mimosa and the first evidence to demonstrate that the rapid and slow leaf-movements of mimosa are caused by different chemical substances, respectively. We were able to find a solution to this seemingly insurmountable problem, which had been attempted since the last century, as follows.

First of all, the assignment of K-PLMF 1 (**1**) with a free sulfate function as a leaf-movement factor seems to be questionable because Schildknecht isolated **1** and other turgorins under acidic conditions.[11,13]



K-PLMF1 (1)



M-LMF1 (2)

In fact, it has not been isolated as a free acid, but as a potassium salt from *M. pudica*, *Cassia mimosoides* and others in our careful isolation experiments without using acids or salts. The potassium salt also effected leaf-closing of *M. pudica*; however, its bioactivity was very weak (approximately 10^{-1} g/L), and its leaf-closing activity was observed only against *M. pudica*.[14] As the leaves are very sensitive to H^+ ions (the leaves close on addition of dilute sulfuric acid (10^{-2} g/L)) and salts, it is assumed that the leaf-closing activity of **1** would be attributed to the free sulfuric acid group. These results showed that **1** would not be a genuine leaf-movement factor of mimosa, and is not a common phytohormone regulating the leaf-movement.[14] Thus, we reexamined the purification of the excitatory substance in *M. pudica*.

RESULTS AND DISCUSSION

The purification of the excitatory substance in *M. pudica* was carefully carried out based on our bioassay, which is similar to the Fitting-Hess-Schildknecht test[11] using the leaves of mimosa. The bioactive fraction induced a rapid leaf-closure. The result of the bioassay was greatly influenced by the environment such as temperature, humidity, and sunshine. Therefore, the bioassay showed good reproducibility only in a greenhouse in midsummer. All the separation procedure must be carried out under neutral conditions without acids or salts. The fresh leaves of *M. pudica* (48.8 kg) were immersed in methanol (234 L) for two weeks and concentrated *in vacuo*. The concentrated aqueous extract was partitioned with *n*–hexane, ethyl acetate, then with *n*–butanol. The aqueous layer showed strong leaf-closing activity. The isolation procedure is shown in **Figure 1**, where each separated fraction completely lost its leaf-closing activity at the 4th purification step after partition using HPLC, as reported by Umrath *et al.*[12] However, we have discovered that the leaf-closing activity recovered by combining the separated fractions. The eluent from Cosmosil column with 50% MeOH aq. was necessary for the recovery of the leaf-closing activity, thus, we searched for the chemical substance that shows bioactivity by combinig with the eluent. The devided fractions were monitored by a combination of the *Mimosa* test and ^{1}H NMR spectroscopy, leading to the finding that three fractions related to the rapid movement of *M. pudica*. Thus, the true excitatory substance consists of three different components: 1) potassium L-malate (**3**), 2) magnesium *trans*-aconitate (**4**), and 3) dimethylammonium salt (**5**) (Figure 1). The metal ion analyses were carried out by XPS (X-ray photo-electronic spectroscopy).

Mimosa leaves closed very rapidly on addition of 10^{-8} - 10^{-9} M of this mixture, all components of which are required for the rapid movement of *M. pudica*. In addition, metal ions are also important for the activity. When calcium salt were used instead of magnesium salt, the resulting mixture of the three components did not show any leaf-closing activity. Compounds **3** and **4**, which were prepared from commercially available L-malic acid and trans-aconitic acid respectively, also showed bioactivity by combining with **5**.

In conclusion, the components shown in **Figure 1** comprise the true excitatory substances related to the rapid movement of *M. pudica*, the activity of which disappears on further purification in accordance with the result obtained by Umrath *et al.*[12] Probably **3** would be a true character of Umrath's "aliphatic oxyacid".

The stomatal movement is also introduced by the same mechanism as the leaf-movement, that is, the movement of water into or out of stomatal cells, which is driven by fluxes of K^+ ions. In this case, malate anion is also reported to serve as a counter anion of K^+ ions.[15]

