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TETRAHEDRON

Leaf-movement Factors of Nyctinastic Plant, *Phyllanthus urinaria* L.; The Universal Mechanism for the Regulation of Nyctinastic Leaf-movement.

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Abstract : Phyllanthurinolactone (**1**) and phyllurine (**2**) were isolated from *Phyllanthus urinaria* L. as bioactive substances for nyctinasty. Compound **1** was quite effective for leaf–closing of the plant at 1×10^{-7} M in the daytime and **2** was quite effective for the leaf–opening at 2.5×10^{-5} M, whereas both **1** and **2** were not effective for other nyctinastic plants even at 1×10^{-4} M. The concentration of **1** increased before leaf-closure in the plant body, whereas that of **2** was constant through a day. Thus, the leaf-movement of *P. urinaria* L. is proposed to be controlled by the change in the balance of concentration between **1** and **2**, which is regulated by a β-glucosidase, similar to the case of *Lespedeza cuneata* G. Don. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: plants; natural products; biologically active compounds

INTRODUCTION

Most leguminosae plants close their leaves in the evening, as if to sleep, and open them in the morning.[1] This is called nyctinastic movement, and is known to be controlled by a biorhythm of the plant regulated by its biological clock. The discovery of a biological clock was based on the observation of nyctinastic movement in *Mimosa pudica* L. In the 18th century, a French scientist discovered that the rhythm involved in nyctinastic leaf-movement was maintained even under continuous darkness in a cave.[2]

Hitherto, we have isolated several chemical substances that induce the leaf-closing movement of nyctinastic plants.[3-10] Different leaf–closing substances exist in each nyctinastic plant. Furthermore, we have identified both leaf-opening and -closing substances from the same plant in several plant species. This implies that the nyctinastic movement would be controlled not only by a change in the concentration of the leaf-closing factor,[11] but also by competitive interaction between leaf–closing and –opening substances.

A nyctinastic plant, *Phyllanthus urinaria* L., belongs to the *Euphorbiaceae* family. We have already isolated two leaf-movement factors of contrasting bioactivities from *P. urinaria*.[6,10] Because of this difference in species, the structures of leaf-closing and -opening substances greatly differed from those of other leguminosae plants. Thus, it is important to clarify the mechanism for the control of nyctinasty in this plant to study the universality of our previously proposed mechanism. And we shall advance the universal mechanism of the chemical control of nyctinasty, which can explain all nyctinastic movement by only one key-word, that is, the control of the β-glucosidase activity by a biological clock.

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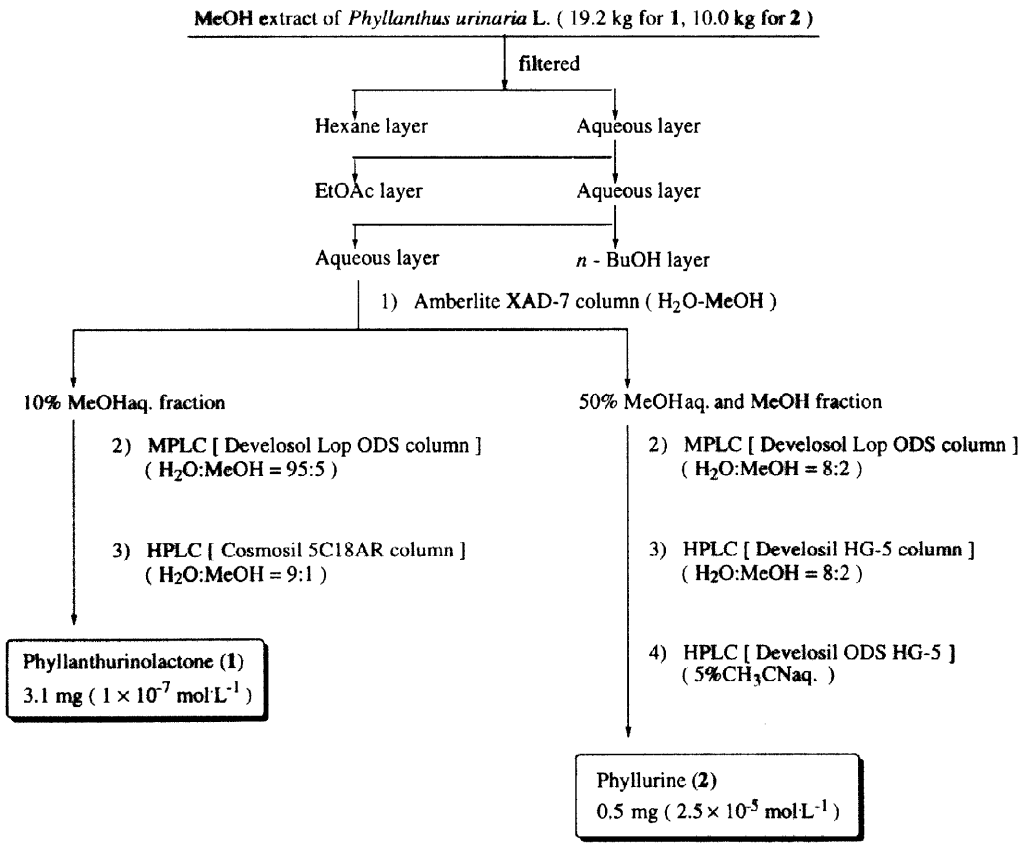
RESULTS AND DISCUSSION

