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PHYLLURINE, LEAF-OPENING SUBSTANCE OF A NYCTINASTIC PLANT, *PHYLLANTHUS URINARIA* L.

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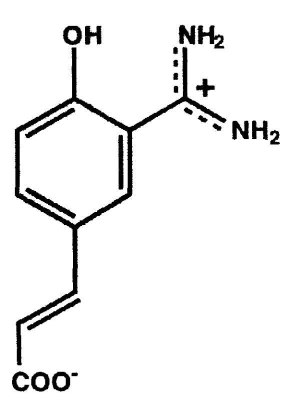
Abstract : We have isolated phyllurine (**1**) as the leaf–opening substance of *Phyllanthus urinaria* L. that keeps the *Phyllanthus* leaves open. **1** was quite effective for the leaf–opening of *Phyllanthus urinaria* L. at 2.5×10^{-5} M, but not effective for other nyctinastic plants even at 1×10^{-4} M. The leaf-closing substance of this plant has already been identified as phyllanthurinolactone (**2**). Thus, the leaf-movement of *Phyllanthus urinaria* L. is proposed to be controlled by the interaction between **1** and **2**, similar to the case of *Lespedza cuneata* G. Don.

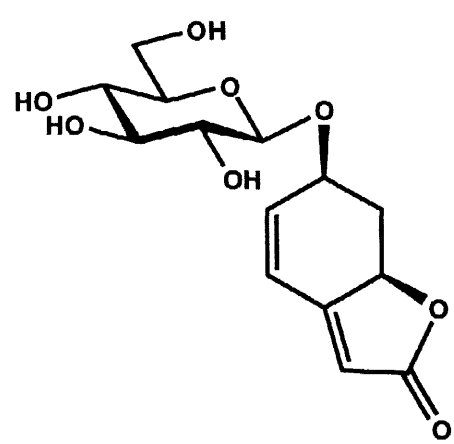
Keywords: plants; natural products; biologically active compounds; zwitter ions.

Most leguminosae plants close their leaves in the evening, as if to sleep, and open them in the morning.[1] This is called nyctinasty, and such a circadian rhythmic movement has been known to be controlled by their biological clocks.[2] Recently, we have identified several bioactive substances that regulate this leaf-movement,[3] and revealed that nyctinastic movement of the plants is controlled by the interaction between leaf-closing and -opening substances.[4] A biological clock controls the balance between these two bioactive substances.

Interestingly, a nyctinastic plant, *Phyllanthus urinaria* L., belongs to the *Euphorbiaceae* family. Because of this difference of species, the structure of phyllanthurinolactone (**2**),[2c] which is the only glycoside-type leaf-closing substance ever isolated, greatly differs from those from other leguminosae plants. Thus, it is important to identify the leaf-opening substance of this plant and reveal the differences in the regulating mechanism for nyctinastic movement.



Phyllurine (1)



Phyllanthurinolactone (2)

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Isolation of the leaf-opening substance was carried out based on a bioassay using a leaf of *Phyllanthus urinaria* L. The bioactive fraction keeps the leaves open until 6:00 PM.[5] The fresh whole plant of *Phyllanthus urinaria* L. (10.0 kg) was extracted with methanol for two weeks and concentrated *in vacuo*. The concentrated extract was partitioned with ethyl acetate, then with *n*–butanol. The bioactive aqueous layer was carefully separated by Amberlite XAD–7 column chromatography eluted with MeOH–H_2O (0:10, 15:85, 25:75, 35:65, 50:50, and 10:0), 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 with 20% MeOH aq., HPLC using preparative Develosil ODS HG–5 column with 20% MeOH aq., and HPLC using analytical Develosil ODS HG–5 column with 5% CH_3CN aq. to give phyllurine (**1**, 0.5 mg).

L-Trp was effective at as low as 1×10^{-4} M on *Phyllanthus* leaves and 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.[6] IAA has been already reported to show weak leaf-opening activity to the leaves of all nyctinastic plants.[7] The bioassay was carried out by the addition of the sample solution at 11:00 PM.; thus, 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, **1** was effective at as high as 2.5×10^{-5} M only for the leaves of *Phyllanthus urinaria* L., and, similar to other leaf-opening substances, 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] thus, the genuine leaf-opening substance of this plant should be **1**.

