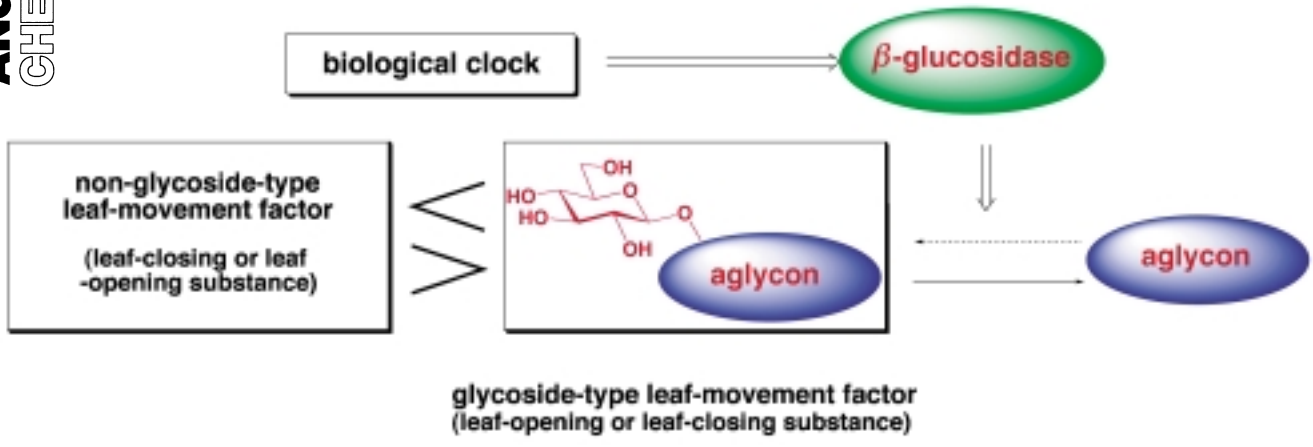
The opening and closing of the leaves of plants such as *Mimosa pudica* are controlled by a biological clock.

β-Glucosidase plays a decisive roll in this mechanism; activation of the enzyme initiates the circadian rhythm which the leaf movement follows.



Chemistry and Biology of Plant Leaf Movements

Minoru Ueda and Shosuke Yamamura*

The leaves of *Mimosa pudica* L. are well known for their rapid movement when touched. Recently, we were able to isolate an excitatory substance in small quantities from this plant, which consists of three different components (potassium L-malate, magnesium *trans*-aconitate, and dimethylammonium salt). Many plants close their leaves in the evening, as if to sleep, and open them early in the morning (nyctinastic leaf movement). This circadian rhythm is known to be controlled by the biological clock of such plants. Extensive studies on other nyctinastic plants led to the isolation of a variety of leaf-opening substances (LOSs) and leaf-closing substances (LCSs). Based on our experiments on these bioactive substances, we found that the circadian rhythmic leaf movement of these plants is initiated by the regulated balance of LOSs and LCSs. The balance of concentration between the two leaf-movement factors (LMFs) is inversed during the day. The glycoside-type LMF is hydrolyzed with β-glucosidase, the activity of which is regulated by the biological clock. The circadian rhythm observed in the leaf movement is introduced by activation of β-glucosidase regulated by the biological clock.

Keywords: bioorganic chemistry · glycosidases · glycosides · phytochemistry · plant physiology

1. Introduction

Plants are rooted and unable to move from one place to another by themselves. However, they are not static; they sensitively respond to a variety of factors such as light, darkness, temperature, and humidity, and also to chemical substances represented by allelopathic compounds. The result is a visual movement with or without cell elongation. Plant movement is mainly classified into three kinds: 1. tropism (movement in a particular direction due to a stimulus), 2. nasty (movement triggered by a stimulus but with no relation to the direction of the stimulus), and 3. taxis (stimulus-triggered movement directed either towards the stimulus or away from it). The movements of *Mimosa pudica* L. (Figure 1) and *Dionaea muscipula* (Venus flytrap), which belong to type 2, are especially famous.

In 1880, Charles Darwin, well known for his biologically important book entitled "On the Origin of Species" also published an invaluable and voluminous book entitled "The Power of Movement in Plants" based on his own experiments, assisted by his son Francis, with more than three hundred different kinds of plants including nyctinastic ones represented by *Mimosa pudica*.[1] From the viewpoints of plant physiology and phytochemistry in particular, their ingenious experiments on phototropism led to the discovery of auxin, the first plant hormone of the six known classes in higher plants: auxins, ethylenes, gibberellins, cytokinins, abscisic acids, and brassinolides. Similar results have also been obtained in the leaf movement of nyctinastic plants including *Mimosa pudica*, *Albizzia julibrissin* and *Cassia mimosoides*.

The present review is focused on a variety of bioactive compounds related to the leaf movement of plants. The plant leaf movement can be divided into two categories; one is the seismonastic rapid movement observed in only a few plants, and the other is the nyctinastic slow movement observed in nyctinastic plants; their leaves fold up at night and unfold by day according to a circadian rhythm with a period of about 24 hours (Figure 2).

Nyctinastic leaf movement is observed in almost all leguminous plants. This periodic leaf movement controlled by an internal biological clock can take place in continuous daylight or continuous darkness.[2] The origin of the biological clock can be traced to careful observation of the nyctinastic leaf movement of *M. pudica* in the 18th century.[26] A French scientist found that *M. pudica* extended its leaves in the daytime ("awake") and folded them at night ("sleep") even if the plant was kept in continuous darkness in a cave. After that, Bünning in Germany developed and expanded the science of biological clocks, and now a biological clock, which

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Figure 1. The rapid leaf movement of *Mimosa pudica* L.

is considered to be one of the main themes of science in the 21st century, has been proved to exist in all living organisms from prokaryote to human beings. Both the rhythmic bioluminescence of dinoflagellate *Gonyaulax polyedra* emitted at night and the jet lag we experience are attributed to an internal clock.

In addition to nyctinastic movement, the plant *Mimosa pudica* shows seismonastic movement. Such an exciting biological phenomenon has been attracting much attention since the fourth century BC. The 2000 year history of research on the movement of *M. pudica* was cited in part of the excellent review by Schildknecht.[3]

2. Excitatory Substances in *Mimosa pudica*

When touched, the plant *Mimosa pudica* L. closes its leaves very rapidly one by one, in a movement like that of animals. When etherized, it did not show any response to stimulation. This rapid movement of the leaf is observed only in the *Mimosa* family, *Biophythum*, and *Dionaea muscipula*. This surprising phenomenon has prompted both biologists and chemists to make great efforts to understand the mechanism of the leaf movement of the Mimosaceae. One of the most interesting problems is how the stimulant is transported from the stimulated point to other areas.

From the viewpoint of electrophysiology, different rates of stimulus conduction were observed by Houwink[4] and Shibaoka.[5] The stimulant, which is transported in the xylem sap stream, elicits a response in the neighboring tissue in the form of a variation potential (slow-wave or s-wave). Either the signal substance itself or the variation potential which is generated by it triggers a rapid action potential (medium or

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Figure 2. *Albizzia julibrissin* Durazz. during the day (left) and at night (right).

rapid wave; m- or r-wave, respectively), which can travel ahead of the slower variation potential, as summarized by Schildknecht.[3] Regardless of the mode of action, some stimulants may interact directly or indirectly with a motor cell.

The actual leaf movement is explained by movement of water into or out of cells which is driven by fluxes of ions, especially K^+ ions.[6] The leaf-movement organ of the plant leaf is the pulvinus. Two groups of the cells in the pulvinus, the flexor and extensor cells, are arranged above and below the central vascular tissue, respectively. The flexor cells swell and the extensor cells shrink to bend the pulvinus and flex or fold the leaf or leaflet, whereas the flexor cells shrink and the extensor cells swell to straighten the pulvinus. Biologists have revealed that the pulvinar cells lose K^+ ions when shrinking and actively take up the K^+ ions when swelling; as many as 60% of the total K^+ ions within the pulvinus move from the flexor side to the extensor side and back again during a complete cycle of leaf movement.[7] K^+ ions can enter and leave plant cells by other channels which are differently regulated.[8] However, the signals by which a biological clock might control the nyctinastically regulated leaf movement, through the change of the K^+ channel state, have long remained unknown.