P. urinaria was extracted with methanol. Purification of bioactive substances was carried out with monitoring the leaf-closing or -opening activity for the leaf of *P. urinaria*. Scheme 1 shows the overall isolation procedures.



Scheme 1. Isolation procedure of phyllanthurinolactone (1) and phyllurine (2) from *Phyllanthus urinaria* L.

The concentrated extract was partitioned with *n*-hexane, ethyl acetate, then with *n*-butanol. The bioactive aqueous layer was carefully separated by Amberlite XAD-7 column chromatography eluted with MeOH–H_2O. The most important point of this isolation procedure is the removal of anthocyanin pigments from the bioactive fractions. These pigments gave a very broad peak in chromatographies under neutral conditions and disturbed the clear fractionation. We could remove these pigments by using an Amberlite XAD-7 which adsorbed these pigments well. Moreover, the Amberlite XAD-7 completely separated the two bioactive fractions with leaf-closing and -opening activities, respectively. The bioactive fractions were further purified by using MPLC with MeOH–H_2O; the final purification by HPLC gave **1** as a yellow powder, and **2** as a colorless powder. Compound **2** was easily adsorbed on a reversed-phase ODS column; thus, the purification by HPLC gave only a small amount of **2**. Besides **2**, L-tryptophan was isolated as a weak leaf-

opening substance. All isolation procedures were carried out under neutral conditions, using no acid or buffer which induces leaf-closure.

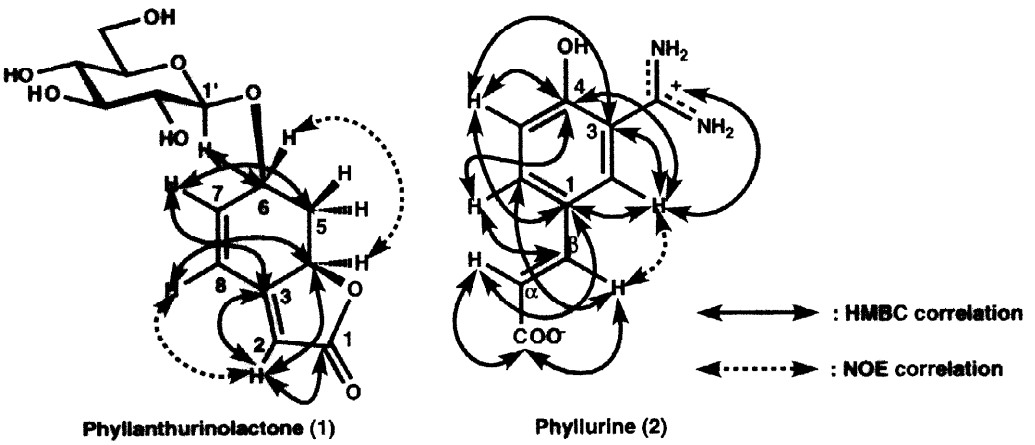
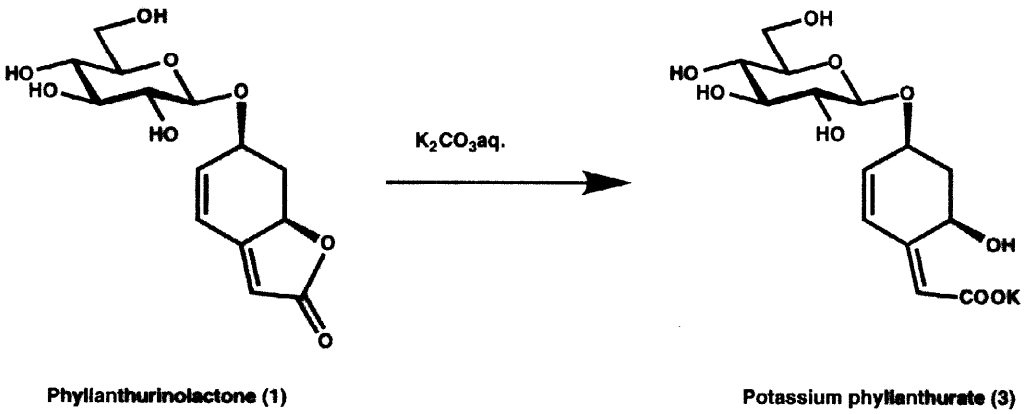


