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Fluorescence study on the nyctinasty of *Cassia mimosoides* L. using novel fluorescence-labeled probe compounds

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Abstract—We synthesized fluorescence-labeled probe compounds bearing 6-((7-amino-4-methylcoumarin-3-acetyl)amino)-hexanoyl (AMCA, **1**), 6-*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-aminohexanoyl (NBD, **2**), and 6-(4-((5-dimethylaminonaphthalene-1-sulfonyl)-amino))-hexanoyl (dansyl, **3**) groups as the fluorescent functionality. In these probe compounds, NBD-type probe, **2**, showed leaf-opening activity at 5×10^{-6} M. The bioactivity of **2** is one-fifth as strong as that of the natural product, potassium isolespedezate (**6**). We carried out the binding experiment using **1** in a plant body. Then, it was suggested that fluorescence-labeled probe compound directly bound to a motor cell in pulvina of *Cassia mimosoides*. And this binding was specific to *C. mimosoides*. Probe compounds cannot bind plant sections of other nyctinastic plants. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Most legumes 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] We have identified several bioactive substances that regulate this leaf-movement,[3–16] and our recent studies revealed the mechanism for the control of nyctinasty by the biological clock.[12–14] The next issue is to determine how these compounds induce leaf-movement. For this reason, our current study is focused on the mode of action of the leaf-movement factor. However, different from the studies using some cell-lines, our bioassay is carried out by using whole plant-leaf. Thus, we should start from determining a cell to which leaf-movement factor binds. Our leaf-movement factor is supposed to induce the swelling and shrinking of motor cells by direct or indirect interaction with motor cells, which exists in the pulvini of the plant and is most important for the leaf-movement. The cell exert their function by swelling and shrinking in the process of leaf-opening and -closing and play a central role in the plant leaf-movement.[17] The ion fluxes followed by massive water fluxes across the plasma membrane of these cells produce the swelling and shrinking behavior of the motor cells. To determine the role of leaf-movement factor in plant leaf-movement, we developed molecular probes based on the leaf-movement factor for identification of its target cell in the plant body, which leads to bioorganic studies of nyctinasty. Investigation of the target cells where bioactive substances are perceived is the first step towards the bioorganic study of their receptor molecule. Here, we report chemical synthesis of novel fluorescence-labeled probe compounds bearing 6-((7-amino-4-methylcoumarin-3-acetyl)amino)-hexanoyl (AMCA, **1**), 6-*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-aminohexanoyl (NBD, **2**), and 6-(4-((5-dimethylaminonaphthalene-1-sulfonyl)-amino))-hexanoyl (dansyl, **3**) groups as the fluorescent functionality, and the direct observation of the target cell for the leaf-movement factor using these probe compounds.

2. Results and discussion

2.1. Synthesis of fluorescence-labeled probe compounds with small fluorescence dye

Recently, we synthesized FITC-labeled potassium galacto-isolespedezate (**4**),[18,19] based on the artificial leaf-opening substance **5**,[20,21] which showed leaf-opening activity against the leaf of *Cassia mimosoides* L. However, the bioactivity of **4** was one-fiftieth as strong as that of the natural product (**6**); thus, a fluorescence labeled probe compound of much stronger bioactivity was required for the bioorganic study of nyctinasty. We examined the length of linker moiety in FITC-labeled probe compounds, to improve their bioactivities. However, according to this methodology, bioactivity of **4**, for example, was at most improved to one-fiftieth of **6**, which is insufficient for bioorganic study. These results suggest that the weak bioactivity of **4** would be attributed to the large size of the FITC group. Based on

Keywords: nyctinasty; leaf-opening substance; fluorescence; probe compound; motor cell.

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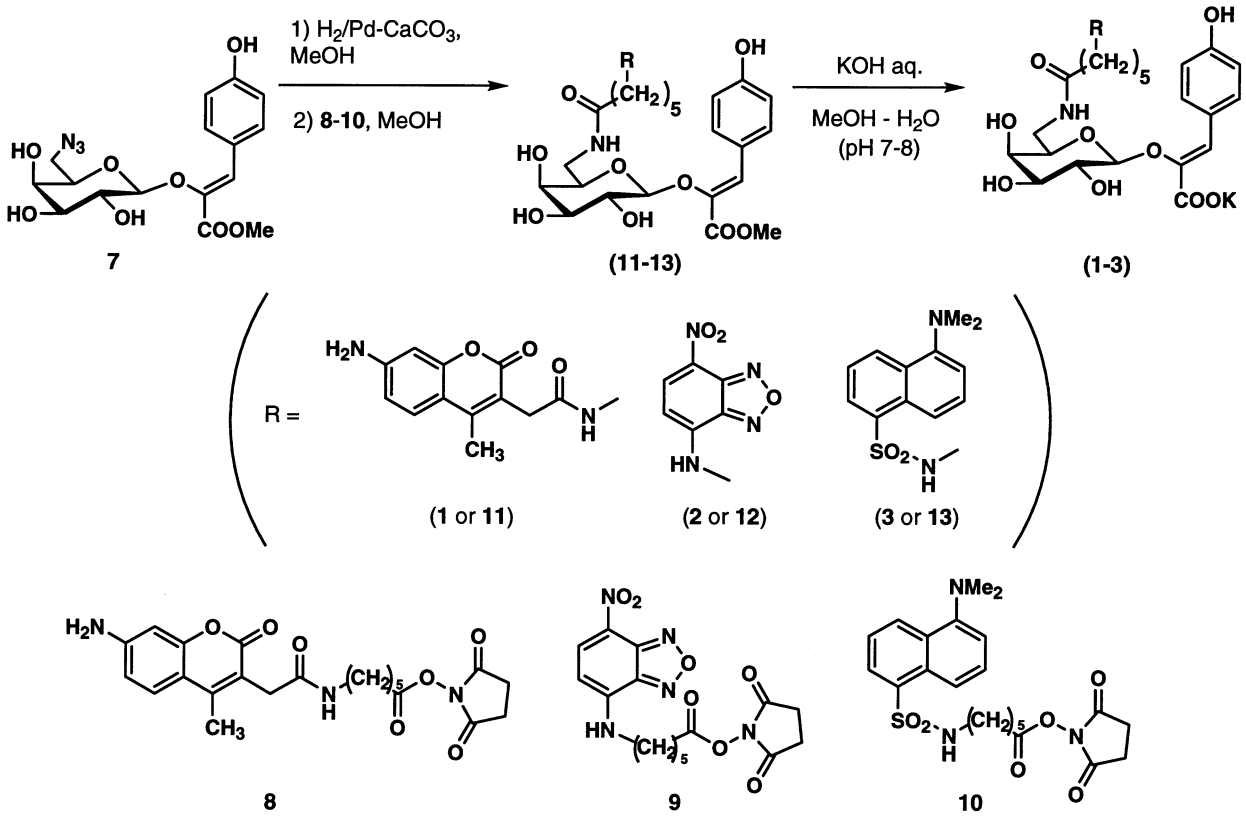
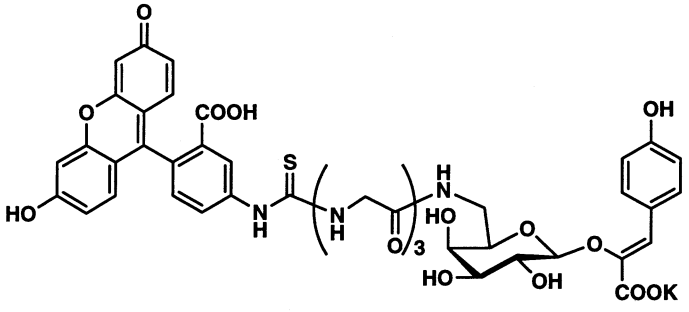