Recently, the water distribution in the pulvinus of *M. pudica* was visualized by an NMR imaging technique, which indicated that water in the lower half of the main pulvinus disappeared after stimulation of a *Mimosa* plant. The water previously contained in this area is apparently transferred to the upper half of the main pulvinus. Movement of the water in conjugation with *Mimosa* movement was also visualized by a noninvasive NMR imaging technique.[9]

In 1916, Ricca strongly suggested that some stimulants related to leaf movement are included in the *Mimosa* plant.[10] This theory was based on his ingenious experiments using two parts of a cut shoot of the *Mimosa* plant, which were connected to each other through a glass tube filled with salt water or water. Since then, many scientists have been attempting to locate the true stimulants of *M. pudica*, and a wide variety of bioactive compounds have been isolated from the *Mimosa* plant, some of which are shown in Figure 3.[3, 11–14]

Many common amino acids were also detected. Of these, interestingly, L-glutamic acid (**1**) caused periodical closure and opening movements at intervals of 5 to 10 min; however, it was proved not to be a true excitatory substance of the *Mimosa* plant.[12] In connection with this phenomenon, it is quite interesting that Nakajima et al. carried out electrophysiological approaches using the stem surface of *M. pudica* and obtained results which indicated that sodium L-glutamate was the active compound responsible for the strong pulse, while D-glutamate was not active.[15] The nitrogen-containing heterocyclic compounds **2**–**4** shown in Figure 3 were not related to the leaf movement.[11, 12]

In 1983, after their extensive efforts in searching for the true leaf-movement factors (LMFs) of *M. pudica*, Schildknecht and Bender finally succeeded in the isolation and structure elucidation of four compounds (K-PLMF 1 (**5**), K-PLMF 2 (**6**), S-PLMF (**7**), and M-LMF (**8**)) from *M. pudica*, *Acacia karroo*, *Oxalis strica*, and others,[16] as shown in Figure 3. K-PLMF 1 (**5**), one of the representative compounds, was also synthesized by Schildknecht et al.[17] Both natural and synthetic samples showed leaf-closing activity at concentrations of 10^{-4} g L^{-1}. In addition, they also obtained some cyclic nucleotides as leaf-movement cofactors (**10** and **11**).[3]

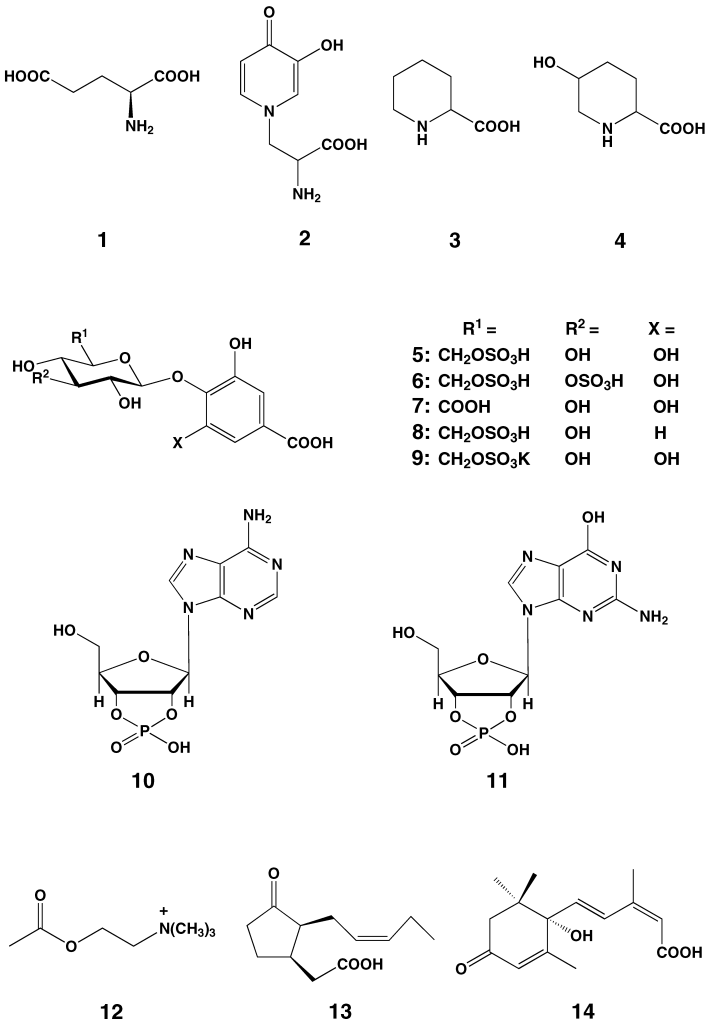


Figure 3. Chemical substances isolated as leaf-movement factors of *M. pudica*.

Through these experiments, they proposed that the leaf-movement factors (**5**–**8**), named turgolins, are a new class of phytohormones which control the nyctinastic and seismonastic movements. However, **5** with a free sulfate function seems to be questionable because **5** has been isolated not as a free acid, but as a potassium salt **9** from *M. pudica*, *Cassia mimosoides*, and others in our careful experiments.[18] This potassium salt was also effective for leaf closing of *M. pudica*. However, its activity (about 10^{-1} g L^{-1}) was very weak in both natural and synthetic samples,[19] and its leaf-closing activity was not observed for the leaf of other nyctinastic plants. Furthermore, in 1984, **5** was proved by Umrath et al. not to be the genuine excitatory substance of *M. pudica*.[20]

Umrath et al. extracted the pinnae of *M. pudica* with boiling water in a short-step process and chromatographed the aqueous extracts on a Sephadex LH 20 column with methanol/water (92/8) as the mobile phase to afford two bioactive fractions (substances E and G).[20] The proportional activities were 80% for E and 20% for G; β-glucosidase was not effective on the former, while the G substance lost its activity when treated with β-glucosidase. This result indicated that the main leaf-closing substance (LCS) E, assigned to be the excitatory substance of *M. pudica*, is not **5**, as the structure of this consists of gallic acid and a D-glucose derivative linked by a β-glycosidic bond. In addition, they reported that the substance E is an unstable oxyacid which completely lost its leaf-closing activity through further purification.[20] Therefore, it was apparently almost impossible to obtain such an unstable substance from *M. pudica*. However, quite recently, we were able to find a solution to this seemingly insurmountable problem, a solution which has been sought since the last century.[21]

The methanol extract of *M. pudica* was carefully separated based on our own bioassay (Figure 4), similar to the Fitting-Hess–Schildknecht test. The test must be carefully carried out, because the leaves are very sensitive to H^+ ions (the leaves fold on addition of dilute sulfuric acid (10^{-2} g L^{-1})), temperature, humidity, and so on. We carried out the bioassay in midsummer because of the good reproducibility. The isolation procedure is shown in Scheme 1, where each separated fraction completely lost its activity during the 4th step (HPLC purification),[21] as previously reported by Umrath et al. The disappearance of the bioactivity in the 4th step was highly reproducible; thus, we assumed that plural components are necessary for the leaf-closing activity. Therefore, each fraction was carefully combined, one by one, and monitored by a combination of the *Mimosa* test and ^{1}H NMR spectroscopy, leading to the finding of three fractions related to the rapid movement of *M. pudica*. Thus, the genuine excitatory substance consists of three different components: 1) potassium L-malate (**15**), 2) magnesium *trans*-aconitate (**16**), and 3) the dimethylammonium salt **17**.

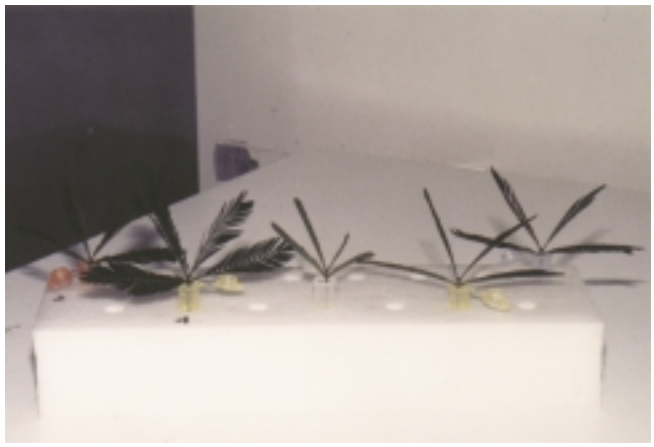
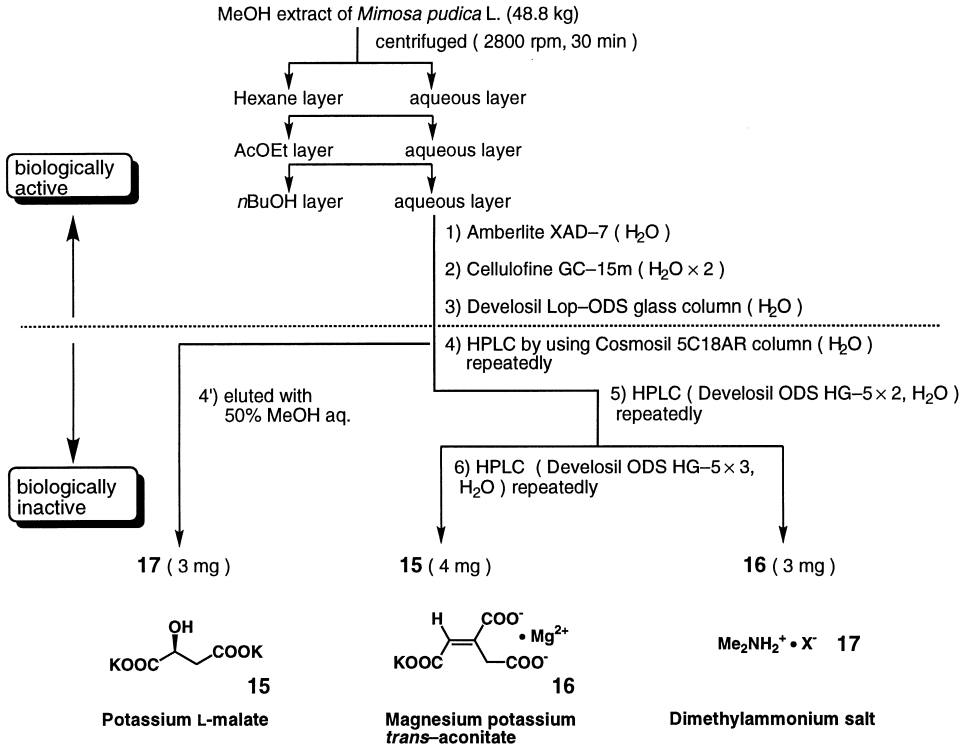