Fig.1. Important correlations in the HMBC and NOE spectra of **1** and **2**

Structure determinations of **1** and **2** were carried out by means of 2D NMR and FAB MS spectroscopy. First, we describe the structure determination of **1**. Positive-mode HR FAB MS analysis of **1** gave the $[M + H]^+$ ion at *m/z* 315.1078, from which molecular formula of $C_{14}H_{18}O_8$ could be deduced. From the ^1H NMR spectrum, **1** was expected to be a glycoside. Characterization of this hexose moiety commenced with the assignment of the glycoside protons in the ^1H NMR spectrum. The vicinal coupling constants between these protons showed that this hexose included in **1** was β- glucopyranoside. The structure for the aglycon part of **1** was determined by COSY andHMBC spectra to contain the conjugated diene lactone (Fig. **1**). Unfortunately, the HMBC correlation between H_4 and C_1 was not observed; however, the IR peak of the carbonyl function at 1737 cm^{-1} and ^{13}C NMR signal at δ 178 ppm suggested the presence of a lactone structure. The glycosilated hydroxyl group was determined as C_6 from the HMBC correlation between the anomeric proton ($H_{1'}$) and C_6. NOESY experiment and coupling constants revealed the stereochemical relationship of H_4–H_{5a}–H_6 to be *syn* (Fig. **1**). Coupling constant between H_6 and H_7 is almost zero; thus, the dihedral angle between them is thought to be nearly equal to $\pi/2$. The configuration of the diene moiety was determined to be *s-trans* by NOE correlation observed between H_2 and H_8 which indicates that these protons are in *syn* relationship (Fig. **1**). The absolute configuration of **1** was determined from the total synthesis by Mori *et al.*[12] They also synthesized all the possible stereoisomers relative to the diol moiety of the aglycon. One of the methylene signals, H_{5a}, is observed in lower-field as compared to H_{5b} ($\Delta\delta$ = 1.2 ppm). However, in potassium phyllanthurinate (**3**) (Fig. **2**), which was obtained by alkaline hydrolysis of **1**, the signal of H_{5a} shifted from 3.0 ppm to 2.4 ppm; thus, the δ-value for the original signal of H_{5a} seemed to be affected by vicinal oxygen atoms. A negative-mode CID MS/MS experiment carried out for the molecular ion peak gave the daughter ions at *m/z* 151 $[deglycosylM–H.]^-$, 133 $[M–H–Glc.]^-$, which supported this structure.



Phyllanthurinolactone (1) **Potassium phyllanthurate (3)**

Fig. 2. Alkaline hydrolysis of phyllanthurinolactone (1) to potassium phyllanthurate (3)

The compound **1** was quite effective for the leaf–closing of *P. urinaria* at 1×10^{-7} M in the daytime, but not effective on the other nyctinastic plants, *Cassia mimosoides* L., *Aeschynomene indica,* and *Mimosa pudica* L. even at 1×10^{-4} M. And the leaf–closing substances of other nyctinastic plants were not effective for *P. urinaria* even at 1×10^{-2} M. The stereochemistry of **1** was also very important for its bioactivity. Only the natural *syn*-(6*S*, 7a*R*) form showed leaf-closing activity.[12] This result suggests that some receptor for **1** would be concerned with the leaf-closing activity.

We also examined the effect of the lactone structure in **1**, because all of our previously isolated leaf-closing substances had the carboxylate group.[3,5,7] Thus, we prepared potassium phyllanthurate (**3**), by hydrolysis using potassium carbonate (Fig. 2). Compound **3** was purified by preparative HPLC, and was used for the bioassay. It was revealed that the bioactivity was diminished to the extent of one–hundredth on hydrolysis. Phyllanthurinolactone (**1**) is supposed to be a unique leaf-closing substance with the lactone and glycoside structures in the molecule.