Scheme 1. Synthetic route of fluorescence probe compounds (**1**–**3**).

this idea, we examine AMCA, NBD, and dansyl groups as the fluorescence dyes.[22] All these fluorescence dyes are much smaller than the FITC. We synthesized probe compounds bearing those fluorescent dyes according to the procedure previously reported in Ref. 19. As shown in Scheme 1, compound **7**, which was prepared according to the previously established procedure,[19] was coupled with succinimide-type fluorescence reagents (**8**–**10**). An aminosugar and a hexanoic acid linker in the fluorescent reagents were connected through an amide bond, which is assumed to be stable against the hydrolysis by an esterase in the plant body. However, the reactivity of the amino group in the aminosugar was so low that secondary hydroxyl groups in the sugar moiety or phenolic hydroxyl group reacted predominantly to give by-products which have plural fluorescent functionalities in a molecule. Addition of some bases, such as DMAP or TEA, did not improve the reaction yield. Moreover, purification of the resulting coupling product (**11**–**13**) in this step caused a serious loss of the yield because of the aggregative properties of **11**–**13** to form an insoluble precipitate; accordingly, the product was subjected to the following hydrolysis reaction without purification to improve the yield. After alkaline hydrolysis in the final step from an ester (**11**–**13**) to a potassium salt (**1**–**3**), pH of the reaction mixtures must be adjusted to weakly basic (pH 7–8) with monitoring pH by a pH meter to circumvent the decomposition of a fluorescent group in the course of work-ups. Probe compounds (**1**–**3**) were mainly obtained as the (*Z*)-form along with a trace amount of the (*E*)-form. The double bond in **1**–**3** isomerized in the purification process to give a trace amount of corresponding geometrical isomer. On these probe compounds, different



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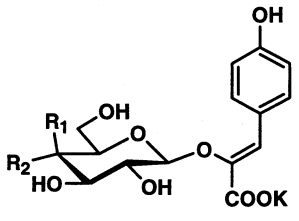


Potassium galactoisolespedezate
(5, R_1=OH, R_2=H)

Potassium isolespedezate
(6, R_1=H, R_2=OH)

Table 1. The leaf-opening activity of each fluorescence-labeled probe (status of leaf at 9:00 p.m.) (++: completely open, +: weakly open, +−: random, −: closed)

	1×10^{-4} M	1×10^{-5} M	5×10^{-6} M	1×10^{-6} M
1	++	++	−	−
2	++	++	++	−
3	++	+	+−	−
17	++	++	−	−

chemical shifts were observed on the olefinic protons of two geometrical isomers. The stereochemistry of the probe compound was determined from the chemical shifts of the olefinic protons compared with that of **4** and its geometrical isomer.[19]

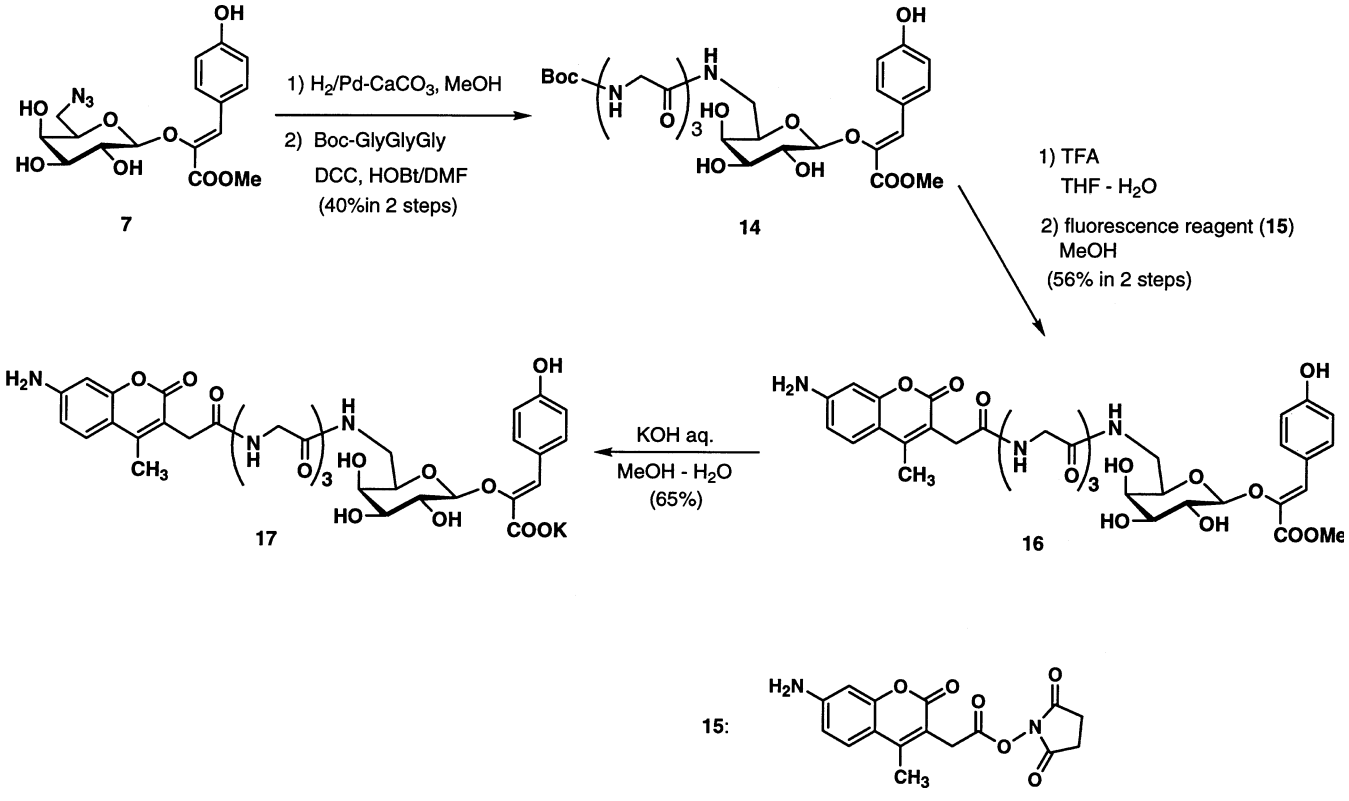
The resulting fluorescence-labeled probe compounds (**1**–**3**) showed leaf-opening activity against the leaf of *C. mimosoides*.[22] The bioactivities of all the probe compounds are shown in Table 1. Especially, the NBD-labeled probe compound (**2**) showed the strongest bioactivity, and was effective at 5×10^{-6} M, which was one-fifth as strong as that of the natural product (**6**). And all these probe compounds retain their leaf-opening activity for a week. This result suggested that probe compounds (**1**–**3**) were not hydrolyzed in the plant body by internal β-glucosidase, and can be used in the fluorescence study using a plant section.