Figure 4. Bioassay for the detection of the chemical substances which induce rapid leaf movement in *M. pudica*.

Mimosa leaves folded very rapidly on addition of this mixture in a concentration of 10^{-8}–10^{-9} M.[21] In addition, metal ions are also important for the activity. When calcium was used instead of magnesium as the counter ion of **16**, the resulting mixture did not show any leaf-closing activity. In conclusion, the mixture of **15**, **16**, and **17** is the genuine excitatory substance 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.[20] We assume that **15** corresponds to the "unstable



Scheme 1. Isolation of the stimulatory substances from *M. pudica*.

oxyacid" of Umrath and co-workers. It is important that our three-component excitatory substance induces only the rapid movement of *Mimosa*, and no slow leaf movement was observed with it.

Parallel to the leaf-closing substances, the leaf-opening substances (LOSs) for the nyctinastic leaf movement of *Mimosa* have also been studied by many groups. In particular, indole-3-acetic acid (IAA) caused the leaf opening of *M. pudica* at night. In the experiments with cut pinnae in the dark, IAA induced optimal opening of the leaflets at concentrations from 3×10^{-3} to 5×10^{-4} M; lower concentrations were ineffective.[22] On the other hand, Tsurumi et al. indicated that the excised pulvinus opened with IAA at concentrations from 10^{-7} to 10^{-4} M.[23] This difference has not been clarified, but the high sensitivity of the excised pulvinus to IAA may be due to direct treatment with the IAA solution. Regarding the circadian rhythm of *M. pudica*, it is suggested that IAA may be necessary for the pulvinus to manifest the effect of day–night cycles or the action of the biological clock through control of osmotic pressure, as well as for maintenance of the permeability of motor cell membranes in *Mimosa* pulvini.[24] In addition, Suda indicated that acetylcholine (ACh, **12**) was able to induce opening of *Mimosa* leaflets at night, but only in high concentrations (5×10^{-3} M).[25]

In connection with IAA-induced leaf opening of *Mimosa* pulvinus, jasmonic acid (**13**) was isolated from the *Mimosa* plant.[13] Jasmonic acid (**13**) and abscisic acid (**14**) each inhibited both IAA- and light-induced opening of the pulvinus at concentrations of 10^{-5} M. Both of them are proposed to be the regulators of *Mimosa* pulvinus movement. However, as previously pointed out by Umrath et al. and Schildknecht, chemical substances which induce the slow leaf-closing and leaf-opening movements and differ from the plant hormones must be included in the *Mimosa* plant.[21] We will describe these bioactive substances of *Mimosa* in Section 3.2. When compared with *M. pudica*, other nyctinastic plants, which open their leaves early in the morning and close them at night according to the circadian rhythm, seem to be much simpler in their leaf movement.

3. Leaf-Movement Factors in Nyctinastic Plants

Nyctinastic movement was believed to be controlled by Schildknecht's turgorins which induce leaf-closing movement of the plants.[3] However, we revealed that turgorin is not a genuine leaf-movement factor, and nyctinastic movement is regulated by a chemical substance that differs depending on the plant species. Our present results are in accordance with the physiologically significant opinion of Umrath et al. that every family or subfamily of plants has its own leaf-movement factor that is effective only for plants belonging to that family, although they have not identified any leaf-movement factors.[20]

We tried to isolate genuine leaf-movement factors of nyctinastic plants. An important problem in the chemical studies of nyctinasty by Schildknecht is the difference between the plants used in the bioassay and for extraction. They used *Mimosa* leaves for the detection of the leaf-closing activity from all nyctinastic plants. If the hypothesis of Umrath et al. is true, the plant used in the bioassay must be the same as the one extracted. In order to detect the leaf-movement factors of nyctinastic plants, we developed a bioassay using the original leaves. The movement of the leaves, which were detached from each stem and immersed in water, was also found to follow the circadian rhythm. The young leaves of the nyctinastic plant to be tested were immersed in distilled water and allowed to stand at room temperature overnight. The leaves that opened again the next morning were used for the bioassay. The bioactivity was judged by the closure of the leaves in the daytime (Figure 5).

The bioactive substance closes the leaves a few hours after the addition. In the course of the isolation, we noted that a leaf-opening substance was contained together with the leaf-closing one in a nyctinastic plant (Figure 5). Thus, we have to divide the leaf-movement factor into two categories, one is leaf-closing substances, which close the plant leaf even in the

Figure 5. Bioassay for the detection of chemical substances responsible for nyctinasty; leaf-closing (above), and leaf-opening (below) substances.

daytime, and the other is leaf-opening substances, which keep the plant leaf open even at night.

The presence of leaf-opening substances indicates that nyctinastic movement is controlled not only by the change in the concentration of the leaf-closing factor, but also by the competitive interaction between leaf-closing and leaf-opening substances. The idea of the coexistence of a leaf-opening substance with a leaf-closing one is very reasonable in terms of the previously mentioned result that K^+ ions enter and leave plant cells through ion channels which are regulated differently.[8]

3.1. The Isolation of Leaf-Movement Factors

The most difficult problem encountered in the chemical studies of nyctinastic movement lies in the isolation of leaf-closing and leaf-opening substances. The difficulty in the isolation comes from the weak biological activity of the plant extract observed by bioassay. We decided that this weak bioactivity is due to the coexistence of the leaf-opening substance with the leaf-closing one in the same fraction. When both of them coexist in the same fraction, the respective bioactivities should cancel each other out, and, as a result, no bioactivity can be monitored by bioassay. All of the previous attempts for the purification of the leaf-closing substance were carried out with a combination of gel filtration chromatography and HPLC. It is supposed that all such studies of the isolation failed because no attempt was made to separate the leaf-closing substance from the leaf-opening one. Thus, it is crucial to separate these inversely bioactive substances from each other in the first step of the isolation procedure.

We attempted to apply polymer gel chromatography to the separation of leaf-opening and leaf-closing substances. After many separation conditions were examined, we found that an Amberlite XAD-7 chromatography column could separate the compounds completely, based on the difference in polarity. The highly polar leaf-closing substance was eluted with 100% H_2O; on the other hand, the comparatively less polar leaf-opening substance was eluted with MeOH-containing eluent. The bioassay gave completely distinct results in the full course of the isolation.

After the separation of the two bioactive fractions with leaf-closing and leaf-opening activities, each fraction was separated with gel filtration chromatography, MPLC (Medium Pressure Liquid Chromatography) using an ODS (octadecyl silyl) glass column, and preparative HPLC using an ODS column. HPLC using a combination of three columns showed a particularly good separation which enabled us to isolate the leaf-closing substance. Thus, we have succeeded in the isolation of several bioactive substances controlling the leaf movement of nyctinastic plants (Figure 6).

3.2. Leaf-Movement Factors of *Mimosa pudica* L.

The nyctinastic movement of *Mimosa*, which is another leaf-movement of this plant, was neglected for a long time, because the slow movement was thought to be caused by the same chemical substance as that in the rapid movement.[3] For this reason, the two leaf-movements of *Mimosa* have often been confused. We have disclosed the true character of the leaf-movement factors of *Mimosa* and demonstrated that the rapid and slow leaf movements are caused by different chemical substances.

Our three-component stimulant, described in Section 2, induced only the rapid movement of the *Mimosa* leaf, and no slow leaf closure was observed even at the threshold-level concentration. Therefore, we assumed that the bioactive substance regulating the nyctinastic leaf movement should also be contained in *Mimosa*. As already mentioned, Umrath et al. obtained weak leaf-closing substance G from *M. pudica*.[20] We assumed that substance G is assigned to be the leaf-closing substance of *M. pudica* which induces a nyctinasty.