Next, we describe the structure determination of **2**. A strong molecular ion was observed in the positive–mode HR FAB MS experiment to give the composition of **2** to be $C_{10}H_{10}O_3N_2$, while no molecular ion was observed in the negative–mode experiment. Despite this formula, **2** gave a negative result in the ninhydrine test; thus, the nitrogen atoms in **2** would exist in ammonium form. The aromatic region of the ^1H NMR spectrum of **2** showed that **2** has a 1,2,4-trisubstituted aromatic ring. There was also observed a conjugated carboxylic acid moiety in this region. HMBC correlations observed between these two parts gave the structure of *p*-coumarate substituted at the C_3-position (Fig. **1**). The residual part of **2** was deduced to be an amidinium ion, which is connected to the C_3-position of **2**. This is supported by the weak correlation between H_2 and the immonium carbon observed in the HMBC experiment (Fig. **1**). The presence of the amidine group was also supported by the IR spectrum. A peak was observed at 1698 cm^{-1} corresponding to the stretching of the imino group. This amidine carbon was difficult to be detected by ^{13}C NMR experiment, probably because of the broadness of its signal, similar to the case of the guanidino function.[8] The FG HMBC experiment gave a cross peak between the amidinium carbon and H_2. The chemical shift of the C_3–position in the ^{13}C NMR spectrum shifted to lower field with the change of the solvent from CD_3OD/D_2O = 6/4 (129 ppm)

into D_2O (148.6 ppm). This is attributed to the inhibition of the zwitter ionic structure of **2** in CD_3OD/D_2O = 6/4, whereas natural **2** is supposed to have a zwitter ionic structure in the plant body.

Interestingly, *P. urinaria* belongs to the *Euphorbiaceae* family. Because of this difference in species, the structures of **1**, which is the only glycoside-type leaf-closing substance ever isolated, and **2** greatly differ from those of other leguminosae plants.

L-Trp was also isolated as a weak leaf-opening substance from *P. urinaria* together with **2**. It is important to determine which is the genuine leaf-opening substance of this plant. We determined that **2** is the genuine leaf-opening substance of this plant from the following results. L-Trp was effective at as low as 1×10^{-4} M for the leaf-opening of *Phyllanthus* leaves. The bioactivity arising from L-Trp was easily distinguishable from that of **2**, because L-Trp opens the leaves of all other nyctinastic plants similar to indole-3-acetic acid (IAA). It is proposed that the bioactivity of L-Trp is attributed to IAA, which is known as an important metabolite of L-Trp.[13] IAA has been already reported to show weak leaf-opening activity to the leaves of all nyctinastic plants.[14] The bioassay was carried out by the addition of the sample solution at 11:00 a.m.; this long period necessary for the bioassay to detect the leaf-opening activity is sufficient for the metabolism of L-Trp into IAA. On the other hand, **2** was effective at as high as 2.5×10^{-5} M only for the leaves of *P. urinaria.*, while it was 250-fold as effective as L-Trp for the leaves of *P. urinaria.* And, **2** was not effective for other nyctinastic plants, such as *Aeschynomene indica* and *Albizzia julibrissin* Durazz., even at 1×10^{-4} M. All of the leaf-movement factors previously isolated by us showed specific bioactivity on a plant species[3-10] and the bioactivity of **2** was much stronger than that of L-Trp; thus, the genuine leaf-opening substance of this plant should be **2**.

We have now isolated both of the bioactive substances, **1** and **2**, controlling nyctinastic leaf-movement from *P. urinaria.* In the case of *Lespedeza cuneata* G. Don,[15] we have shown that a change in the balance between leaf-opening and leaf-closing substances controls the nyctinasty, and the balance is controlled by a biological clock through the regulation of the activity of β-glucosidase which hydrolyzes the glycosidic bond of the leaf-opening substance in this plant.[15] However, in this case, we could analyze only the concentration of the leaf-opening substance, because of the difficulty in the analysis of the highly polar leaf-closing substance. A most important problem is whether a similar model would be applicable in the case of *P. urinaria.* In this case, the leaf-closing substance **1**, which is a glucoside, would be hydrolyzed by the β-glucosidase whose activity is regulated by a biological clock.

The plant extracts of *P. urinaria* were collected every four hours through a day. We have discovered that the extracts collected in the daytime and at night respectively exhibited opposite bioactivity to each other; the ones collected in the daytime exhibited leaf-opening activity and kept the leaves open even at night, while the ones collected at night exhibited weak leaf-closing activity and kept the leaves closed even during the daytime (Table **1**). It is important that the bioactivity of the plant extract completely reflected the status of the collected leaves: the extract collected when the leaves were closed showed leaf-closing activity and vice versa. This result suggested that the balance of concentration between **1** and **2** inversed through a day in the plant body.