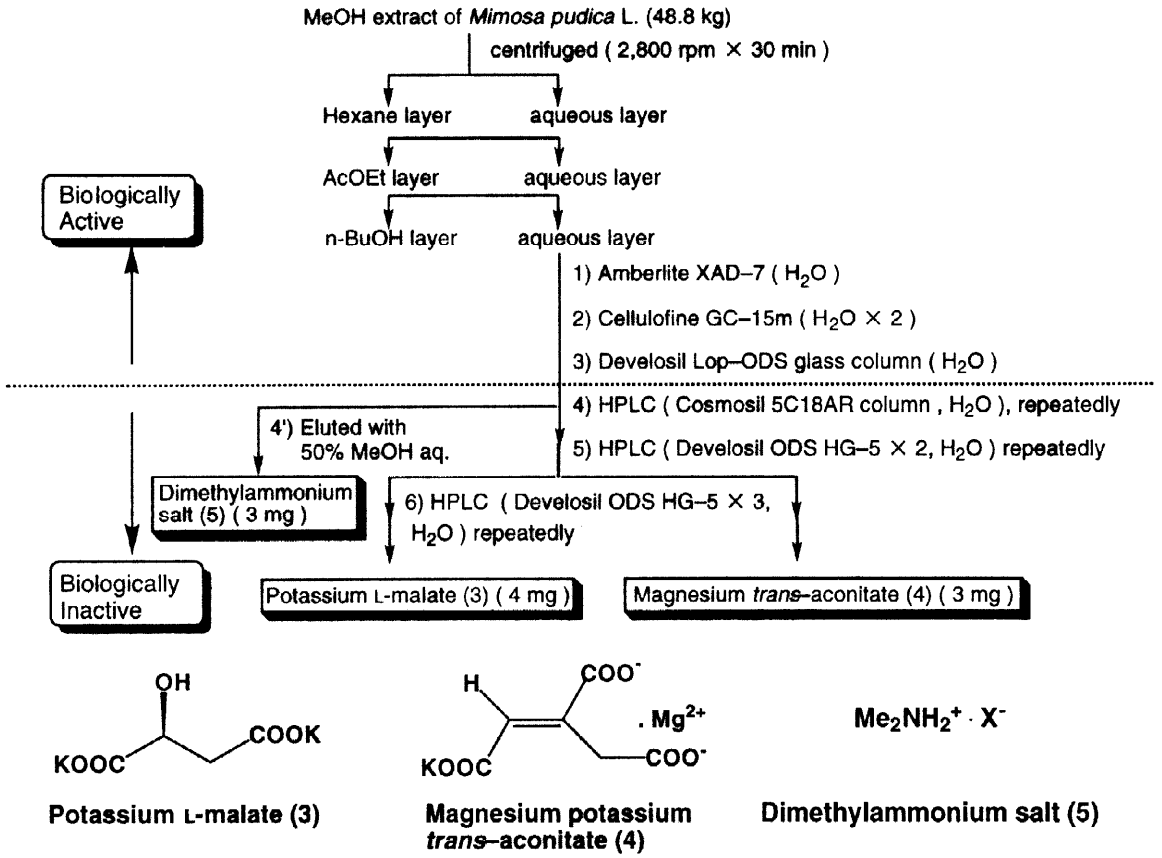
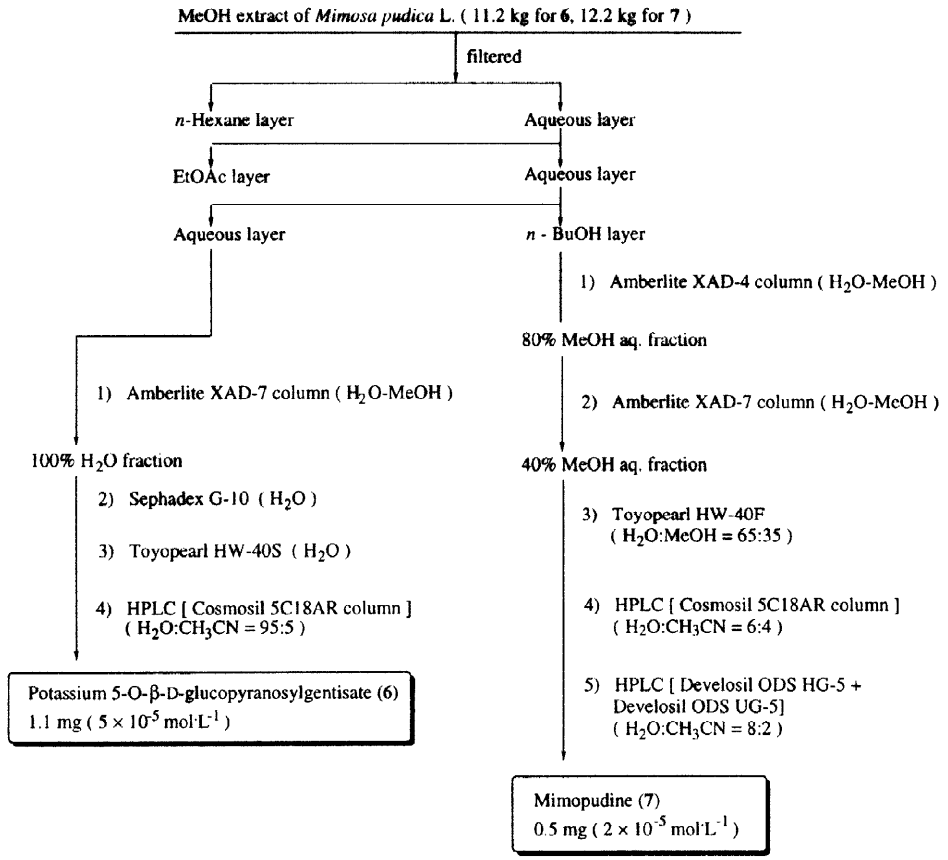