A modified bioassay was used for the separation of leaf-movement factors. We used a *Mimosa* leaf, cut at the lamina, instead of a four-pinnae leaf and carried out the bioassay in a glass tube, because the *Mimosa* leaf folded easily in gentle wind through the long period needed for bioassay. This method was inferior in sensitivity to the previous method using the four-pinnae leaf; however, its reproducibility was satisfactory.

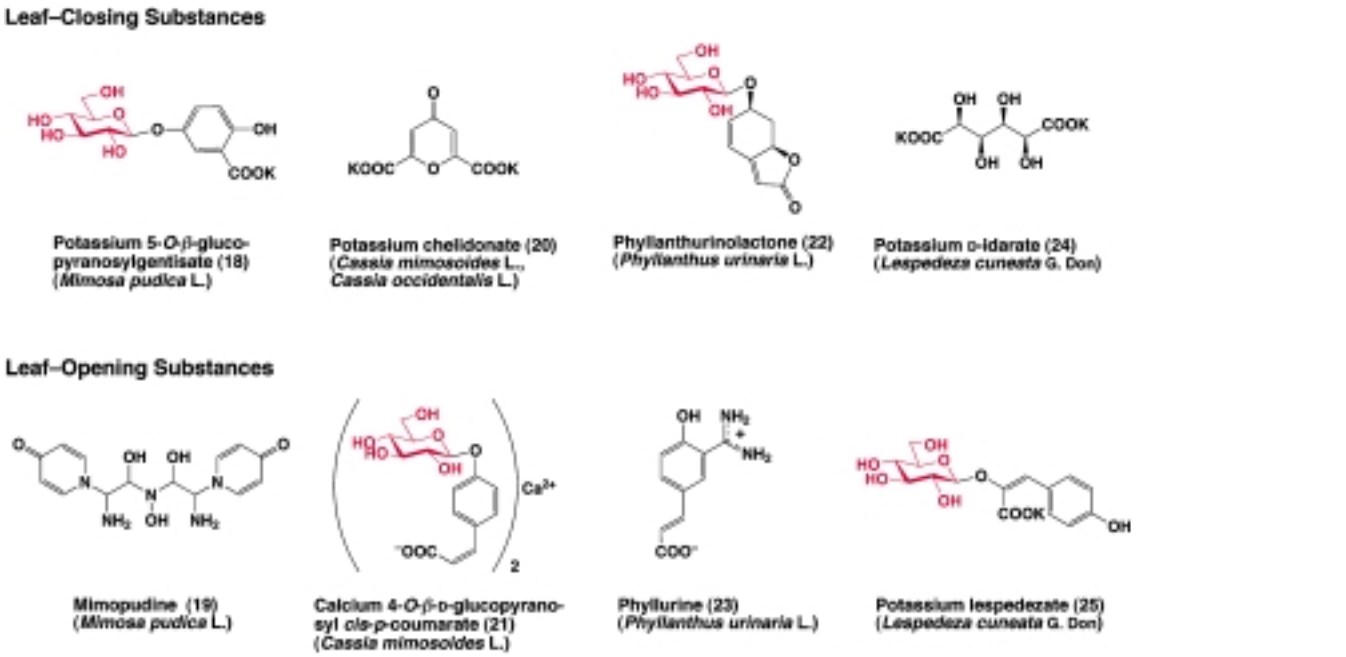


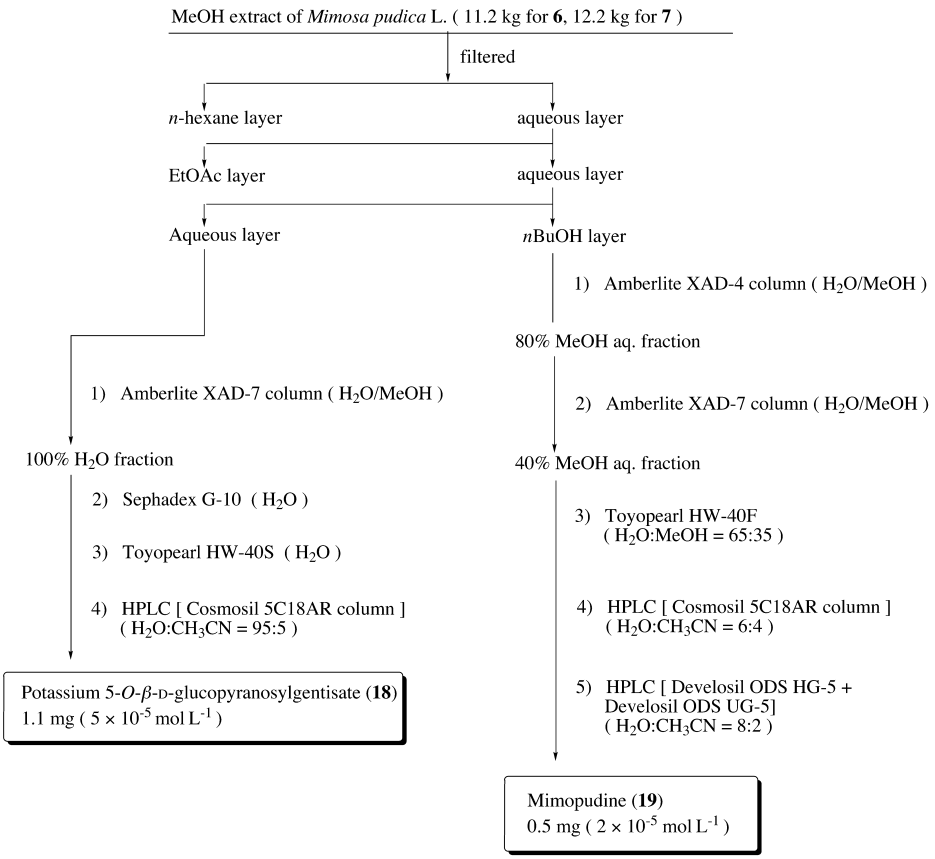
Figure 6. Four pairs of leaf-movement factors. Each pair was isolated from the same nyctinastic plant.

After the separation with XAD-7, fractions with strong leaf-closing activity were obtained. However, only the seismonastic activity that induces the rapid leaf closure was observed. We assumed that the nyctinastic activity which induces the slow leaf closure could be masked by the strong activity attributed to the excitatory substances. It was crucial to separate these two bioactivities for the detection of nyctinastic leaf-closing activity (Scheme 2).

Based on the report by Umrath et al.,[20] we examined the application of the gel filtration chromatography using Toyopearl HW-40S columns for the separation of the excitatory and leaf-closing substances.[27] We thought that the excitatory substance corresponded to substance E and the leaf-closing substance **18** was substance G, as described by Umrath et al. On the other hand, the leaf-opening substance was also isolated from *M. pudica*.[28] It was supposed that strong leaf-closing activity masked the bioactivity of the leaf-opening substance. Thus, the *n*-butanol layer, which contains almost no excitatory substances, was separated with HPLC to give mimopudine (**19**).

Structure determinations of **18** and **19** were carried out by means of NMR spectroscopy and ESI mass spectrometry experiments. Compound **19** was unstable in aqueous solution, and decomposed easily to give pyridone. An ESI-linked scan experiment was useful in the structure determination of **19** (Figure 7).[28]

Compound **18** was quite effective for leaf closing of *M. pudica* at night at concentrations of 5×10^{-5} M and **19** was effective for the leaf opening of the same plant in the daytime at concentrations of 2×10^{-5} M, but neither was



Scheme 2. Procedures for the isolation of the leaf-closing and leaf-opening substances of *M. pudica*.

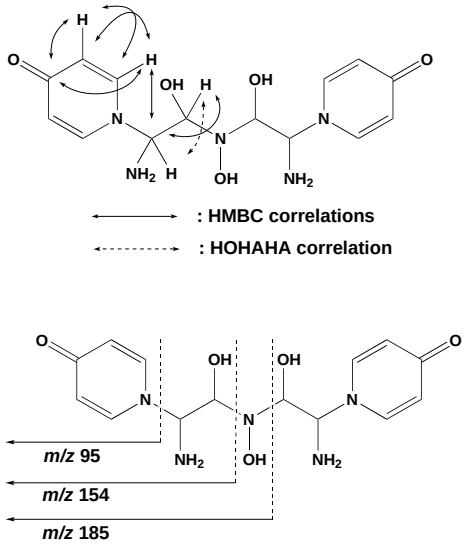


Figure 7. HMBC and HOHAHA correlations (above) and linked-scan ESI MS (below) for the structure determination of mimopudine (**19**).

effective for other nyctinastic plants. Compound **18** induced only the slow leaf closure of the *Mimosa* leaf.[27]

Interestingly, the leaves of *M. pudica* kept open with **19** at night were sensitive to physical stimulus by touch, as observed in the daytime. This result suggests that **19** is effective only for the obstruction of the slow nyctinastic leaf closing movement, but not effective against the rapid seismonastic movement. We have now been able to separate these two leaf movements at the molecular level, and to demonstrate that the slow nyctinastic movement of *Mimosa* is initiated by a different mechanism from that of the rapid seismonastic movement.[28]

We have succeeded in the identification of the chemical substances concerning rapid and slow leaf movements of *Mimosa*, which have been discussed for more than 80 years.