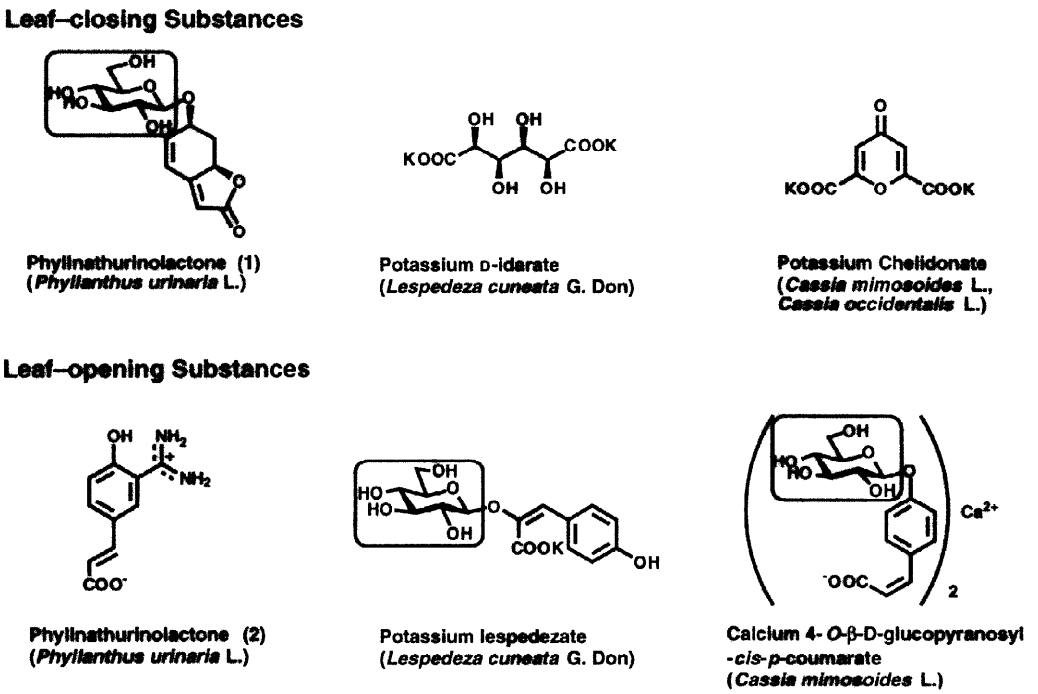


Fig. 4 The universality of glucosides among the leaf-movement factors

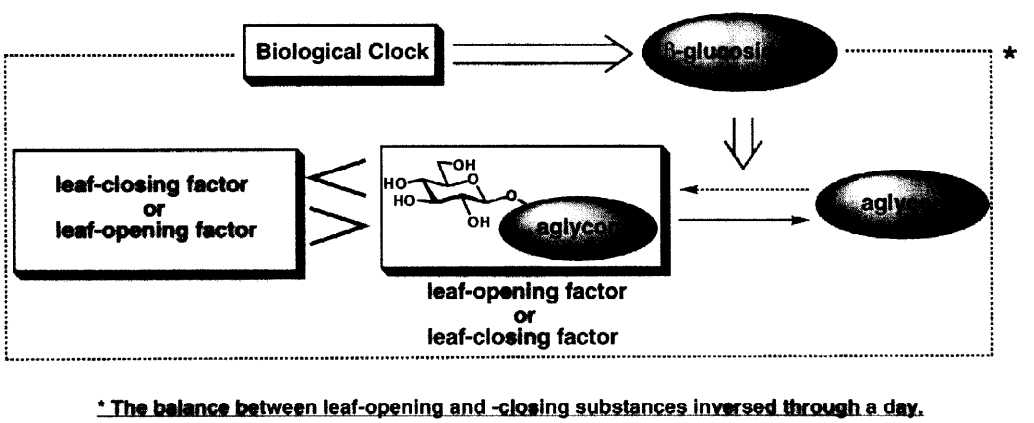


Fig. 5 The universal mechanism for nyctinasty

We thought that *Albizzia. julibrissin* DURAZZ[8] and *Mimosa pudica* L., [16] whose leaf-opening substances are non-glycoside type, have a glycoside-type leaf-closing substance, and *Aeshynomene indica.* whose leaf-closing substance is a non-glycoside type has the corresponding glycoside-type leaf-opening substance. The search for these bioactive substances and the measurement of the β-glucosidase activity concerned with nyctinasty are now in progress, in a variety of nyctinastic plants.