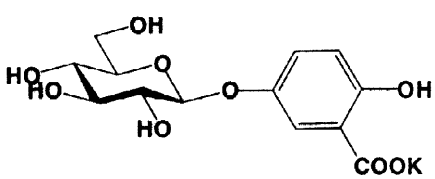
Figure 1. The isolation procedure of the excitatory substances of *Mimosa pudica L.*

Interestingly, these compounds induced only the rapid movement of the leaf, on the other hand, no slow leaf-movement was observed even at the threshold-level concentration. Therefore, we assumed that the bioactive substance regulating the nyctinastic leaf-movement would be also contained in mimosa.

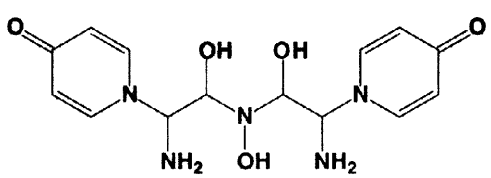
We tried to isolate the leaf-closing substance of mimosa, which induces the slow leaf-closure of the mimosa leaf. Together with the leaf-closing substance, the leaf-opening substance was assumed from our previous studies to co-exist in *M. pudica*.[14] The most important problem in the isolation of the leaf–closing and -opening substances from *M. pudica* was the difficulty of bioassay. Because of its high sensitivity, the mimosa leaf folded easily in gentle wind through the long period needed for bioassay. Thus, we used a mimosa leaf cut at the lamina instead of a four-pinna leaf and carried out the bioassay in a glass tube to overcome this difficulty. This method was inferior in sensitivity to the previous method using a four-pinna leaf; however, its reproducibility was satisfactory. The leaf-opening activity was judged by keeping the leaf open at 9:00 p.m.; on the other hand, the leaf-closing activity was judged by the leaf-closure within two hours after the addition of the sample.

The fresh leaves of *M. pudica* (11.2 kg) were immersed in methanol (60 L) for two weeks and concentrated *in vacuo*. The concentrated aqueous extract was partitioned with organic solvents, then the aqueous layer showed strong leaf-closing activity. The aqueous layer was separated efficiently by using the Amberlite XAD-7 column chromatography. Our isolation procedures are shown in **Figure 2**.





Potassium 5-*O*-β-D-glucopyranosylgentisate (6)



Mimopudine (7)

Figure 2. The isolation procedures of leaf-closing and -opening substances of *M. pudica*. L.

After the separation with XAD-7, fractions with strong leaf-closing activity were obtained. With regard to the leaf-closing activity, only the thigmonastic activity, which induces a rapid leaf-closure, was observed. We assumed that the nyctinastic activity, which induces a slow leaf-closure, could not be monitored due to the interference of strong bioactivity attributed to the previously mentioned excitatory substances. It was crucial to separate these two bioactivities for the detection of nyctinastic leaf-closing activity. Based on the report by Umrath,[12] we examined the application of gel filtration chromatography for the separation of the excitatory and

leaf-closing substances. With Sephadex G-10 and Cellulofine GC-15m, sufficient separation could not be achieved. However, the Toyopearl HW-40S with H_2O showed good separation of these bioactive substances, and the bioactive fraction inducing slow leaf-movement was eluted after the fraction inducing rapid movement. We thought that the excitatory substances correspond to the Umrath's substance E, and the leaf-closing substance is the Umrath's substance G. Further purification using HPLC with Cosmosil 5C18AR gave **6** (1.1 mg) as a yellow syrup.[16]