3.3. Leaf-Movement Factors of *Cassia mimosoides* L.

As shown by its name, *C. mimosoides* resembles *Mimosa*. However, its leaves do not respond to physical stimulus. Only the nyctinastic movement is observed in this plant. Potassium chelidonate (**20**) was isolated as a leaf-closing substance from *C. mimosoides*.[18] Compound **20** was also found in *Cassia occidentalis* L., which belongs to the same genus as *C. mimosoides*.[8] This result suggests the universality of the leaf-movement factor among a plant genus.

The natural and synthetic **20** induced the closure of *Cassia* leaves in the daytime at concentrations of 10^{-6}–10^{-7} M. Interestingly, the corresponding sodium salt, as well as the free acid, showed no bioactivity. Also, the potassium acetate, potassium oxalate, and potassium salt of phloroglucinol did not show any leaf-closing activity in the plant. Thus, both the potassium ion and chelidonate anion are necessary for bioactivity. Potassium chelidonate (**20**) as a "turgorin" plays an important role in the leaf movement of nyctinastic plants *C. mimosoides* and *C. occidentalis*.

On the other hand, calcium 4-*O*-β-D-glucopyranosyl-*cis*-*p*-coumarate (**21**) was isolated as the leaf-opening substance of *C. mimosoides*.[29] Compound **21** lost its bioactivity under strongly polar isolation conditions. This is because the counter cations that dissociated from the carboxylate of **21** were exchanged with other cations during the separation using polar solvents. We depressed the dissociation of the carboxylate group by using apolar solvents in the isolation of **21**. Compound **21** effectively opened the leaves of *C. mimosoides* at night at concentrations of 4×10^{-6} M. The potassium salt of **21**, which was isolated together with **21**, exhibited no biological activity, and calcium acetate was effective only at a concentration of 1×10^{-2} M. Thus, both the calcium ion and its counter ion were essential for the bioactivity of **21**.

3.4. Leaf-Movement Factors of *Phyllanthus urinaria* L.

Phyllanthurinolactone (**22**)[30] and phyllurine (**23**)[31] were isolated as the leaf-closing and leaf-opening substances, respectively, from the nyctinastic plant *Phyllanthus urinaria* L., which belongs to the *Euphorbiaceae* family. Since other leaf-closing substances have been isolated from the *Fabaceae* family, it would be interesting to study the difference in the chemical structure of the leaf-movement factors between these two plant families. Due to this difference in the plant family, the structure of bioactive substances greatly differs from those of other nyctinastic plants belonging to the *Leguminosae* plants.

The structure determinations of **22** and **23** were carried out by means of 2D NMR experiments. The absolute configuration of **22** was determined from the total synthesis by Mori et al.[32, 33] They also synthesized all the possible stereoisomers relative to the diol moiety of the aglycon. Bioactivity was observed only in the natural *syn*-6*S*,7a*R* form. Phyllanthurinolactone (**22**) was effective for the leaf closure of *P. urinaria* in the daytime at a concentration of 1×10^{-7} M, and phyllurine (**23**) was effective for leaf opening at a concentration of 5×10^{-5} M.

3.5. Leaf-Movement Factors of *Lespedeza cuneata* G. Don.

Potassium D-idarate (**24**) as a leaf-closing factor,[34] and both potassium lespedezate (**25**) and potassium isolespedezate (**28**, geometrical isomer of **25**) as leaf-opening substances[35] were isolated from *L. cuneata*, according to the bioassay using the leaves of *C. mimosoides*.

Due to the stiffness of the stem, the leaves of *L. cuneata* transported the sample solution poorly, and were insufficient for use in the bioassay. We used the leaves of *C. mimosoides* for the bioassay instead of the leaves of *L. cuneata* to overcome this experimental difficulty. The bioactivities of **24**, **25**, and **28** were quite specific for the leaves of *C. mimosoides* in the daytime at a concentration of 5×10^{-7} M.

The stereochemistry of **24** was determined by comparing its spectroscopic data and bioactivity with those of various potassium tetrahydroxydicarboxylates prepared from D-hexo-

ses. All other diastereomers are biologically inactive or only very weakly active;[34] thus, the bioactivity of **24** was specific with respect to its stereochemistry.

Compounds **25** and **28** were the first leaf-opening substances which were isolated from nyctinastic plants. To investigate structure–activity relationships, various analogues of **25** were synthesized and subjected to bioassay (Figure 8).[36] The bioactivities of sodium salts and free acids

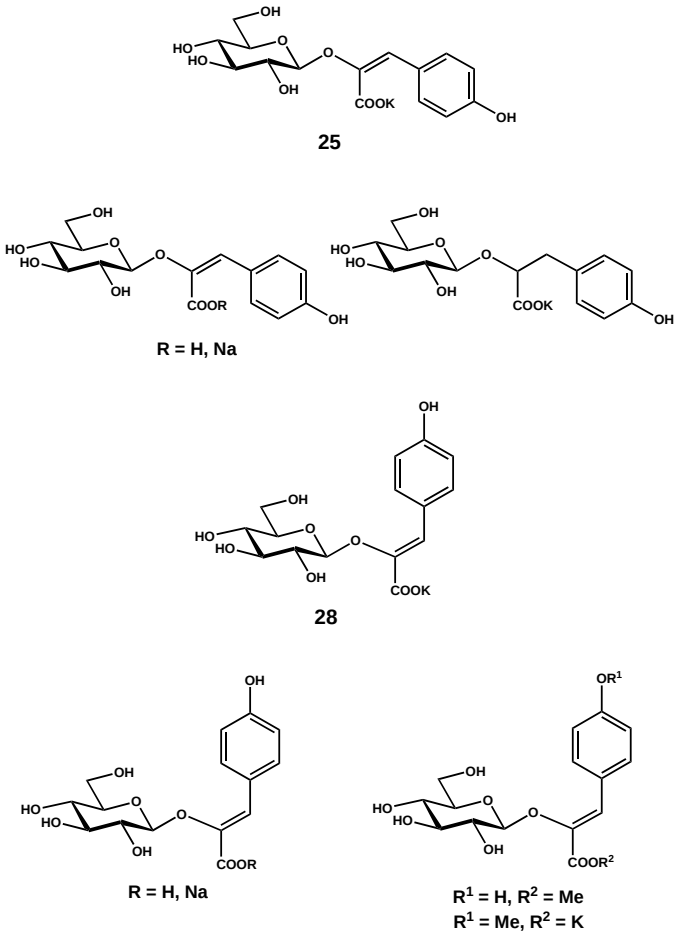
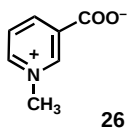


Figure 8. Potassium lespedezate (**25**), potassium isolespedezate (**28**), and their analogues.

were as strong as those of the natural products. Interestingly, *p*-hydroxyphenylpyruvic acid (**29**) and its potassium salt (**30**) opened the leaves at concentrations of 10^{-4} M even at night. Moreover, variation of the sugar moiety in **25** gave compounds which exhibited almost the same bioactivity as **25**.[37] From these results, it is obvious that one of the most important structures for leaf-opening activity is a *p*-hydroxyphenylpyruvate unit in the enol form.[36, 37] The sugar moiety would serve only to improve the solubility and stabilize the enol double bond.

3.6. Trigonelline from *Aeschynomene indica*

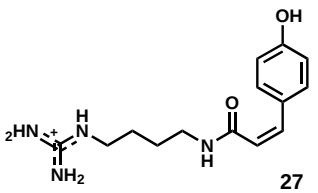
We isolated trigonelline (**26**) as a leaf-closing factor of the nyctinastic plant *Aeschynomene indic*a.[38] Trigonelline (**26**) was effective for the leaf closure of *A. indic*a at a concentration of 1×10^{-7} M.



Trigonelline (**26**) was also reported to be a G2 factor, present in the cotyledons of garden peas, that promotes cell arrest in the G2 stage (DNA postsynthetic stage) of both roots and shoots.[39] Compound **26** promotes cell arrest in the G2 stage in 40 % of all root cells. In the absence of **26**, this cell population is arrested in the G1 stage. Tramontano et al. reported that **26** is the first chemically identified "hormone" that controls the cell cycle in plants or animals.[39c] These results are very interesting in terms of the relationship between the cell cycle and the biological clock.