We have carried out a quantitative HPLC analysis of both **1** and **2** to measure the internal change in their concentration through a daily cycle, using the plant extracts prepared every four hours during a day, with HPLC equipped with a photodiode array detector. The moderately low polarities of **1** and **2** were suitable for the quantitative analysis.

Table 1.
Bioactivity of the extract collected every four hours

Time of collection (status of the leaves)	Daytime	Night
0:00 a.m. (– –)	–	– –
4:00 a.m. (– –)	–	– –
8:00 a.m. (++)	++	+
0:00 p.m. (++)	++	+
4:00 p.m. (–)	–	– –
8:00 p.m. (– –)	–	– –

concentration: 0.1 g/L, ++ completely open ; + nearly open ; + – at random ; – nearly closed ; – – completely closed

Under acidic conditions using 1% MeOHaq. containing 1% AcOH as a mobile phase, good separation was achieved on the HPLC analysis. The result showed that the content of **2** was almost constant through a day; on the other hand, the content of **1** remarkably changed through a day (Table. **2**).[16] The extract collected at 8:00 p.m. contained about ten-fold as much **1** as the one collected at 0:00 p.m. As the leaves kept 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. There are two possibilities to deactivate **1**, by hydrolysis of the ester or glycoside linkage. The content of **3** proved to be constant through a day (Table 2); thus, it is revealed that **1** was not decomposed by the hydrolysis of the ester linkage in the plant body. This result suggests that **1** would be hydrolyzed to its aglycon similar to the case of *L. cuneata* (Fig. **3**).[15]

Table 2.
Quantitative HPLC analysis of the concentration of **1**, **2**, and **3** collected every four hours.

Time of collection	Concentration [mol·L^{-1}]			**2/1**
	1	**2**	**3**	
0:00 a.m.	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
0:00 p.m.	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

From the HPLC analysis, the content of **1** was one-twentieth of that of **2** in the daytime, and increased to one-half at night. The competition experiment between **1** and **2** was compatible with this result. When the existence of **1** was one-half of that of **2**, the leaf was closed; on the other hand, when it was one-twentieth of that of **2**, the leaf opened (Table 3). Thus, the difference in bioactivity between the plant extracts has been attributable to the difference in the content of the leaf-closing substance, **1** (Fig. 3).

Table 3.
Competitive interaction between **1** and **2**

Concentration of (**1**) [$mol \cdot L^{-1}$]		Ratio of (**1**) and (**2**)						Control
		1 : 0.5	1 : 1	0.5 : 1	1 : 5	1 : 10	1 : 20	
Day time	1×10^{-4}	– –	– –	–	–	++	++	++
Night	1×10^{-4}	– –	– –	– –	– –	– –	++	– –

++ completely open ; + nearly open ; + – at random ; – nearly closed ; – – completely closed

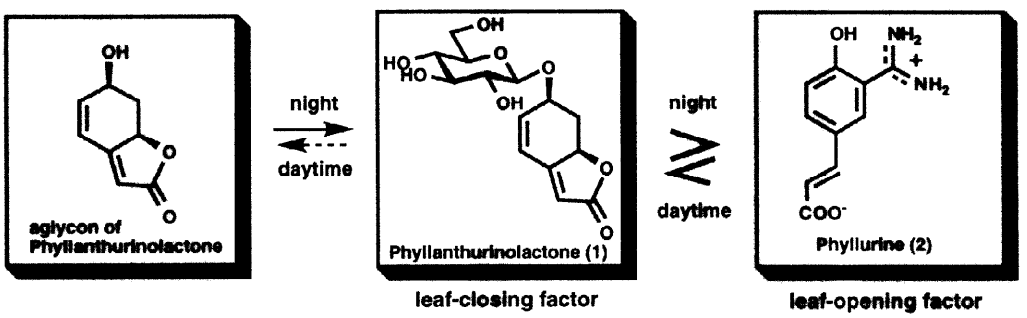


Fig.3 The chemical mechanism for the regulation of nyctinastic leaf-movement in *P. urinaria*.

In our previous paper, we described that there were two plausible mechanisms for the control of the balance between leaf-opening and -closing substances: (1) the concentrations of both the leaf-closing and -opening substances change by the control of the biological clock.; (2) the concentration of only one bioactive substance changes by the control of the biological clock, while the concentration of the other one is constant through a day. Now, we can conclude that mechanism (2) operates in the case of *P. urinaria*. This result shows the complete mechanism for the chemical control of nyctinasty. The transformation of **1** into its aglycon by the activation of a 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* (Fig. 4).