According to our recent report,[18] an improvement of bioactivity for the probe compound was expected by the introduction of a triglycine linker. That is because the hydrophilic property of oligoglycine chain would be more effective than carboxylic acids with long-chain alkyl group as a linker part to improve their bioactivity. Thus, we synthesized AMCA-labeled compound with a triglycine linker (Scheme 2), and compared the bioactivity with that of **1**. The azide was reduced with Lindler's catalyst and was coupled with Boc-triglycine using the DCC–HOBt method. The product (**14**) was deprotected and coupled with an activated-ester type fluorescence reagent (**15**) to give **16**. After hydrolysis with KOH, AMCA-labeled probe compound with the triglycine linker (**17**) was obtained as a potassium salt. Compound **17** was mainly obtained as the (*Z*)-form along with a trace amount of the (*E*)-form. The stereochemistry of **17** was determined from the chemical shifts of the olefinic protons compared with that of **4** and its geometrical isomer.[19]

However, probe compound (**17**) showed leaf-opening activity against the leaf of *C. mimosoides* at 1×10^{-5} M, which is as strong as **1** (Table 1). From these results, it was concluded that the size of the fluorescence functionality is more important than the structure of the linker moiety for the bioactivity of probe compounds. Thus, fluorescence-labeled probe compounds with a flexible chain, such as **1**, **2**, and **3**, would be sufficient for the fluorescence study of nyctinasty.

2.2. Direct observation of the target cell for the leaf-movement factor

We used probe **1** for the detection of the target cell for the leaf-movement factor. On the other hand, probe **2** and **3**, the fluorescence of which are similar to the background one (autofluorescence) of the plant tissue under a fluorescence microscope, was inappropriate for the fluorescence study.



Scheme 2. Synthetic route of fluorescence probe compound with triglycine linker (**17**).

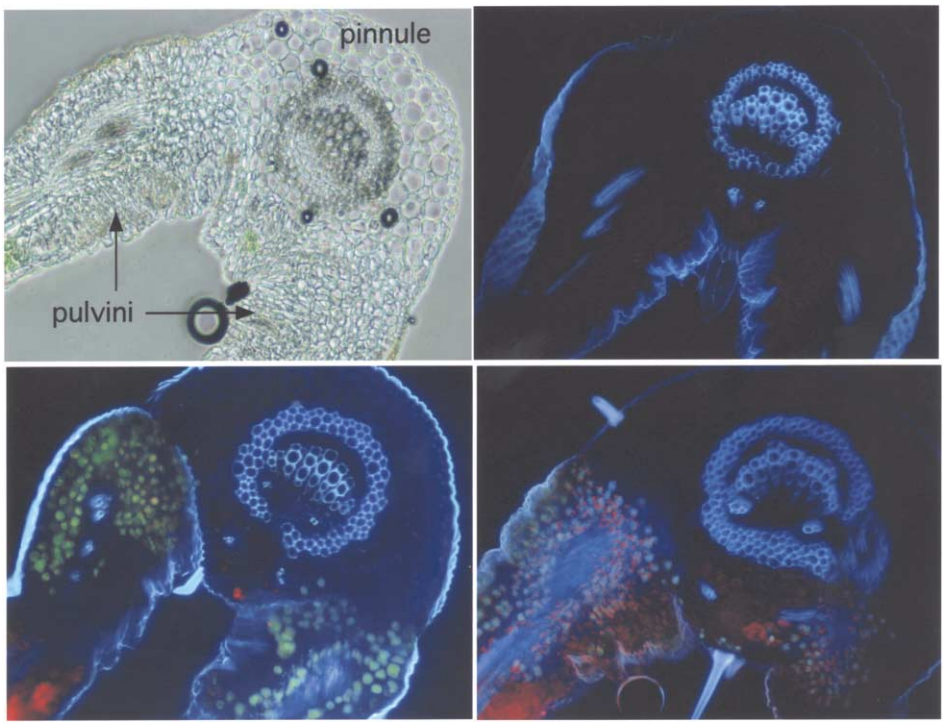


Figure 1. Fluorescence study using probe compound (**1**, **17**); upper left: DIG image of plant section, upper right: blank fluorescence image of plant section, lower left: fluorescence image of plant section treated with **1**, lower right: fluorescence image of plant section treated with **17**.

And FITC labeled probe (**4**) did not give a sharp and bright fluorescence image compared with **1** because of its weak binding ability.

A leaf of *C. mimosoides* was cut by a microslicer perpendicular to the petiole to a thickness of 30 μm. Then the section was incubated overnight in an aqueous solution containing 5×10^{-5} M of **1** under dark condition to avoid photobleaching (fading of fluorescence) of fluorescent dye. When the incubation period was too short, such as 30 min, no staining was observed in the plant section. After staining, the stained section was incubated for 15 min with a washing buffer to remove excess fluorescent probes. Careful examination on washing time revealed that very long washing time decreased the reproducibility of staining. Then, the stained section was monitored by using a fluorescence microscope with an appropriate filter. The use of an antifadant reagent was essential to prevent photobleaching. Fig. 1 shows photographs of the plant sections under a fluorescence microscope. Strong autofluorescence was observed in the plant section. However, when the section was treated with probe compound (**1**), the staining pattern for **1** was observed only in the motor cells contained in pulvini (Fig. 1).[23] No other part of the plant section showed the fluorescent stain for **1**. Also, no stain was observed in the control section, which was treated with a solution without **1** (Fig. 1). And probe compound **17** gave the same result (Fig. 1). Moreover, no stain was observed in the binding experiment using AMCA reagent (**8**) itself, which is easily hydrolyzed to give corresponding potassium carboxylate under the incubation condition. Thus, the binding of **1** and motor cell would be specific to the structure of leaf-opening substance moiety in **1**, and not non-specific resulting from AMCA moiety. These results suggest that the binding site for **1** (and thus, **5**) should exist on the surface of the motor cell.