3.7. *cis-p*-Coumaroylagmatine, the Leaf-Opening Substance in *Albizzia julibrissin* Durazz

The nyctinastic movement of the leaves of the silk tree, *Albizzia julibrissin* Durazz, is as well known as the rapid movement of *Mimosa* leaves. We isolated *cis-p*-coumaroylagmatine (**27**) from *A. julibrissin* as the leaf-opening substance.[40] Compound **27** kept the leaves open at a concentration of 5×10^{-6} M. We also revealed that an unknown leaf-closing substance is also contained in the extract of this plant.



It is interesting that *A. julibrissin* contains only the thermodynamically unstable *Z* isomer, and no *E* isomer. *cis-p*-Coumaroylagmatine (**27**) and its *trans* isomer were synthesized from agmatine and *cis-p*-coumaric acid.[41] Synthetic **27** shows the same level of activity as the authentic sample of natural *cis-p*-coumaroylagmatine, and the synthetic *trans* isomer of **27** opened the leaves of *A. julibrissin* at concentrations as high as 1×10^{-3} M.

4. Common Properties of Leaf-Movement Factors

It has been believed that nyctinastic movement is controlled by turgorin, a leaf-closing plant hormone common to all nyctinastic plants. However, the leaf-closing substances of nyctinastic plants were proved to be different from each other. There also exist leaf-opening substances that compete with leaf-closing ones in controlling the leaf movement of nyctinastic plants.

It is most important that the bioactivity of all leaf-movement factors is specific to the original plant; these substances were not effective for other nyctinastic plants. Therefore, contradictory to Schildknecht's theory, each nyctinastic plant has its own leaf-opening and leaf-closing substances, and nyctinasty is controlled not by a plant hormone, but by these specific compounds.

It is also important that both leaf-closing and leaf-opening substances have been isolated from some nyctinastic plants, as shown in the cases of *Mimosa pudica*,[27, 28] *Cassia mimo-*

soides,[18, 29] *Lespedeza cuneata*,[34, 35] and *Phyllanthus urinaria*.[30, 31] One of the leaf-movement factors is always a glycoside (Figure 6). There are two types of nyctinastic plants: one has a glycoside-type leaf-closing substance, examples are *M. pudica* and *P. urinaria*; the other has a glycoside-type leaf-opening substance, as in *C. mimosoides* and *L. cuneata*. Leaf-opening substances differ in certain aspects from indole-3-acetic acid (IAA) which is believed to induce the leaf opening of nyctinastic plants:[22] 1. the bioactivities of the leaf-opening substances (active at concentrations of approximately 1×10^{-6} M) were much stronger than that of IAA ($> 1 \times 10^{-4}$ M), and 2. the bioactivities of the leaf-opening substances were specific to the genus of the plant while that of IAA was nonspecific.

L-Trp, which was also isolated as a weak leaf-opening substance from some nyctinastic plants,[28, 31] was effective on the leaves of all nyctinastic plants at concentrations as low as 1×10^{-4} M in a similar manner to IAA. It is proposed that the bioactivity of L-Trp is attributed to IAA, which is known as an important metabolite of L-Trp. The bioassay was carried out by the addition of the sample solution at 11.00 a.m., and the bioactivity was checked by study of the leaf opening at 9:00 p.m.; the long period necessary for the bioassay to detect the leaf-opening activity is sufficient for the metabolism of L-Trp into IAA. Thus, L-Trp is not a genuine leaf-opening substance. Moreover, these leaf-movement factors have six properties in common:

1) The amount is always very low; a few milligrams of the leaf-movement factor were isolated from approximately ten kilograms of plant material.
2) At a concentration of approximately 10^{-6}–10^{-7} M, all bioactive substances exert their effect, but only on the plants where they originate. This concentration is almost the same as that of known phytohormones, such as IAA and gibberellin.
3) The bioactivity of all leaf-movement factors is specific to the original plant from which they were isolated.
4) All leaf-closing substances have a common physiological property, in that they compete with IAA at 10^{-6} M. However, much more IAA (approximate concentration of 10^{-4} M) is required to achieve a competitive state than occurs in natural abundance in a plant body.
5) The bioactivity of these compounds is dependent on their stereochemistry. Optically active **22** and **24** were the only bioactive compounds among their stereoisomers. This suggests that a specific receptor participates in the process of signal transduction by these substances.
6) The leaf-opening substance competes with the leaf-closing substance (Tables 1 and 2). When the concentration of the leaf-closing substance was higher than that of the leaf-opening substance, the leaves were closed during the day and vice versa.

Table 1. Competitive interaction between **20** and **21**.[a]

Concentration of **20** [mol L^{-1}]		ratio of **20** to **21** 1:0	1:0.2	1:1	1:5	0:1	control sample
daytime	1×10^{-3}	−−	−−	++	++	++	++
	1×10^{-6}	−−	−−	++	++	++	
night	1×10^{-3}	−−	−−	+−	++	++	−−
	1×10^{-6}	−−	−−	−−	++	++	

[a] The scale reaches from "completely open" (++) to "completely closed" (−−).

Table 2. Competitive interaction between **24** and **25**.[a]

Concentration of **24** [mol L^{-1}]		ratio of **24** to **25** 1:0	1:0.5	1:1	0.5:1	0:1	control sample
daytime	1×10^{-4}	−−	−−	−−	++	++	
	1×10^{-5}	−−	−−	+−	++	++	
	1×10^{-6}	−−	−	+−	++	++	
night	1×10^{-4}	−−	−−	++	++	++	
	1×10^{-5}	−−	−−	+−	+	++	−−
	1×10^{-6}	−−	−−	+−	+−	++	

[a] The scale reaches from "completely open" (++) to "completely closed" (−−).

5. Chemical Control of Leaf Movement in Nyctinastic Plants

From the viewpoint of chemical studies, the most important problem to be solved is how a biological clock controls the nyctinastic leaf movement with these compounds. We have isolated two leaf-movement factors of contrasting bioactivities from the same nyctinastic plant. This was unexpected, and is not compatible with the theory proposed by Schildknecht.[3] The discovery of leaf-opening substances from nyctinastic plants prompted us to assume that nyctinastic leaf movement is controlled by a competitive interaction between leaf-closing and leaf-opening substances. It was confirmed that leaf-opening and leaf-closing substances actually compete with each other. As described above, four sets of leaf-movement factors were isolated from four plants, *Mimosa pudica* L., *Lespedeza cuneata* G. Don, *Cassia mimosoides* L., and *Phyllanthus urinaria* L. We assumed that the concentrations of the two substances change through a day.

Moreover, we have made an important discovery that plant extracts of all the nyctinastic plants collected in the daytime and at night exhibited opposite bioactivity to each other; the former exhibited leaf-opening activity, while the latter exhibited leaf-closing activity.[41–44] It is important that the bioactivity of the plant extract completely reflects the status of the collected leaves; the extract collected when the leaves were closed showed leaf-closing activity and vice versa.

Presumably, these bioactive substances are not stored, but are metabolized in the course of time. The enzymatic transformation during metabolism should be controlled by a biological clock. Thus, chemical studies on this transformation should give us an important clue to understanding the chemical control of leaf movement by a biological clock.

As examples, we will discuss the chemical mechanisms for the control of nyctinasty of *Lespedeza cuneata* G. Don[42] and *Phyllanthus urinaria* L.[44] in the next two sections.

5.1. Chemical Control of the Nyctinasty in *Lespedeza cuneata* G. Don

The extracts of *L. cuneata* collected in the daytime (around 10.00 a.m.) and at night (around 7.00 p.m.) showed inverse

Table 3. Quantitative HPLC analysis of the concentration of **25**, **28**, and **29** collected in the daytime and at night from *Lespedeza cuneata* G. Don.

Time of collection	concentration [mol L^{-1}]		
	25	**28**	**29**
daytime	2.7×10^{-5}	4.1×10^{-5}	2.5×10^{-5}
night	2.7×10^{-5}	1.8×10^{-5}	1.2×10^{-4}

bioactivity; in other words, the former showed leaf-opening activity but the latter showed leaf-closing activity. This result suggests that the balance of concentration between **24** and **25** (or **28**, *cis* isomer of **25**) is reversed in these two extracts.[41, 42] HPLC analysis revealed that the extract collected in the daytime contained twice as much **25** and **28** as the extract collected at night (Table 3). This twofold increase in the concentrations of **25** and **28** is sufficient to inverse the bioactivity of the extract from the competition experiment between **24** and **25** (or **28**). As shown in Table 2, potassium D-idarate (**24**), a leaf-closing substance of *L. cuneata*, interacts competitively with potassium lespedezate (**25**), a leaf-opening substance of the same plant. When the concentration of **24** was higher than that of **25**, the leaves were closed during the day, and vice versa. Thus, both **25** and **28** are metabolized in the evening, and biosynthesized in the morning.