Taking this result into account, we can advance a universal mechanism for the chemical control of nyctinasty, namely, that 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. Our universal model for the regulation of leaf-movement is shown in Fig. 5. There would 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 activated in the morning.

Materials and Methods

General Procedures. UV-VIS spectra were obtained in an aqueous solution on a Jasco UVIDEC-610A spectrophotometer at room temperature. 2D NMR, ^{1}H NMR (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on 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 spectra were measured on a Jeol JMS-700 spectrometer, using glycerol as a matrix. CID MS/MS experiments were carried out on a Finnigan MAT TSQ 700 mass spectrometer using glycerol as a matrix. The CID experiments were performed with argon as a collision gas at the collision energy of -29 eV. 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 photodiode-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. P. urinaria that was used for extraction was purchased from Aroma Engei in Kagoshima, Japan. *P. urinaria* 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 *P. urinaria.* with a sharp razor blade were used for the bioassay. One leaf was placed in H_2O (*ca* 1.0 ml) using a 20–ml glass tube in the greenhouse at 30 °C and allowed to stand overnight. The leaves which opened again 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 6:00 p.m. and leaf-closing within two hours after the addition of the sample.

Extraction of Plants Collected during a 24-h Day. Fresh leaves of *P. urinaria* (50 g) were collected every four hours throughout the day (4:00 a.m., 8:00 a.m., 12:00 p.m., 1:00 p.m., 4:00 p.m., 8:00 p.m., and 12:00 a.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, are then partitioned between *n*-hexane (150 mL × 3). After centrifugation (2,000 rpm × 20 min), they were evaporated to 30 ml under reduced pressure. These residues were used for quantitative analyses of **1**, **2** and **3**.

Quantitative Analysis of bioactive substances. The amounts of **1** and **2** in each aqueous layer were determined by an HPLC analysis using a Develosil ODS HG-5 column (ϕ 4.6 × 250 mm, Nomura Chemicals Co.) with 1% MeOH aq. containing 1% AcOH as the mobile phase (flow rate, 0.8 mL/min; detection at 260 nm for **1** and 348 nm for **2**) at 40 °C. 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.

Isolation of Phyllanthurinolactone (1). The fresh whole plant of *P. urinaria* L. (19.2 kg) was extracted with methanol 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 *P. urinaria* L. 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, Organo Co.) eluted with MeOH–H_2O (0 : 10, 1 : 9, 2 : 8, 3 : 7, and 10 :0 × 1 L each). Bioactive 10% MeOH eluate was further purified by using Develosil Lop-ODS glass column (ϕ 55 × 500 mm, Nomura Chemicals, Co) with MeOH–H_2O (5 : 95). Final purification by HPLC using Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co.) with MeOH–H_2O (10 : 90) gave **1** (3.1 mg) as a yellow powder. Phyllanthurinolactone (**1**): ^{1}H NMR (400 MHz, D_2O) δ 6.75 (1H, br.d, J = 10 Hz, H_8), 6.46 (1H, br.d, J = 10 Hz, H_7), 5.95 (1H, br.s., H_2), 5.14 (1H, ddd, J = 1.5, 4.6, and 12 Hz, H_4), 4.85 (1H, ddd, J = 1.5, 5.7, and 10.5 Hz, H_6), 4.68 (1H, d, J = 8 Hz, $H_{1'}$), 3.91 (1H, dd, J = 2.0, 12.5 Hz, $H_{6'a}$), 3.72 (1H, dd, J = 6.0, 12.5 Hz, $H_{6'b}$), 3.50 (1H, t, J = 9 Hz, $H_{3'}$), 3.47 (1H, ddd, J = 9.0, 6.0, 2.0 Hz, $H_{5'}$), 3.39 (1H, t, J = 9.0 Hz, $H_{4'}$), 3.27 (1H, dd, J = 8.0, 9.0 Hz, $H_{2'}$), 3.04 (1H, br.td, J = 5.7, 11 Hz, H_{5a}), 1.78 (1H, td, J = 11, 12 Hz, H_{5b}).; ^{13}C NMR (100 MHz, D_2O, 30 °C) δ 178. 5, 167.0, 142.3, 122.3, 112.0, 103.5, 80.9, 77.6, 77.3, 76.1, 74.6, 71.1, 62.3, 39.2.; IR (film) 1737, 1643, 1161, 1100, 1077 cm^{-1}; UV-VIS spectrum (H_2O) λ_{max} (ε) 257 (26000) nm; $[\alpha]_D^{21}$ –6.0 ° (c 0.20, H_2O). HR FAB MS (positive): $[M+H]^+$ Found *m/z* 315.1078, $C_{14}H_{19}O_8$ requires *m/z* 315.1080.