On the other hand, the isolation of the leaf-opening substance was carried out as shown in **Figure 2**. The fresh leaves of *M. pudica* (12.2 kg) were immersed in methanol (ca. 58 L) for two weeks and concentrated *in vacuo*. The aqueous layer showed strong leaf-closing activity because of the excitatory substances that cause rapid leaf–movement of mimosa. It was supposed that this strong leaf-closing activity masked the bioactivity of the leaf-opening substance. Thus, the *n*–butanol layer, which is supposed to contain no excitatory substances, was carefully separated by Amberlite XAD–4 column chromatography eluted with MeOH–H_2O, and the 80% MeOH eluate showed weak leaf–opening activity. Then, the 80% MeOH aq. eluate was carefully separated by Amberlite XAD–7 column chromatography eluted with MeOH–H_2O, and both the 30% and 40% MeOH aq. eluate showed weak leaf–opening activities. The fraction with strong leaf–closing activity, which was eluted with H_2O, was completely separated from the fraction with leaf–opening activity. The bioactive 30% MeOH aq. fraction was further purified by Toyopearl HW-40 F column chromatography with 35% MeOH aq., HPLC using preparative Develosil ODS HG–5 column with 40% MeOH aq., and HPLC using analytical column (CAPCELL PAK UG80) with 40% MeOH aq. to give L-Tryptophan (6.1 mg) as a colorless powder. On the other hand, the other bioactive fraction, 40% MeOH eluate, was purified by Toyopearl HW-40 F column chromatography with 35% MeOH aq., HPLC using preparative Cosmosil 5C18AR column with 40% CH_3CN aq., and HPLC using a combination of two analytical columns (Develosil HG-5 and Develosil UG-5) with 20% CH_3CN aq. to give mimopudine (**7**, 0.5 mg) as a white powder.[17]

Structure determination of **6** and **7** was carried out by means of NMR and ESI MS experiments. The structure of the leaf-closing substance was determined to be potassium 5-*O*-β-D-glucopyranosylgentisate (**6**) from the ^1H NMR spectrum and negative mode ESI MS analysis. The NOE spectrum gave a strong correlation between the anomeric proton ($H_{1'}$) and H_6; thus, the hydroxyl group at the C_5 position was glycosilated (**Figure 3**). The chemical shifts of **6** corresponded with the previous report.[18] The structure of **6** was similar to that of Schildknecht's M-LMF 1 (**2**) which was reported not to be bioactive for the rapid leaf-closing of mimosa.[11]

The structure of the leaf-opening substance was determined as follows[17]: Positive ESI MS experiment gave the ions corresponding to $[M+Na]^+$, $[M+H]^+$, $[M+2Na]^{2+}$, $[M+2H]^{2+}$. HR ESI MS experiment was carried out against the peak of $[M+H]^+$ at *m/z* 338.1434 to give the formula of $C_{14}H_{19}O_5N_5$. As the ^1H NMR spectrum of **7** was very simple, **7** was concluded to have a symmetric dimer structure. FG–HMQC, FG–HMBC experiments gave the structure of **7**. Weak coupling between $H_{1'}$ and $H_{2'}$ was observed in the HOHAHA experiment. **Figure 4** contains important correlations observed in the HMBC and HOHAHA experiments.

The chemical shifts of the $C_{2'}$ (90.5 ppm) and $H_{2'}$ (5.30 ppm) positions suggested that this carbon connects to both oxygen and nitrogen. An ESI linked-scan experiment for the strong peak of $[M+Na]^+$ ion gave the fragment ions shown in **Figure 4**.

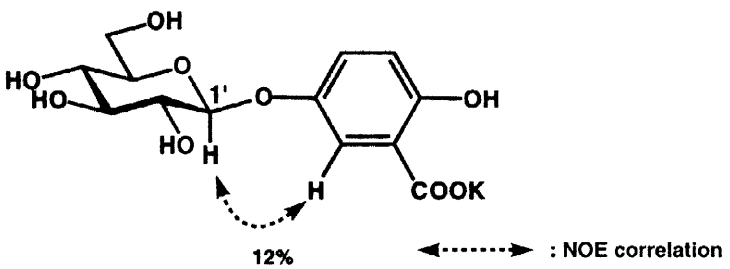


Figure 3. The NOE experiment in the 5-*O*-β-D-glucopyranosylgentisate (**6**)