Structural determination of **1** was carried out by means of NMR and FAB MS experiments. HMQC, HMBC, and NOE experiments gave the structure of **1**.[8] The aromatic region of the ^1H NMR spectrum of **1** showed that **1** has a 1,2,4-trisubstituted aromatic ring. There was also observed a conjugated carboxylic acid moiety in this region. Correlations observed between these two parts gave the structure of *p*-coumarate substituted at the C_3-position (Fig. 1). A strong molecular ion was observed in the positive–mode HR FAB–MS experiment to give the composition of **1** to be $C_{10}H_{11}O_3N_2$, while no molecular ion was observed in the negative–mode experiment. Thus, the residual part of **1** was deduced to be an amidinium ion, which is connected to the C_3-position of **1**. This is supported by the weak correlation between H_2 and immonium carbon observed in HMBC experiment (Fig. 1). The presence of the amidine group was also supported by IR spectrum. A peak was observed at 1698 cm^{-1} corresponding to the stretching of the imino group, together with a peak at 1603 cm^{-1} corresponding to the stretching of the carboxylate group. This amidine carbon was difficult to detect by ^{13}C NMR experiment, probably because of the broadness of its signal, similar to the case of the guanidino function. The chemical shift of the C_3–position in ^{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).[9] This is attributed to the inhibition of the zwitter ionic structure of **1** in CD_3OD/D_2O = 6/4, whereas natural **1** is supposed to have zwitter ionic structure in the plant body.

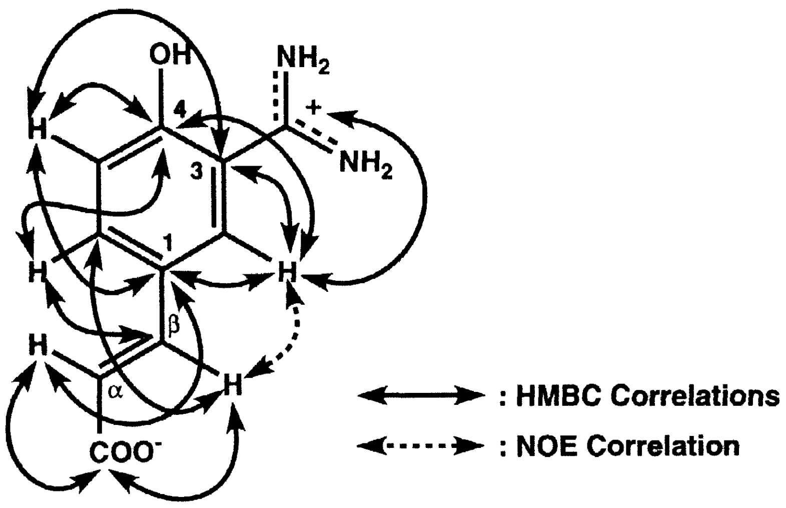

Fig. 1 Important Correlations Observed in HMBC and NOE Experiments.

Similar to other nyctinastic plants, the nyctinastic leaf-movement of ***Phyllanthus urinaria*** is assumed to be controlled by the competitive interaction between leaf–closing and –opening substances.[4, 10, 11] We have now isolated both of these substances, **1** and **2**, from *Phyllanthus urinaria*. Further studies on the regulation of the nyctinastic movement by a biological clock are now in progress. Our previous study on the nyctinastic movement of *Lespedeza cuneata* G. Don revealed that nyctinastic leaf-movement is controlled by a biological clock through the regulation of the activity of β-glucosidase which hydrolyzes the leaf-opening substance of this plant.[4, 10] A similar model would be applicable in the case of *Phyllanthus urinaria* L. In this case, the leaf-"closing" substance, which is a glucoside, would be hydrolyzed by the β-glucosidase whose activity is regulated by a biological clock. There is some possibility that the regulation of all nyctinastic leaf-movements can be explained by only one mechanism that either the leaf-closing or –opening substance is a glucoside in all nyctinastic plants. The biological clock regulates the activity of β-glucosidase which deactivates the glucoside to control the internal balance of concentration between leaf–closing and –opening substances.

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5. Nyctinastic plants belonging to legminosae close their leaves around 6:00 PM. On the other hand, Phyllanthus urinaria L. closed their leaves around 3:00 PM. Leaf-opening activities are usually judged by the time lag of about three hours in the leaf-closing movement, thus, in this plant, we have judged the leaf-opening activity by the leaf-opening until 6:00 PM.

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8. Phyllurine (**1**) : ^{1}H NMR (400 MHz, D_2O, 35 °C): 7.75 (1 H, d, J = 16 Hz, Hα), 7.25 (1 H, d, J = 2 Hz, H2), 7.15 (1 H, dd, J = 2 and 8 Hz, H6), 6.95 (1 H, d, J = 8 Hz, H5), 6.50 (1 H, d, J = 16 Hz, Hβ) ppm.; ^{13}C NMR (100 MHz, D_2O, 35 °C): 170.5 ($C_{carbonyl}$), 148.6 (C3), 147.0 (Cβ), 147.0 (Cα), 146.1 ($C_{immonium}$), 145.8 (C4), 128.7 (C6), 124.3 (C1), 117.9 (C5), 116.8 (C2), ppm.; UV–vis (H_2O) $\lambda_{max}(\varepsilon)$ 324 (7200), 302 (6600), 212 (12000) 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.

9. The chemical shift of the C_4–position in ^{13}C NMR spectrum also shifted to higher field with the change of the solvent from CD_3OD/D_2O = 6/4 (151.5 ppm) to D_2O (145.8 ppm).

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