Alkaline hydrolysis of Phyllanthurinolactone (1). Phyllanthurinolactone (**1**) (1.8 mg, 5.7 × 10^{-6} mol) and potassium carbonate (2 mg, 1.45 × 10^{-5} mol) were dissolved in 250 µL of H_2O. After being stirred for 24 hrs at room temperature, the reaction was quenched by the addition of AcOH to adjust the pH to 5. The solution was dried up and dissolved in 50 µL of 5% MeOHaq. The reaction mixture was separated by HPLC using Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co.) with 5% MeOHaq. as eluent to give potassium phyllanthurate (**3**, 0.5 mg, 32.7%): ^{1}H NMR (400 MHz, D_2O) δ 6.22 (1H, d, J = 10 Hz, H_8), 6.17 (1H, br.d, J = 10 Hz, H_7), 5.83 (1H, br.s., H_2), 5.00 to 4.70 (H_4, H_6), 4.65 (1H, J = 8 Hz, $H_{1'}$), 3.90 (1H, dd, J = 2.0, 12.5 Hz, $H_{6'a}$), 3.71 (1H, dd, J = 6.0, 12.5 Hz, $H_{6'b}$), 3.49 (1H, t, J = 9 Hz, $H_{3'}$), 3.47 (1H, ddd, J = 9.0, 6.0, 2.0 Hz, $H_{5'}$), 3.38 (1H, t, J = 9.0 Hz, $H_{4'}$), 3.26 (1H, dd, J = 8.0, 9.0 Hz, $H_{2'}$), 2.39 (1H, br.td, J = 5.9, 12

Hz, H_{5a}), 1.83 (1H, td, J = 12, 11 Hz, H_{5b}). Signals of H_4 and H_6 proton could not be observed because of the large signal of HOD in D_2O.; ESI MS (negative) *m/z* 331.1 $[M-H]^-$.

Isolation of Phyllurine (2). Isolation of the leaf-opening substance was carried out based on a bioassay using the leaves of *P. urinaria*. The bioactive fraction kept the leaves open until 6:00 PM.[5] The fresh whole plant of *P. urinaria* (10.0 kg) was extracted with methanol for two weeks and concentrated *in vacuo*. The concentrated extract was partitioned with ethyl acetate (200 mL × 5), then with *n*–butanol (200 mL × 5). 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, 15:85, 25:75, 35:65, 50:50, and 10:0 × 1 L each), and the 25%, 50%, and 100% MeOH aq. fractions showed weak leaf-opening activity. The 25% MeOH aq. fraction was analyzed, and it was revealed that this contained L-tryptophane as a leaf-opening substance. The 50% and 100% MeOH aq. fractions were further purified by MPLC using Develosil Lop ODS glass column (Nomura Chemicals Co.) with 20% MeOH aq., HPLC using preparative Develosil ODS HG–5 column (ϕ 20 × 250 mm, Nomura Chemicals Co.) with 20% MeOH aq., and HPLC using analytical Develosil ODS HG–5 column (ϕ 4.6 × 250 mm, Nomura Chemicals Co.) with 5% CH_3CN aq. to give phyllurine (**2**, 0.5 mg): Phyllurine (**2**) : ^1H NMR (400 MHz, D_2O, 35 °C): 7.75 (1 H, d, *J* = 16 Hz, H_α), 7.25 (1 H, d, *J* = 2 Hz, H_2), 7.15 (1 H, dd, *J* = 2 and 8 Hz, H_6), 6.95 (1 H, d, *J* = 8 Hz, H_5), 6.50 (1 H, d, *J* = 16 Hz, $H\beta$) ppm.; ^{13}C NMR (100 MHz, D_2O, 35 °C): 170.5 ($C_{carbonyl}$), 148.6 (C_3), 147.0 (C_α), 146.1 ($C_{immonium}$), 145.8 (C_4), 128.7 (C_6), 124.3 (C_1), 117.9 (C_5), 116.8 (C_2), 116.0 ($C\beta$) ppm.; UV–vis (H_2O) $\lambda_{max}(\varepsilon)$ 324 (8100), 302 (7400), 212 (13000) nm.; IR ν : 1698, 1603 cm^{-1}; HR FAB MS (positive): $[M + H]^+$ Found *m/z* 207.0807, $C_{10}H_{11}O_3N_2$ requires *m/z* 207.0844.

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