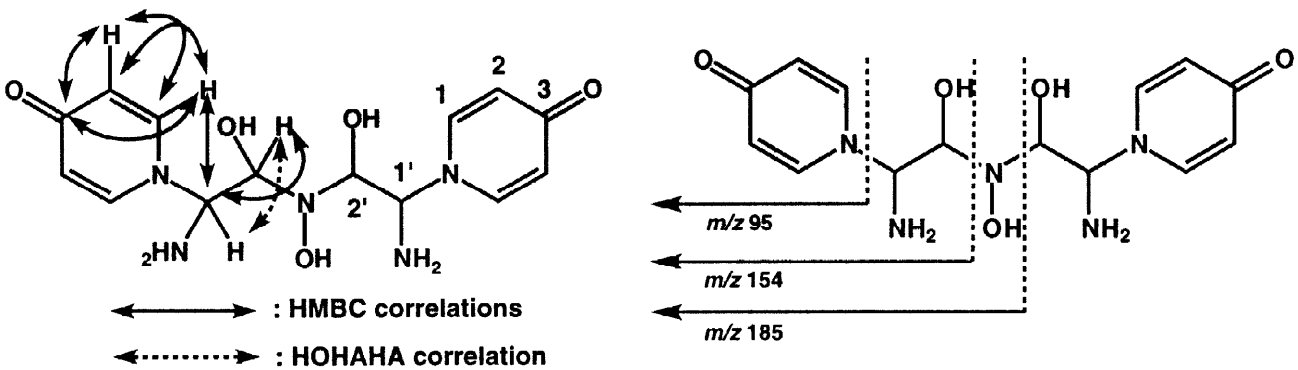


Figure 4. The structure determination of mimopudine (**7**) by 2D NMR (left) and ESI linked-scan analysis (right)

Compound **7** was unstable in aqueous solution, and was easily decomposed in D_2O during an over-weekend NMR experiment to give pyridone. The low availability of **7** from *M. pudica* would be due to this unstability in aqueous media.

Compound **6** was quite effective for leaf–closing of *M. pudica*. at 5×10^{-5} M in the daytime, and **7** was effective for the leaf-opening of the same plant at 2×10^{-5} M at night; however, both compounds were not effective for other nyctinastic plants, *Aeschynomene indica*L., *Phyllanthus urinaria* L., *Cassia mimosoides* L. and *Albizzia julibrissin* Durazz. even at 1×10^{-4} M. Interestingly, all the leaf-closing and –opening substances of other plants were not effective for the leaves of mimosa even at 1×10^{-4} M. Therefore, **6** and **7** are specific leaf-movement factors of *M. pudica*.

On the other hand, L-Trp, isolated as a leaf-opening substance together with **7**, was also effective at as low as 5×10^{-4} M on the leaves of mimosa and all other nyctinastic plants as observed in indole-3-acetic acid (IAA).[19] It is proposed that the bioactivity of L-Trp is attributed to IAA, which is an important metabolite of L-Trp.[20] The leaf-opening activity of indole-3-acetic acid (IAA) has been studied by many groups. However, IAA showed much weaker bioactivity than our leaf-opening substance. IAA caused the leaf-opening of *M. pudica* at night at concentrations ranging from 3×10^{-3} to 5×10^{-4} M.[21] The long period needed for the

bioassay of L-Trp to detect the leaf-opening activity is sufficient for the metabolism of L-Trp into IAA. All leaf-movement factors previously isolated by us showed specific bioactivity on each plant species;[22-29] thus, the genuine leaf-opening substance of mimosa should be **7**.

Interestingly, the leaves of *M. pudica* kept open with **7** at night were sensitive to physical stimulus by touch, as observed in the daytime. This result suggests that **7** is effective only for the obstruction of the slow nyctinastic leaf–closing movement, but not effective for the rapid thigmonastic movement. It is also important that **6** induced only the slow leaf-closure of the mimosa leaf. Now, we have been able to separate these two leaf-movements at the molecular level, and demonstrate that the slow nyctinastic movement of mimosa has a different mechanism from that of the rapid thigmonastic one.

In a previous paper,[30] we advanced a common mechanism for the regulation of nyctinastic leaf-movement: either the leaf-closing or -opening substance is a glycoside, and the glycoside is deactivated by a β-glucosidase whose activity is controlled by a biological clock. It is worthy of notice that all nyctinastic plants whose leaf-movement factors we have isolated so far, have the glucoside-type leaf-closing or -opening substance (**Figure 5**).