2.3. Specific binding ability of fluorescent probe compound to *C. mimosoides*

From our previous studies, it was revealed that each nyctinastic plant has different leaf-movement factor whose bioactivity is specific to the original plant.[3–16] Thus, synthetic probe compounds is expected to show specific leaf-opening activity against the leaves of *C. mimosoides*, and not to be effective for the leaf of other plants even at higher concentrations. We examined the specificity of bioactivity on the probe compounds **1** and **17**. Probe compound **1** and **17** did not show leaf-opening activity against the leaves of *Leucaena leucocephala*, *Albizzia julibrissin* Durazz., and *Aeschynomene indica* L. at 5×10^{-5} M. On the other hand, **1** and **17** showed leaf-opening activity against the leaves of *C. mimosoides* even at 1×10^{-5} M. These results showed that the bioactivities of **1** and **17** were specific to the leaves of *C. mimosoides*. From this result, the binding of probe compound is expected to be specific against the section of *C. mimosoides* and no binding would be observed in the experiment using the section of other plants.

Then, we used probe **1** for the binding experiment with the sections of *C. mimosoides*, *L. leucocephala*, *A. julibrissin*, and *A. indica*. The binding experiments were carried out in absolutely the same manner described in Section 2.2. An aqueous solution containing 5×10^{-5} M of probe **1** was used for the staining of each plant section. After the treatment with **1**, section of each plant was monitored by fluorescence

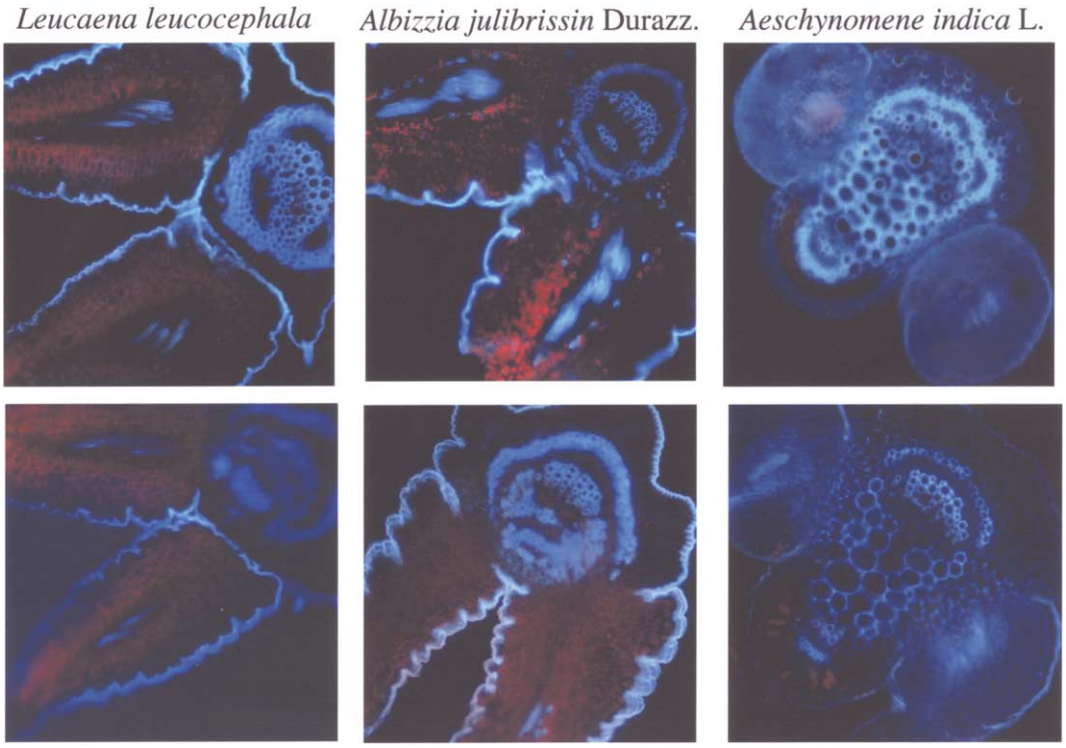


Figure 2. Fluorescence images of plant sections treated with **1** (upper: sections before treatment, lower: sections after treatment).

microscope. Thus, it was revealed that only the section of *C. mimosoides* gave the fluorescence image resulting from **1** (Fig. 1) and no other sections did not (Fig. 2). Moderately strong red fluorescence observed on each plant section is due to the autofluorescence from chlorophylls contained in the plant section, which was also observed on the blank sample (Fig. 2). This result showed that the binding of probe compound with a motor cell is also specific to the plant species and suggested that the specific receptor molecule would be involved in the binding of **1** with a motor cell. And, it was estimated that each plant would have different receptor molecule on a motor cell which is specific to each leaf-movement factor.

3. Conclusion

Because of its high hydrophilicity, leaf-movement factors would not be able to be transmitted into the plasma membrane. Thus, it was assumed that some receptors would exist on a plasma membrane of the target cell for the leaf-movement factor.[24] Now, we have succeeded in the visualization of binding between the leaf-movement factor and the motor cell, and demonstrated that the target cell for the leaf-movement factor is a motor cell.

Also, it was revealed that the binding of fluorescent probe compound (**17**) is specific to *C. mimosoides*. Fluorescent probe compound (**17**) cannot bind with the section of other nyctinastic plants. This result strongly suggest that each nyctinastic plant has different receptor molecule on the motor cell to perceive external chemical signal for introduction of swelling and shrinking of motor cell. However, more examples of fluorescence study should be carried out on other nyctinastic plants, such as *L. leucocephala*, *A. julibrissin* and *A. indica*, etc. to confirm this hypothesis by using fluorescence probe compounds designed on the structure of the leaf-movement factor of each plant. The systematic studies on the identification of the receptor protein for leaf-movement factor and investigations on the difference of receptor molecule in each nyctinastic plants will give a clue for the question: why different leaf-movement factor operates in each nyctinastic plants?

