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Enantio-differential approach to identify the target cell for glucosyl jasmonate-type leaf-closing factor, by using fluorescence-labeled probe compounds

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Abstract—Potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**) is a leaf-closing factor of *Albizzia* plants that induces nyctinastic leaf closure. In this paper, we synthesized probe **3** and its congener **4** by using a pair of enantiomerically pure methyl jasmonate that was prepared by using optical resolution, and carried out fluorescence studies using **3** and **4** to identify the target cell of **1**. The probe **3** bound to the motor cells of two *Albizzia* plants, whereas it could not bind to the motor cells of plants belonging to other genus. On the other hand, probe **4** did not bind to the motor cell at all. These results suggested that a specific receptor for **1** is involved in the motor cell of *Albizzia* plants.

1. Introduction

Most leguminous plants close their leaves in the evening, as if to sleep, and open them in the morning according to the circadian rhythm controlled by a biological clock. Charles Darwin, well known for his theory of evolution, carried out the pioneering study in this field.[1] And in the 1970s, physiological studies by Satter using *Albizzia saman* revealed that nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvini, a small organ located in the joint of the leaf to the stem.[2] The flux of potassium ions through a potassium channel on the plasma membranes of the motor cells is followed by water flux into the cell, which results in swelling and shrinking of these cells. Since then, most of the physiological studies on nyctinasty were carried out using plants belonging to the genus *Albizzia*. Recently, channel proteins, which would be concerned with volume change in motor cell, were identified by a genetic approach.[3] We found that a pair of leaf-movement factors, leaf-opening and leaf-closing factors, controlled nyctinasty.[4] Each nyctinastic plant has a pair of leaf-movement factors whose bioactivities are specific to the plant genus.[5] These factors would be involved in the regulation of potassium channels concerning nyctinasty. Considering the difference of leaf-movement factors between plant genuses,[5] bioorganic studies of nyctinasty using *Albizzia* plants, especially *A. saman*, would be important for the coordination of results between bioorganic and physiological studies. Potassium β-D-glucopyranosyl 12-hydroxyjasmonate (**1**)[6] and *cis-p*-coumaroylagmatine (**2**)[7] were isolated as a leaf-closing and leaf-opening factor of the genus *Albizzia*, respectively. Leaf-movement factors **1** and **2** were not effective for plants belonging to other genuses, such as *Cassia mimosoides*, *Phyllanthus urinaria*, and *Mimosa pudica*.[6,7] We have already revealed that the target cell of **2** is a motor cell by using fluorescence-labeled **2**.[8] And we reported the synthesis of a diastereo-mixture of fluorescent probes (**3** and **4**) from racemic methyl jasmonate, and fluorescence studies using them.[9]

However, the use of a molecular probe in the bioorganic study requires serious attention. It was often discussed whether molecular probes containing a larger and less polar functional unit such as fluorescence dye can bind to the genuine binding protein of a natural product in living organisms, because the addition of a larger and less polar functional unit to a natural product increases affinity with the membrane or some abundant proteins in the cell, which resulted in the observation of nonspecific bindings. Thus, in the study using probes, use of a 'negative' probe, which is synthesized from the biologically inactive analog of a bioactive natural product is highly important, because a biologically inactive 'negative' probe gave only nonspecific bindings due to a larger and less polar functional unit.[10] Comparison of the results using a biologically active 'positive' probe and a biologically inactive 'negative' probe would give exact results (Fig. 1). The 'negative' probe should have a highly similar nature with the 'positive' probe besides the affinity to its binding protein. Then, the enantiomer of a biologically active natural product can be used as an ideal 'negative' probe because all physical properties except optical rotation and affinity to

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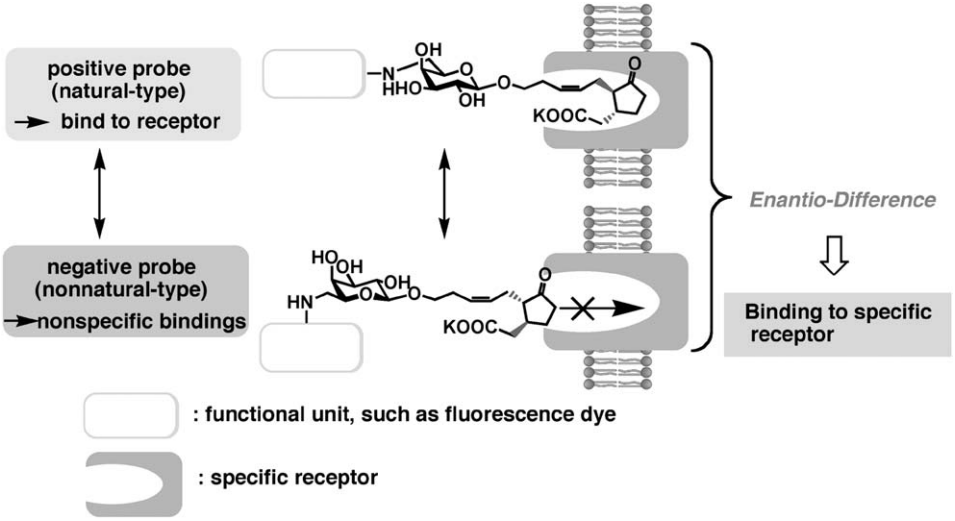
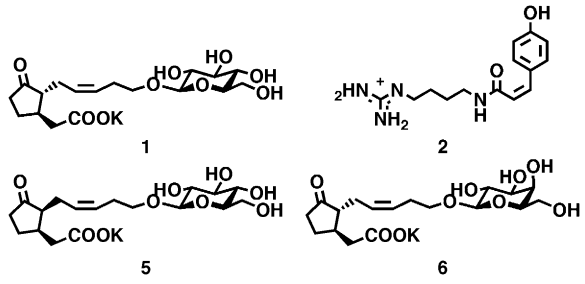


Figure 1. Enantio-differential approach using an enantio-pair of molecular probes.

binding proteins are identical between a pair of enantiomers (Fig. 1). Thus, enantio-differential approach that compares the results using a pair of probes that was prepared from a pair of enantiomers would be an ideal method in the bioorganic study using a molecular probe.