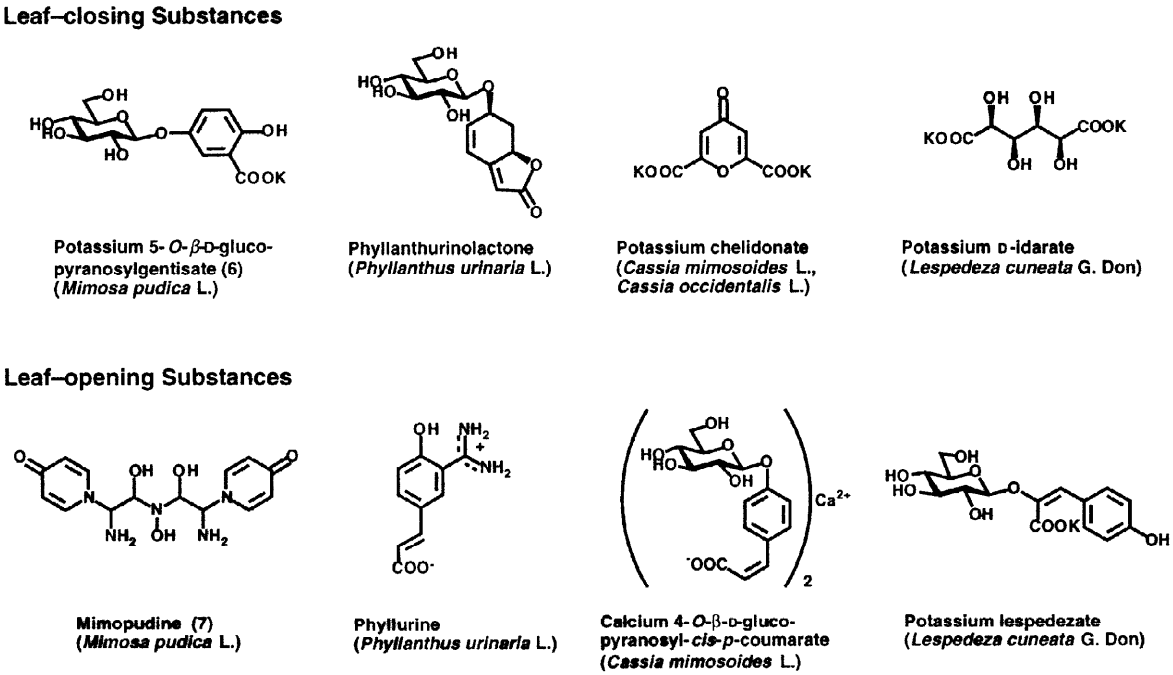


Figure 5. The glycoside-type leaf-movement factors commonly exist in all nyctinastic plants.

Our common mechanism is strongly supported by the isolation of **6**. There would exist two types of plants; one has a glycoside-type leaf-opening substance which is deactivated by β-glucosidase activated in the evening: the other has a glycoside-type leaf-closing substance which is deactivated by β-glucosidase activated in the morning. *M. pudica* belongs to the latter type. As potassium gentisate (**8**), an agycon of **6**, was not effective even at 1×10^{-2} M for mimosa leaves, it is assumed that **6** would convert into the corresponding biologically inactive aglycon (**8**) with β-glucosidase in the morning, similar to the case of *P. urinaria*.[30] The

balance between **6** and **7** would change through a day, as in the case of *Lespedeza cuneata*[31, 32] and *Phyllanthus urinaria*.[30]

From these results, we have shown that the genuine excitatory substance of *M. pudica*, which was reported 80 years ago by Ricca, is a mixture of three different compounds. This result would be important with respect to the discovery of the bioactive substance which exerts its bioactivity through cooperation of the plural components. Also, we have shown that the nyctinastic leaf-movement is induced by other chemical signals. These two leaf-movements are regulated by different chemical substances and would be regulated by different mechanisms. The nyctinastic leaf-movement is assumed to be controlled by the competitive interaction between leaf–closing and -opening substances.[30, 32] We have succeeded in the identification of the chemical substances related to two leaf-movements of mimosa, which have been discussed for over 80 years. Our results would give the molecular level basis to the plant science of leaf-movement.

MATERIALS AND METHODS

General Procedures. 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 ESI MS spectra were measured by a Jeol JMS-700 spectrometer, using methanol as a mobile phase. The spectrometer was operated at 5 kV (positive mode) or -5 kV (negative mode) acceralation voltage. The HPLC analyses were 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. Potassium gentisate (**8**) was prepared from gentisic acid purchased from Aldrich Chem. Co. Metal ion analyses were carried out by PHI ESCA-5600 using Mg-kα ray (1253.6 eV) operated at 300 W.

Plant Materials. Young plants of *M. pudica* were purchased from Yoneyama Plantation Co. (Yokohama, Japan), and cultured in our plantation at Keio University in 1996. The grown *M. pudica* was collected in September 1996, and used for the extraction. *M. pudica* that was used for the bioassay was grown in a greenhouse at Keio University at 25 - 33 °C.

Bioassay. The young leaves detached from the stem of the plant *M. pudica* with a sharp razor blade were used for the bioassay.

For the detection of rapid movement, a four-pinnae leaf was detached from the stem with a sharp razor blade and placed in H_2O (*ca* 1.0 mL), using a 1.5–mL plastic tube, in the greenhouse at over 30 °C. The leaf which recovered from the cutting injury after a while and opened again was used for the bioassay. Each test solution was carefully poured into the test tubes with a microsyringe around 11:00 a.m. The bioactive fraction was judged by the leaf–closure within 20 min. This bioassay was reproducible only in midsummer.