In our previous works,[12,14] we have shown two examples of nyctinastic plants in which the concentration of the leaf-movement factor in a plant body changes through a day according to a circadian rhythm, and this rhythmic change in concentration agrees with the nyctinastic leaf-movement. And now, we have shown that our leaf-movement factor binds to the motor cell. So far, the behavior of our leaf-movement factor can fully account for this physiological phenomenon in the molecular level. The next issue of our research is focused on investigation of the role of leaf-movement factors in this biological event, especially, the interaction between leaf-movement factor and its receptor molecule which invokes the signal transduction in the motor cell which leads to the swelling and shrinking of motor cell.

4. Experimental

4.1. General Procedures

^{1}H NMR (270 MHz) was recorded on a Jeol EX270 spectrometer, and ^{13}C NMR spectra (100 MHz) were recorded on a

Jeol ALPHA 400 spectrometer equipped with microprobe NTH3-FG (NAROLAC) using TMS in $CDCl_3$ or *t*-BuOH (^1H; 1.23 ppm, ^{13}C; 32.1 ppm) in D_2O as internal standards at various temperatures. The FAB-MS and HR FAB-MS spectra were measured on a Jeol JMS-700 spectrometer, using glycerol or *m*-nitrobenzylalcohol as a matrix. The IR spectra were measured by JASCO FT/IR-410. The specific rotations were measured by JASCO DIP-360 polarimeter. The HPLC purification was carried out with a Shimadzu LC-6A pump equipped with an SPD-6A detector using Cosmosil 5C18AR column (∅ 20×250 mm^2) (Nakalai Tesque). The solvents used for HPLC were available from Kanto Chemical and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 μm pore size, 47 mm. dia.) before use. Silica gel column chromatography was performed on Silica Gel 60 K070 (Katayama Chemical) or BW-300 (Fuji Silicia). Reversed-phase open-column chromatography was performed on Cosmosil 75C18-OPN (Nakalai Tesque). TLC was performed on Silica gel F_{254} (0.25 or 0.5 mm, MERCK) or RP-18F_{254S} (0.25 mm, MERCK). Fluorescence reagents were purchased from Funakoshi.

4.1.1. Synthesis of methyl (*Z*)-2-[6′-(6-((7-amino-4-methylcoumarin-3-acetyl)amino)-hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (11). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (10.0 mg, 25.6 μmol) was dissolved in MeOH (300 μL), and TEA (4.2 μL, 29 μmol) was added to this solution. A solution of AMCA **8** (5.0 mg, 11 μmol) in MeOH (1.2 mL) was added to this solution, and the reaction mixture was allowed to stand for 4 h. After evaporation, the residue was separated by pTLC repeatedly using MeOH–H_2O=3:1 to give **11** (4.7 mg, 32% in 2 steps).

Compound **11**: ^1H NMR (270 MHz, CD_3OD, rt): 7.80 (2H, d, *J*=6.8 Hz), 7.50 (1H, d, *J*=8.9 Hz), 7.03 (1H, s), 6.78 (2H, d, *J*=7.1 Hz), 6.67 (1H, dd, *J*=7.8, 2.1 Hz), 6.52 (1H, d, *J*=2.3 Hz), 3.82 (4H, s), 3.74 (1H, d, *J*=2.8 Hz), 3.55 (4H, s), 3.52–3.14 (2H, m), 2.39 (3H, s), 2.07 (2H, t, *J*=7.3 Hz), 1.55–1.47 (4H, m), 1.30 (4H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, 35°C): 176.5, 172.9, 167.0, 164.9, 160.0, 155.9, 153.9, 152.9, 140.1, 134.0, 127.5, 127.4, 125.9, 116.2, 114.6, 113.3, 111.9, 104.1, 100.6, 74.8, 74.6, 72.8, 70.5, 52.7, 41.0, 40.4, 36.9, 35.2, 30.7, 30.0, 27.5, 26.4, 15.4 ppm; HR FAB-MS (positive): $[M+H]^+$. Found *m/z* 684.2816, $C_{34}H_{42}O_{12}N_3$ requires *m/z* 684.2768; IR (film) *ν*: 3350, 3239, 1683, 1633, 1604, 1556, 1514 cm^{-1}; $[\alpha]_D^{22}$=+84.4° (*c* 0.60, MeOH).

4.1.2. Synthesis of potassium (*Z*)-2-[6′-(6-((7-amino-4-methylcoumarin-3-acetyl)amino)-hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (1). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (1.9 mg, 4.8 μmol) was dissolved in DMF (30 μL), and TEA (0.68 μL, 4.8 μmol) was added to this solution. A solution of AMCA **8** (0.8 mg, 1.9 μmol) in DMF (30 μL) was added to this solution, and the reaction mixture was allowed to stand overnight. After the addition of TEA (0.68 μL, 4.9 μmol), the reaction mixture was allowed to stand for one more day. After evaporation, the reaction mixture was dissolved in MeOH–H_2O=1:1 (200 μL), and 0.1 M KOHaq. (9.6 μL, 9.6 μmol) was added to this mixture at 0°C. After 3.5 h of stirring, additional 0.1 M KOHaq. (9.6 μL, 9.6 μmol) was added to this solution and the reaction mixture was stirred for a day. Then, Amberlite IR-120B (H^+) was added to this solution to adjust pH to 6.5–6.8. Then the solution was filtered, neutralized with 0.1 M K_2CO_3, and evaporated. Separation of the reaction mixture with ODS–pTLC (CH_3CN–H_2O=1:1) gave **1** (0.7 mg, 24% in 3 steps). Repeated coupling and hydrolysis reactions gave 3.7 mg of **1** in total amount.

Compound **1**: ^1H NMR (270 MHz, CD_3OD, rt): 7.80 (2H, d, *J*=8.7 Hz), 7.51 (1H, d, *J*=8.7 Hz), 6.98 (1H, s), 6.76 (2H, d, *J*=8.9 Hz), 6.67 (1H, dd, *J*=8.6, 2.1 Hz), 6.52 (1H, d, *J*=2.1 Hz), 3.82 (1H, t, *J*=8.7 Hz), 3.73 (1H, d, *J*=2.3 Hz), 3.65–3.23 (4H, m), 3.17 (2H, t, *J*=6.9 Hz), 2.40 (3H, s), 2.03 (2H, t, *J*=7.1 Hz), 1.50–1.45 (4H, m), 1.31–1.27 (4H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, rt): 177.3, 160.1, 156.7, 154.8, 153.7, 134.3, 128.2, 127.4, 126.9, 125.3, 115.8, 115.3, 114.1, 112.6, 105.7, 101.3, 76.0, 75.6, 73.7, 71.4, 41.8, 41.2, 37.6, 35.9, 31.6, 30.8, 28.2, 27.2, 16.2 ppm; HR FAB-MS (negative): $[M-K]^-$. Found *m/z* 668.2471, $C_{33}H_{38}O_{12}N_3$ requires *m/z* 668.2455; IR (film) *ν*: 3341, 1677, 1605, 1557, 1515 cm^{-1}; $[\alpha]_D^{22}$=+60.0° (*c* 0.14, MeOH).