Moreover, we discovered that the concentrations of **25** and **28** in the plant are inversely proportional to that of 4-hydroxyphenylpyruvic acid (**29**) (Table 3). The effectiveness of **29** was one-hundredth that of **25** and **28** (effective concentrations of **29** and **25**, **28** were 5×10^{-5} and 8×10^{-7} M, respectively) for inducing leaf-opening movement. The extract collected at night contained fivefold as much **29** as the extract collected in the daytime. This result strongly suggests that **25** is biosynthesized from **29** in the morning, and is metabolized (and deactivated) to **29** by enzymatic hydrolysis in the evening, as shown in Figure 9. The mechanism of cell contraction in the nyctinastic leaf movement is not clear, but we assume that it is similar to that of seismonstic movement of *Mimosa*.[45]

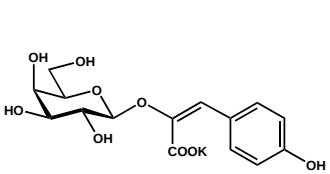
This new model for the regulation of leaf movement was strongly supported by the measurement of β-glucosidase activities of the crude plant preparations collected in the daytime and evening.[42, 43] We collected the leaves of *L. cuneata* and prepared acetone extracts.[46] We used **25** and **28** as substrates of the enzyme and quantitatively analyzed **29**, produced from the acetone extracts, by HPLC. β-Glucosidase activity was observed only in the acetone extract prepared from the plant collected in the evening (Table 4). The balance of concentration between **24** and **25** (or **28**) was inversed by the hydrolysis of **25** (and **28**) into **29** (Figure 9).

Table 4. Transformation from **25** into **29** by the action of crude enzyme.

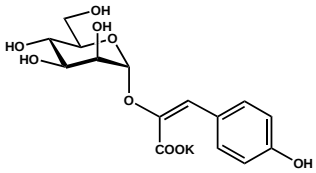
Conditions	concentration [mol L^{-1}]	
	25	**29**
a) crude enzyme (from leaves collected in the daytime) + **25**	1.0×10^{-3}	ND[a]
b) crude enzyme (from stems collected in the daytime) + **25**	1.0×10^{-3}	ND
c) crude enzyme (from leaves collected at night) + **25**	1.0×10^{-3}	2.2×10^{-3}
d) crude enzyme (from stems collected in the daytime) + **25**	1.0×10^{-3}	ND
e) crude enzyme (from leaves collected in the daytime) + buffer	1.0×10^{-3}	ND
f) buffer + **25**	1.0×10^{-3}	ND

[a] ND = not determined.

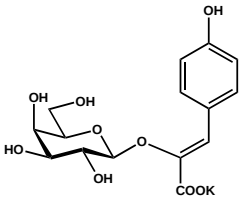
Interestingly, the artificial leaf-opening substances **31**–**34**, sugar derivatives of **25** which were synthesized by us (Scheme 3), were effective at a concentration of 8×10^{-7} M in the bioassay, making them as strong as **25**.[37, 47] The leaf-opening activity of **25** lasted for only two days; after that, the leaf closed at night again. On the other hand, that of **31**–**34** lasted even after a week. This result suggested that **25** would be completely hydrolyzed into **29** by β-glucosidase; however, the enzyme could not hydrolyze **31**–**34**. The importance of β-glucosidase in the regulation of nyctinasty is also verified by these results.[37, 47]



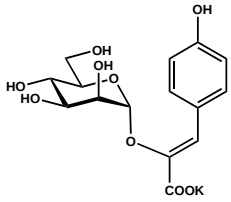
Potassium galactolespedezate (31)



Potassium mannolespedezate (33)



Potassium galactoisolespedezate (32)



Potassium mannoisolespedezate (34)

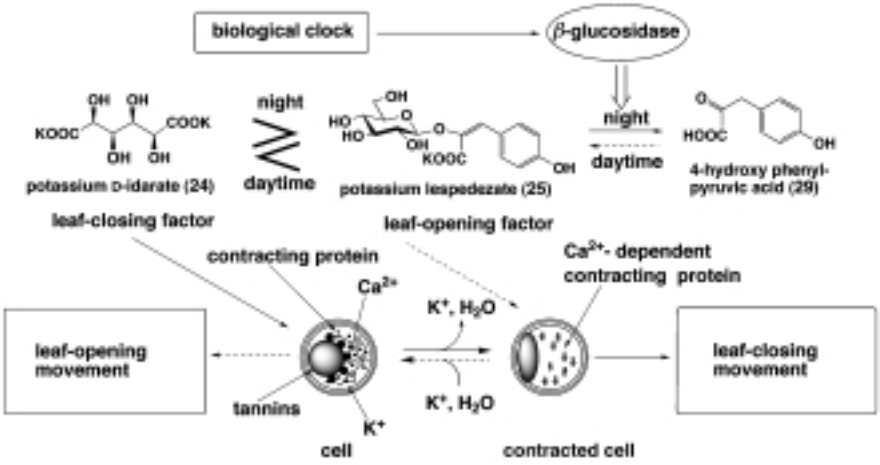
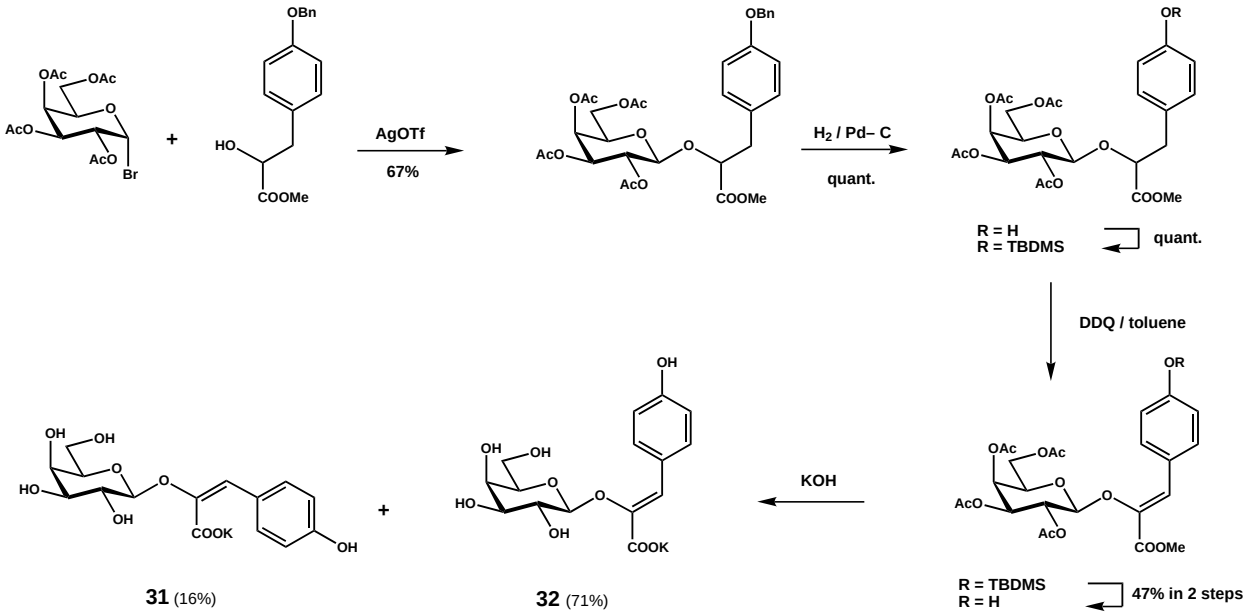


Figure 9. The chemical control of nyctinasty in *Lespedeza cuneata* G. Don.

We propose a new model for the regulation of nyctinastic movement in *L. cuneata*; enzymatic transformation of leaf-movement factors **25** and **28** to **29** inversed



Scheme 3. Chemical synthesis of potassium galactolespedezate (**31**) and potassium galactoisolespedezate (**32**). Ac = acetyl, AgOTf = silver trifluoromethanesulfonate, Bn = benzyl, TBDMS = *tert*-butyldimethylsilyl, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

the balance of concentration between **24** and **25** and **28**, wherein a biological clock should control leaf-movement by activation or expression of the enzyme concerned in this step.

5.2. Chemical Control of the Nyctinasty in *Phyllanthus urinaria* L.

Our study[44, 48] on the nyctinastic movement of *L. cuneata* revealed that nyctinastic leaf movement is controlled by a biological clock through regulation of the activity of β-glucosidase which hydrolyzes the leaf-opening substance of this plant. On the other hand, *P. urinaria* has a glycoside-type leaf-closing substance, and another type mechanism should operate in this plant.

We prepared six extracts collected from the plant *P. urinaria* every four hours through a day, and discovered that the extracts collected in the daytime and at night exhibited opposite bioactivity; the ones collected in the daytime exhibited leaf-opening activities and kept the leaves open even at night, while the ones collected at night exhibited weak leaf-closing activities and kept the leaves closed even during the daytime (Table 5). It is important that the bioactivity of the plant extract completely reflected the status of the collected leaves through a day; the extract collected when the leaves were closed showed leaf-closing activity and vice versa. This result suggested that the balance of concentration between **22** and **23** inversed through a day in the plant body.