For the detection of slow movement, a young mimosa leaf detached from a four-pinnae leaf with a sharp razor blade was used for the bioassay. One leaf was placed in H_2O (*ca* 1.0 mL), using a 5–mL glass tube, in

the greenhouse at 30 °C and allowed to stand overnight. The leaves which opened again the next morning (around 10:00 a.m.) were used for the bioassay. Each test solution was carefully poured into the test tubes with a microsyringe around 11:00 a.m. The bioactive fraction was judged by the leaf–opening up to 8:00 p.m. and leaf-closure within two hours after the addition of the sample.

Isolation of the components of the stimulant in Mimosa pudica L. The fresh whole plant of *M. pudica* (48.8 kg) was extracted with methanol (234 L) for two weeks and concentrated *in vacuo*. Purification of the bioactive substance was carried out with monitoring the rapid leaf–closing activity for the leaf of *M. pudica*. The concentrated extract was partitioned with *n*-hexane (500 mL × 12), ethyl acetate (500 mL × 10), then with *n*–butanol (500 mL × 10). The bioactive aqueous layer was carefully separated by Amberlite XAD–7 column chromatography (ϕ 55 × 500 mm, Organo Co.) eluted with MeOH–H_2O (0 : 10, 1 : 9, 2 : 8, 3 : 7, and 10 : 0 × 1 L each), repeatedly. The bioactive H_2O eluate was further purified by using Cellulofine GC-15m column chromatography (ϕ 55 × 400 mm, Seikagaku Kogyo Co.) with H_2O. The bioactive fraction with slow leaf-closing activity was further purified with HPLC equipped with Cosmosil 5C18Ar (ϕ 20 × 250 mm, Nakalai Tesque Co.) with H_2O (flow rate: 4.0 mL/min, detection: 220 nm), and then HPLC using a combination of two analytical Develosil HG-5 columns (ϕ 4.6 × 250 mm, Nomura Chemical Co.) with H_2O (flow rate: 0.8 mL/min, detection: 220 nm),. The final purification by HPLC using a combination of three analytical Develosil HG-5 columns (ϕ 4.6 × 250 mm, Nomura Chemical Co.) with H_2O (flow rate: 0.45 mL/min, detection: 220 nm), repeatedly gave **3** (4 mg), **4** (3 mg). Dimethylammonium salt (**5**) was contained in the eluent from Cosmosil 5C18Ar column (ϕ 20 × 250 mm, Nakalai Tesque Co.) with 50% MeOH aq. Compound 5 was isolated from the eluent by using HPLC using analytical Develosil HG-5 columns (ϕ 4.6 × 250 mm, Nomura Chemical Co.) with 50% MeOH aq. (flow rate: 1.0 mL/min, detection: 220 nm). Potassium L-malate (**3**) : ^{1}H NMR (400 MHz, D_2O) δ 4.38 (1H, dd, J = 4.0 and 8.0 Hz), 2.80 (1H, dd, J = 4.0 and 16 Hz), 2.65 (1H, dd, J = 8.0 and 16 Hz). ^{13}C NMR (100 MHz, D_2O, 35 °C) δ 181.7, 180.4, 43.3.; magnesium *trans*-aconitate (**4**) : ^{1}H NMR (400 MHz, D_2O) δ 6.60 (1H, br.s), 3.40 (2H, br.s). ^{13}C NMR (100 MHz, D_2O, 35 °C) δ 181.4, 177.7, 177.4, 140.2, 132.7, 39.0.; dimethylammonium salt (**5**) : ^{1}H NMR (400 MHz, D_2O) δ 2.70 (6H, s). ^{13}C NMR (100 MHz, D_2O, 35 °C) δ 32.1.

*Isolation of potassium 5-O-β-D-glucopyranosylgentisate (**6**).* The fresh whole plant of *M. pudica* (11.2 kg) was extracted with methanol (60 L) for two weeks and concentrated *in vacuo*. Purification of the bioactive substance was carried out with monitoring the leaf–closing activity for the leaf of *M. pudica*. The concentrated extract was partitioned with ethyl acetate (200 mL × 5), then with *n*–butanol (200 mL × 8). The bioactive aqueous layer was carefully separated by Amberlite XAD–7 column chromatography (ϕ 55 × 500 mm) eluted with MeOH–H_2O (0 : 10, 1 : 9, 2 : 8, 3 : 7, and 10 :0 × 1 L each). The bioactive H_2O eluate was further purified by using Sephadex G-10 column chromatography (ϕ 55 × 320 mm, Pharmacia Co.) with H_2O, then Toyopearl HW-40S (ϕ 30 × 290 mm, Tosoh Co.) with H_2O. The bioactive fraction with slow leaf-closing activity was further purified by HPLC using Cosmosil 5C18Ar (ϕ 20 × 250 mm) with 5% CH_3CNaq. (flow