4.1.3. Synthesis of methyl (*Z*)-2-[6′-(6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanamide)-β-D-galacto-pyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (12). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (10.0 mg, 25.6 μmol) was dissolved in MeOH (500 μL), and TEA (3.6 μL, 26 μmol) was added to this solution. A solution of NBD **9** (3.0 mg, 7.7 μmol) in DMF (500 μL) was added to this solution, and this reaction mixture was stirred overnight. After evaporation, the residue was separated by pTLC using MeOH–H_2O=2:1 to give **12** (2.3 mg, 17% in 2 steps).

Compound **12**: ^1H NMR (270 MHz, CD_3OD, rt): 8.53 (1H, d, *J*=8.9 Hz), 7.79 (2H, d, *J*=7.0 Hz), 7.00 (1H, s), 6.77 (2H, d, *J*=7.0 Hz), 6.34 (1H, d, *J*=8.9 Hz), 4.94 (1H, d, *J*=7.8 Hz), 3.82 (3H, s), 3.85–3.51 (6H, m), 3.43 (1H, dd, *J*=13.7, 5.4 Hz), 3.24 (1H, dd, *J*=13.7, 5.4 Hz), 2.12 (2H, t, *J*=7.3 Hz), 1.78 (2H, quintet, *J*=7.3 Hz), 1.61 (2H, quintet, *J*=7.3 Hz), 1.43 (2H, quintet, *J*=7.3 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD, 35°C): 176.3, 167.0, 160.1, 140.1, 138.5, 134.0, 127.3, 125.8, 116.2, 104.1, 74.8, 74.6, 72.8, 70.6, 52.7, 41.1, 36.8, 30.7, 29.0, 27.5, 26.4 ppm; HR FAB MS (positive): $[M+H]^+$. Found *m/z* 632.2173, $C_{28}H_{34}O_{12}N_5$ requires *m/z* 632.2204; IR (film) *ν*: 3322, 1699, 1618, 1605, 1584, 1528, 1509 cm^{-1}; $[\alpha]_D^{24}$=+76.0° (*c* 0.45, MeOH).

4.1.4. Synthesis of potassium (*Z*)-2-[6′-(6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino) hexanamide)-β-D-galacto-pyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (2). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (9.5 mg, 24.3 μmol) was dissolved in DMF (500 μL), and TEA (3.4 μL, 24.3 μmol) was added to this solution. A solution of NBD **9** (3.5 mg, 8.7 μmol) in DMF (500 μL) was added to this solution, and this reaction mixture was stirred overnight. After evaporation, the residue was dissolved in MeOH–H_2O=1:1 (1.0 mL), then, 1.0 M KOHaq. (48.6 μL, 48.6 μmol) was added at 0°C. After 2 h of stirring, 1.0 M KOHaq. (48.6 μL, 48.6 μmol) was added. After 3 h of stirring, Amberlite IR-120B (H^+) was added to the reaction mixture to adjust pH among pH 6.5–6.8. Then the solution was filtered, neutralized with 0.1 M K_2CO_3, and evaporated. Twice separation of the reaction mixture with ODS–pTLC (MeOH–H_2O=1:1) gave **2** (3.1 mg, 23% in 3 steps). Repeated coupling and hydrolysis reactions gave 6.7 mg of **2** in total amount.

Compound **2**: ^{1}H NMR (270 MHz, CD_3OD, rt): 8.54 (1H, d, *J*=8.9 Hz), 7.80 (2H, d, *J*=8.7 Hz), 7.04 (1H, s), 6.76 (2H, d, *J*=8.7 Hz), 6.35 (1H, d, *J*=8.9 Hz), 3.82 (1H, t, *J*=8.9 Hz), 3.74 (1H, d, *J*=3.1 Hz), 3.65–3.44 (4H, m), 2.09 (2H, t, *J*=7.3 Hz), 1.76 (2H, quintet, *J*=7.3 Hz), 1.59 (2H, quintet, *J*=7.2 Hz), 1.41 (2H, quintet, *J*=7.1 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 179.3, 178.1, 176.3, 163.9, 159.8, 138.6, 133.8, 126.5, 126.4, 126.1, 116.2, 104.7, 75.0, 74.8, 72.9, 70.6, 41.1, 36.8, 30.8, 28.9, 27.5, 26.4, 23.7 ppm; HR FAB-MS (negative): $[M-K]^-$. Found *m/z* 616.1937, $C_{27}H_{30}O_{12}N_5$ requires *m/z* 616.1891; IR (film) ν: 3341, 1584, 1510 cm^{-1}; $[\alpha]_D^{19}$=+62.5° (*c* 0.26, MeOH).

4.1.5. Synthesis of methyl (*Z*)-2-[6′-(6-(4-((5-dimethyl-aminonaphthalene-1-sulfonyl)-amino))-hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (13). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (12.2 mg, 31.2 μmol) was dissolved in MeOH (500 μL), and TEA (4.3 μL, 32 μmol) was added to this solution. Dansyl reagent (**10**, 9.8 mg, 25.0 μmol) in MeOH (500 μL) was added to this solution, and the reaction mixture was allowed to stand overnight. After evaporation, the residue was purified by HPLC with Cosmosil 5C18Ar column using 80% MeOHaq. to give **13** (6.0 mg, 32% in 2 steps).

Compound **13**: ^{1}H NMR (400 MHz, CD_3OD, 35°C): 8.49 (1H, d, *J*=8.8 Hz), 8.30 (1H, d, *J*=8.8 Hz), 8.13 (1H, d, *J*=7.3 Hz), 7.70 (2H, d, *J*=8.3 Hz), 7.55–7.49 (2H, m), 7.20 (1H, d, *J*=7.3 Hz), 6.93 (1H, s), 6.70 (2H, d, *J*=8.8 Hz), 4.86 (1H, d, *J*=7.3 Hz), 3.73 (3H, s), 3,78–3.12 (6H, m), 2.81 (6H, s), 2.78 (2H, t, *J*=6.8 Hz), 1.83 (2H, t, *J*=7.3 Hz), 1.24 (4H, m), 1.06 (2H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, rt): 176.3, 167.0, 160.1, 153.2, 140.1, 137.2, 134.0, 133.7, 131.4, 131.3, 131.1, 131.0, 130.2, 129.1, 127.4, 125.8, 124.3, 120.6, 116.5, 116.3, 104.1, 74.8, 74.6, 72.8, 70.5, 52.8, 45.8, 43.7, 40.9, 36.8, 30.3, 27.1, 26.2 ppm; HR FAB-MS (positive): $[M+H]^+$. Found *m/z* 702.2678, $C_{34}H_{44}O_{11}N_3S$ requires *m/z* 702.2697; IR (film) ν: 3348, 1698, 1637, 1604, 1586, 1513 cm^{-1}; $[\alpha]_D^{23}$=+68.3° (*c* 0.60, MeOH).