Table 5. Bioactivity of the leaf extracts from *P. urinaria*.[a]

Time of collection (status of leaves)	daytime	night
midnight (− −)	−	−−
4.00 a.m. (− −)	−	−−
8.00 a.m. (+ +)	++	+
noon (+ +)	++	+
4.00 p.m. (−)	−	−−
8.00 p.m. (− −)	−	−−

[a] Concentration of leaf extracts = 0.1 g L^{-1}. The scale reaches from "completely open" (+ +) to "completely closed" (− −).

We have carried out a quantitative HPLC analysis of both **22** and **23** to measure the internal change in their concentration through a daily cycle, using the plant extracts prepared every four hours during a day. The moderately low polarities of **22** and **23** were suitable for the quantitative analysis. The result showed that the content of **23** was almost constant throughout a day; on the other hand, the content of **22** changed remarkably during a day (Table 6). The extract collected at 8.00 p.m. contained about tenfold as much **22** as the one collected at midnight. As the leaves stayed open from 4.30 a.m. until 4.30 p.m. and closed during the rest of the day, the concentration of the leaf-closing substance increases before the closure of the leaves, and decreases before the opening of them.

Table 6. Quantitative HPLC analysis of the concentration of **22**, **23**, and **35** in the leaf extracts from *P. urinaria*.

Time of collection	concentration [mol L^{-1}]			
	22	**23**	**35**	**23:22**
midnight	3.1×10^{-4}	7.8×10^{-4}	1.4×10^{-3}	2.5
4.00 a.m.	1.1×10^{-4}	9.1×10^{-4}	1.4×10^{-3}	8.3
8.00 a.m.	0.6×10^{-4}	8.3×10^{-4}	1.5×10^{-3}	13.8
noon	0.5×10^{-4}	9.7×10^{-4}	1.5×10^{-3}	19.4
4.00 p.m.	0.9×10^{-4}	8.2×10^{-4}	1.4×10^{-3}	9.1
8:00 p.m.	5.0×10^{-4}	7.6×10^{-4}	1.4×10^{-3}	1.5

There are two plausible mechanisms to deactivate **22**: by hydrolysis of the ester or the glycoside linkage. The content of phyllanthuric acid (**35**) proved to be constant through a day (Table 2); thus, it is revealed that **22** was not decomposed by the hydrolysis of the ester linkage in the plant body. This result suggests that **22** would be hydrolyzed to its aglycon similar to the case of *L. cuneata* (Figure 10).

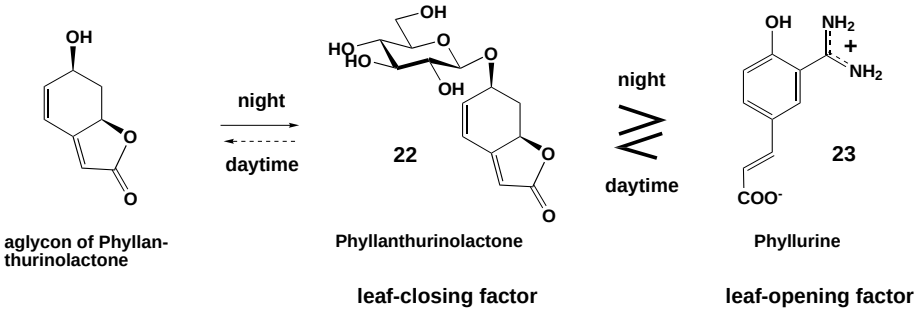


Figure 10. A model for the chemical control of nyctinasty in *Phyllanthus urinaria* L.

The HPLC analysis shows that the content of **22** was one-twentieth that of **23** in the daytime and increased to a half at night. The competition experiment between **22** and **23** was compatible with this result. When the existence of **24** was half that of **23**, the leaf was closed; on the other hand, when it was one-twentieth that of **23**, the leaf opened (Table 7). Thus, the difference in bioactivity between the plant extracts has been attributable to the difference in the content of the leaf-closing substance **22**.

Table 7. Competitive interaction between **22** and **23**.[a]

c (**22**) [mol L^{-1}]		ratio **22:23**						control
		1:0.5	1:1	0.5:1	1:5	1:10	1:20	sample
daytime	1×10^{-4}	−−	−−	−	−	++	++	++
night	1×10^{-4}	−−	−−	−−	−−	−−	++	−−

The scale reaches from "completely open" (++) to completely closed (−−).

The above results show the complete mechanism for the chemical control of nyctinasty. The transformation of **22** into its aglycon by the activation of β-glucosidase should be controlled by a biological clock. It is noteworthy that the concentration of glycoside-type bioactive substances changed through a day in both the cases of *L. cuneata* and *P. urinaria*. A common mechanism would operate in both plants. An important difference between them lies in the fact that the leaf-closing substance, which is a glucoside, would be hydrolyzed by the β-glucosidase in the case of *P. urinaria*.

5.3. The Universal Mechanism for the Regulation of Nyctinasty by a Biological Clock

We have demonstrated that the regulation of all nyctinastic leaf movements can be explained by one mechanism, namely, that either the leaf-closing or leaf-opening substance is a glycoside, and the glycoside is deactivated by a β-glucosidase, the activity of which is controlled by a biological clock.[21, 44] Our universal model for the regulation of leaf movement is shown in Figure 11. According to this model, there exist two types of plants; one has a glycoside-type leaf-opening substance which is deactivated by the activation of a β-glucosidase in the evening, the other has a glycoside-type leaf-closing substance which is deactivated by β-glucosidase in the morning.

6. Summary and Outlook

Leaf movement in nyctinastic plants has long been believed to be controlled by a common plant hormone. However, our results contradict this theory. Indeed, we advance a new theory of the chemical control of nyctinastic leaf movement; nyctinastic leaf-movement is controlled by the balance of concentration between two bioactive substances, leaf-opening and leaf-closing substances. This balance is inversed through the day according to the rhythm of the biological clock. The biological clock regulates this balance through the control of the β-glucosidase activity.

Our leaf-movement factors, whose concentrations have been proved to change according to a circadian rhythm, are thought to be genuine chemical signals that control leaf

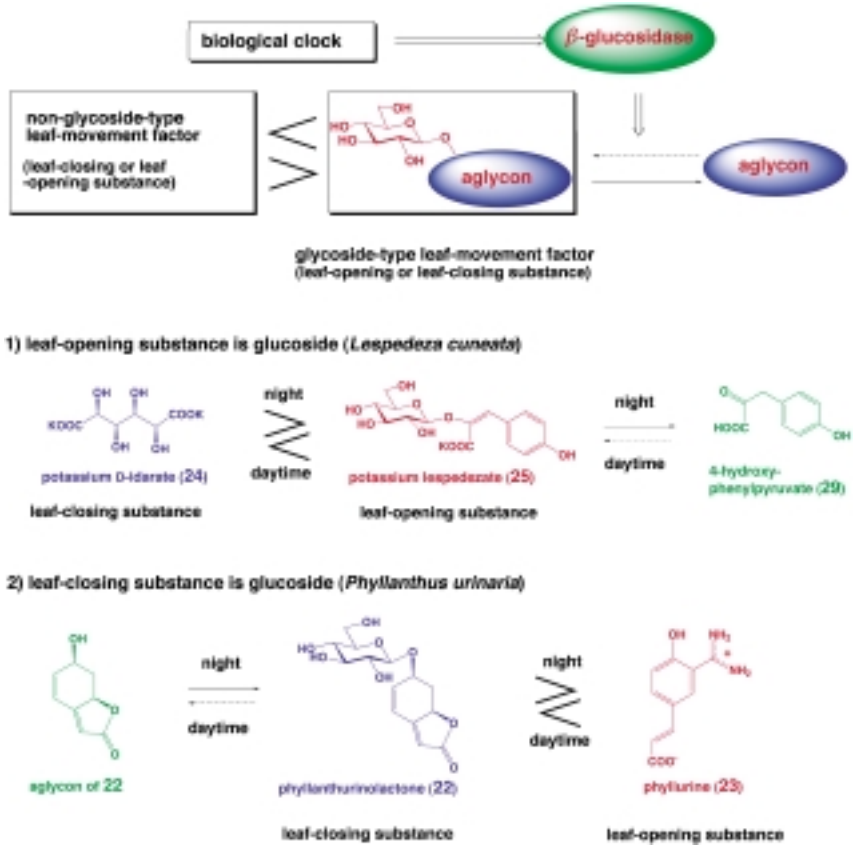


Figure 11. Model for a universal mechanism of chemical control of nyctinasty.

movement in nyctinastic plants under physiological conditions. They can be used as a tool for understanding the mechanism of leaf movement. Our present result will develop the science of this field from plant physiology into bioorganic chemistry.

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