rate: 4.0 mL/min, detection: 260 nm) to give potassium 5-*O*-β-D-glucopyranosylgentisate (**6**) (1.1 mg). Potassium 5-*O*-β-D-glucopyranosylgentisate (**6**): ^{1}H NMR (400 MHz, D_2O) δ 7.50 (1H, d, J = 3 Hz, H_6), 7.24 (1H, dd, J = 3 and 9 Hz, H_4), 6.86 (1H, d, J = 9 Hz, H_3), 4.95 (1H, d, J = 8 Hz, $H_{1'}$), 3.89 (1H, dd, J = 2.0, 12.5 Hz, $H_{6'a}$), 3.70 (1H, dd, J = 5.5, 12.5 Hz, $H_{6'b}$), 3.56 (1H, ddd, J = 2.2, 5.5, 9.0 Hz, $H_{5'}$), 3.53 (1H, t, J = 9 Hz, $H_{3'}$), 3.46 (1H, br.t, J = 9.0 Hz, $H_{2'}$), 3.44 (1H, d, J = 8.0, 9.0 Hz, $H_{4'}$). δ ; ESI MS (negative): $[M-H]^-$ *m/z* 315.

Isolation of mimopudine (7). The n-BuOH layer corresponding to 12.2 kg of *M. pudica* was used for the isolation of **7**. The *n*– BuOH layer was carefully separated by Amberlite XAD–4 column chromatography (ϕ 55 × 320 mm, Organo Co.) eluted with MeOH–H_2O (8 : 2, 9 : 1 and 10 :0 × 1 L each), and the 80% MeOH eluate showed weak leaf–opening activity. Then, the 80% MeOH eluate was carefully separated by Amberlite XAD–7 column chromatography (ϕ 55 × 320 mm) eluted with MeOH– H_2O (0 : 10, 1 : 9, 3 : 7, 4 : 6, 5 : 5, and 10 :0 × 1 L each), and the 30% MeOH and 40% MeOH eluates showed weak leaf–opening activities. The bioactive 30% MeOH aq. fraction was further purified by Toyopearl HW-40 Fine column chromatography (ϕ 30 × 300 mm) with 35% MeOH aq., HPLC using preparative Develosil ODS HG–5 column (ϕ 20 × 250 mm) with 40% MeOH aq. (flow rate: 4.0 mL/min, detection: 260 nm) and purification by HPLC using analytical CAPCELL PAK UG80 column (ϕ 4.6 × 250 mm, Shiseido Co.) with 40% MeOH aq. (flow rate: 1.0 mL/min, detection: 260 nm) to give L-Tryptophan (6.1 mg) as a colorless powder. On the other hand, the other bioactive fraction, 40% MeOH eluate, was purified by Toyopearl HW-40 Fine column chromatography (ϕ 30 × 300 mm) with 35% MeOH aq., HPLC using preparative Cosmosil 5C18AR column (ϕ 20 × 250 mm) with 40% CH3CN aq. (flow rate: 4.0 mL/min, detection: 260 nm), and HPLC using a combination of two analytical columns (Develosil HG-5 and Develosil UG-5) (ϕ 4.6 × 250 mm) with 20% CH_3CN aq. (flow rate: 0.5 mL/min, detection: 260 nm) to give mimopudine (**7**, 0.5 mg) as a yellow powder. mimopudine (**7**) : ^{1}H NMR (400 MHz, D_2O, rt) δ 7.90 (2 H, d, J = 7 Hz, H_1), 6.60 (2 H, d, J = 7 Hz, H_2), 5.30 (1 H, br.s, $H_{2'}$), 4.05 (1 H, d, br.s, $H_{1'}$); ^{13}C NMR (100 MHz, D_2O, 35 °C) δ 182.2 (C_3), 146.0 (C_1), 119.5 (C_2), 90.5 ($C_{2'}$), 65.0 ($C_{1'}$).; ESI MS (positive): *m/z* 360 $[M+Na]^+$, 338 $[M+H]^+$, 191 $[M+2Na]^{2+}$, 169 $[M+2H]^{2+}$; HR ESI MS (positive): calcd. for $[M+H]^+$ ($C_{14}H_{20}O_5N_5$) 338.1464, found *m/z* 338.1434; IR λ 1636, 1537, 1400 cm^{-1}.

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