4.1.6. Synthesis of potassium (*Z*)-2-[6′-(6-(4-((5-dimethyl-aminonaphthalene-1-sulfonyl)-amino))-hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (3). Compound **7** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under a hydrogen atmosphere. After filtration with celite and then evaporation, crude amine (69.1 mg) was obtained. The resulting crude amine (1.9 mg, 4.8 μmol) was dissolved in DMF (30 μL), and TEA (0.68 μL, 4.8 μmol) was added to this solution. Dansyl reagent (**10**, 2.2 mg, 3.3 μmol) in DMF (70 μL) was added to this solution, and the reaction mixture was allowed to stand overnight. After the second addition of TEA (0.68 μL, 4.9 μmol), the reaction mixture was allowed to stand one more day. After the third addition of TEA (0.68 μL, 4.9 μmol), the reaction mixture was allowed to stand one more day. After evaporation, the reaction mixture was dissolved in MeOH–H_2O=1:1 (200 μL), and 0.1 M KOHaq. (9.6 μL, 9.6 μmol) was added to this mixture at 0°C. After 3 h of stirring, another 0.1 M KOHaq. (9.6 μL, 9.6 μmol) was added to this solution and the reaction mixture was stirred for a day. Then, Amberlite IR-120B (H^+) was added to this solution to adjust pH among 6.5–6.8. Then the solution was filtered, neutralized with 0.1 M K_2CO_3, and evaporated. Separation of the reaction mixture with ODS–pTLC (CH_3CN–H_2O=1:1) gave **3** (1.0 mg, 34% in 3 steps). Repeated coupling and hydrolysis reactions gave 3.9 mg of **3** in total amount.

Compound **3**: ^{1}H NMR (270 MHz, CD_3OD, rt): 8.57 (1H, d, *J*=8.6 Hz), 8.37 (1H, d, *J*=8.7 Hz), 8.20 (1H, d, *J*=7.4 Hz), 7.73 (2H, d, *J*=8.9 Hz), 7.63–7.55 (2H, m), 7.28 (1H, d, *J*=6.8 Hz), 6.95 (1H, s), 6.73 (2H, d, *J*=8.7 Hz), 3.81 (1H, t, *J*=9.7 Hz), 3.70 (1H, d, *J*=3.1 Hz), 3.58–3.40 (3H, m), 3,28–3.23 (1H, m), 2.88 (6H, s), 2.82 (2H, t, *J*=6.9 Hz), 1.84 (2H, t, *J*=7.3 Hz), 1.31–1.05 (6H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, rt): 176.3,170.4, 159.2, 153.2, 144.2, 137.2, 133.5, 131.2, 131.1, 131.0, 130.2, 129.1, 126.6, 124.3, 120.6, 116.4, 116.0, 104.9, 75.2, 74.8, 72.9, 70.6, 45.8, 43.6, 41.0, 36.7, 30.8, 30.2, 27.0, 26.1 ppm; HR FAB-MS (negative): $[M-K]^-$. Found *m/z* 686.2334, $C_{33}H_{40}O_{11}N_3S$ requires *m/z* 686.2384; IR (film) ν: 3307, 1651, 1607, 1575, 1514 cm^{-1}; $[\alpha]_D^{20}$=+54.9° (*c* 0.11, MeOH).

4.1.7. Synthesis of methyl (*Z*)-2-[6′-(*N*-*t*-butoxycarbonyl-glycylglycylglycylamino)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (14). Azide **7** (68.5 mg, 180 μmol) was dissolved in MeOH (1.0 mL), and to this solution was added Pd–$CaCO_3$ (15 mg). After stirring for 2 h under a hydrogen atmosphere, the reaction mixture was filtered with celite and the filtrate was dried up to give crude amine (59.9 mg). The amine (22.6 mg) was dissolved in DMF (0.5 mL) without purification, and to this solution, Boc-GlyGlyGly (22.1 mg, 76.5 μmol), 1-hydroxybenzo-triazole monohydrate (12.9 mg, 95.6 μmol), and DCC

(19.7 mg, 95.6 μmol) was added at 0°C. The reaction mixture was stirred under an argon atmosphere at room temperature overnight. The reaction mixture was evaporated to dryness. The resulting residue was dissolved in MeOH, and filtered with hyflo-supercell to remove DCUrea. The filtrate was evaporated and purified with ODS–TLC (MeOH–H_2O=1:1) to give **14** (15.6 mg, 40% in 2 steps).

Compound **14**: ^{1}H NMR (400 MHz, CD_3OD, rt): 7.76 (2H, d, *J*=8.8 Hz), 7.00 (1H, s), 6.76 (2H, d, *J*=8.8 Hz), 4.93 (1H, d, *J*=7.8 Hz), 3.90 (1H, m), 3.88 (2H, s), 3.81 (3H, s), 3.77 (2H, s), 3.74 (1H, m), 3.73 (2H, s), 3.50–3.55 (2H, m), 3.40 (1H, dd, *J*=13.7, 6.8 Hz), 3.26 (1H, m), 1.43 (9H, s) ppm; ^{13}C NMR (100 MHz, CD_3OD, rt): 173.5, 172.0, 171.7, 166.7, 159.7, 139.8, 133.8, 127.1, 125.7, 116.1, 103.6, 81.0, 74.6, 74.3, 72.7, 69.9, 52.7, 49.8, 44.8, 43.8, 43.4, 40.6, 28.7 ppm; HR FAB-MS (positive): $[M+H]^+$. Found *m/z* 627.2516, $C_{27}H_{39}O_{13}N_4$ requires *m/z* 627.2514; IR (film) ν: 3327, 1663, 1605, 1514 cm$^{-1;}$ $[\alpha]_D^{22}$=+76.6° (*c* 0.2, MeOH).

4.1.8. Synthesis of methyl (*Z*)-2-[6′-((7-amino-4-methylcoumarin-3-acetyl)amino)glycylglycylglycyl)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (16). Compound **14** (15.6 mg, 24.9 μmol) was dissolved in 180 μL of THF–H_2O (30:1). To this solution, TFA (180 μL) was added at 0°C. After being stirred for 1 h, the reaction mixture was evaporated to dryness with H_2O to remove TFA as an azeotrope to give TFA salt of crude amine (**15**). Crude **15** was dissolved in DMF (0.5 mL) without purification, and to this solution, AMCA–NHS (3.3 mg, 10 μmol) was added at 0°C. After being stirred at room temperature for 2 h, the reaction mixture was evaporated and purified with ODS–TLC (MeOH–H_2O=1:2) to give **16** (4.2 mg, 56% in 2 steps).

Compound **16**: ^{1}H NMR (400 MHz, CD_3OD, 30°C): 7.75 (2H, d, *J*=8.8 Hz), 7.48 (1H, d, *J*=8.8 Hz), 6.98 (1H, s), 6.74 (2H, d, *J*=8.8 Hz), 6.64 (1H, dd, *J*=2.2, 8.8 Hz), 6.49 (1H, d, *J*=2.2 Hz), 4.95 (1H, d, *J*=7.8 Hz), 3.89 (1H, d, *J*=16.6 Hz), 3.87 (1H, d, *J*=16.6 Hz), 3.86 (2H, s), 3.72–3.81 (4H, m), 3.79 (3H, s), 3.61 (2H, s), 3.49–3.54 (2H, m), 3.38 (1H, dd, *J*=13.4, 6.8 Hz), 3.28 (1H, m), 2.38 (3H, m) ppm; ^{13}C NMR (100 MHz, DMSO-d^6, rt): 169.9, 169.5, 169.1, 169.0, 164.2, 161.7, 158.2, 154.1, 152.3, 150.1, 138.7, 132.2, 126.2, 124.1, 122.9, 115.1, 113.2, 111.3, 109.3, 101.2, 98.3, 72.9, 72.8, 72.2, 70.9, 60.2, 51.9, 42.2, 42.0, 41.9, 33.7, 33.6 ppm. HR FAB-MS (positive): $[M+H]^+$. Found *m/z* 742.2582, $C_{34}H_{40}O_{14}N_5$ requires *m/z* 742.2572; IR (film) ν: 3342, 1659, 1603, 1552 cm^{-1}; $[\alpha]_D^{24}$=+76.9° (*c* 0.77, DMF).

4.1.9. Synthesis of potassium (*Z*)-2-[6′-((7-amino-4-methylcoumarin-3-acetyl)amino)glycylglycylglycyl)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (17). Compound **16** (2.6 mg, 3.5 μmol) was dissolved in 0.3 mL of MeOH–H_2O (1:1), and to this solution, 1.0 M KOHaq. (14 μL, 14 μmol) was added at 0°C. After stirring overnight, the reaction mixture was acidified by the addition of Amberlite IR-120B (H^+), filtered, and evaporated to dryness. The residue was purified with ODS–TLC (acetonitrile–H_2O=1:2), dissolved in H_2O (5 mL), and neutralized with 10% K_2CO_3. After evaporation to dryness, **17** (1.7 mg, 65%) was obtained.

Compound **17**: ^{1}H NMR (400 MHz, D_2O, rt): 7.65 (2H, d, *J*=8.8 Hz), 7.44 (1H, d, *J*=8.3 Hz), 6.77 (2H, d, *J*=8.8 Hz), 6.72 (1H, s), 6.71 (1H, m), 6.52 (1H, d, *J*=2.4 Hz), 4.89 (1H, d, *J*=7.8 Hz), 3.97 (1H, d, *J*=17.6 Hz), 3.95 (1H, d, *J*=17.6 Hz), 3.94 (2H, s), 3.84 (1H, d, *J*=3.4 Hz), 3.75–3.81 (3H, m), 3.66 (1H, dd, *J*=10.3, 3.4 Hz), 3.60 (1H, m), 3.59 (2H, s), 3.43 (1H, dd, *J*=14.2, 3.9 Hz), 3.29 (1H, dd, *J*=14.2, 8.3 Hz), 2.29 (3H, s) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 174.9, 173.2, 172.7, 172.0, 165.4, 156.9, 156.6, 154.6, 154.4, 152.2, 146.1, 132.6, 127.5, 126.7, 121.1, 116.0, 114.1, 113.6, 112.3, 102.9, 101.6, 73.9, 73.8, 72.1, 69.8, 65.4, 43.7, 43.4, 43.2, 40.5, 34.6 ppm; HR FAB-MS (negative): $[M-K]^-$. Found *m/z* 726.2283, $C_{33}H_{36}O_{14}N_5$ requires *m/z* 726.2259; IR (film) ν: 3352, 1652, 1605, 1557 cm^{-1}; $[\alpha]_D^{24}$=+35.0° (*c* 1.0, H_2O).

4.1.10. Bioassay. The young leaves detached from the stem of the plant *C. mimosoides*, which was grown in the greenhouse of Keio University, 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 kept at 25–35°C and allowed to stand overnight. The leaves which opened again the next morning (around 10:00 a.m.) were used for the bioassay. Each test solution was carefully poured into test tubes with a microsyringe around 10:00 a.m. The bioactive fraction was judged by the leaf-opening after the leaf-closing of the plant leaf in the blank solution containing no sample. Other nyctinastic plants, *L. leucocephalam*, *A. julibrissin* and *A. indica*, used in bioassay were also grown in the greenhouse of Keio University.

4.1.11. Fluorescence study using a fluorescence microscope. The leaf of *C. mimosoides* opening in the daytime was cut in an appropriate size and fixed in agar. The agar was sliced perpendicular to the petiole by a microslicer (DSK-1000, DOUSAKA EM) by 20–30 μm, and the sections containing the pulvini were floated on distilled water. The sections were immersed in a solution containing the various concentration of fluorescent-labeled probe compound, and allowed to stand overnight under shielded condition at room temperature for staining. After staining, the sections were washed by being incubated with equilibration buffer (SlowFade™ Light Antifadant Kit, Molecular Probes) for 15 min. This section was placed on a slide glass and covered by a coverglass after adding a drop of antifade reagent (SlowFade™ Light Antifadant Kit, Molecular Probes). The observation of these sections was carried out by using an ECLIPSE E-800 microscope (Nikon) equipped with VFM fluorescence instrument. Appropriate filter was used against each fluorescent dye: UV-2A (Nikon) filter for NBD, B-2A (Nikon) filter for FITC and NBD. The plant sections of other nyctinastic plants, *L. leucocephalam*, *A. julibrissin* and *A. indica*, were prepared and treated with fluorescent-labeled probe compound in the same procedure.

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