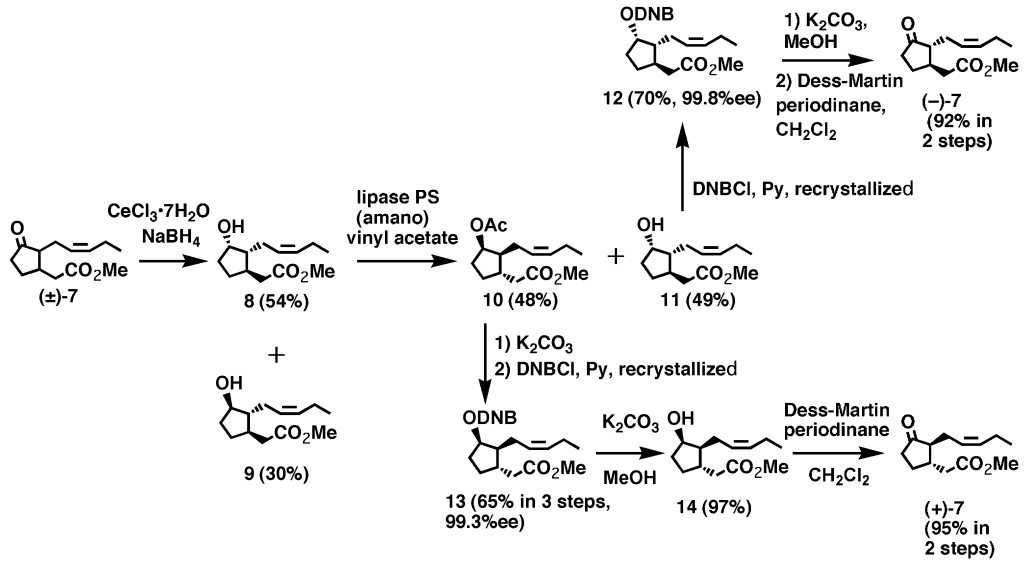
In this paper, we synthesized probe **3** and its congener (**4**) by using a pair of enantiomerically pure methyl jasmonate that was prepared by using optical resolution,[11] and carried out fluorescence studies using **3** and **4** to identify the target cell of **1**.



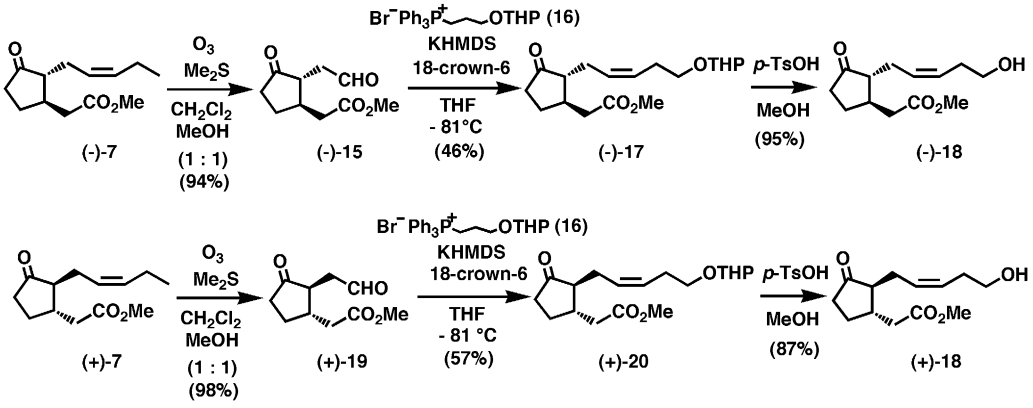
The molecular design of probes **3** and **4** was based on the following results on a structure–activity relationship study: potassium β-D-glucopyranosyl tuberonate (**5**),[12] which is a cis-isomer of **1** on the cyclopentanone ring, had no leaf-closing activity for the *Albizzia* plant,[6] although β-D-galacto-pyranosyl 12-hydroxyjasmonate (**6**)[9] showed leaf-closing activity for *Albizzia julibrissin* at 1×10^{-5} M. These results strongly suggested that an aglycon moiety of **1** would be important for leaf-closing activity, whereas the leaf-closing activity of **1** would not be affected by the structure modification in the sugar moiety similar to other glycoside-type leaf-movement factors.[13,14] Thus, we developed a pair of 'positive' and 'negative' probes from D-galactopyranosyl bromide[13,14] and both enantiomers of aglycon in **1**, which can be prepared from optically pure methyl jasmonate. Introduction of FITC into **1** was designed according to the molecular design of previously developed probes.[13,14] FITC was introduced to the amino group on the 6′-position of the sugar moiety through a glycylglycylglycyl linker by an amide linkage. We synthesized diastereomers **3** and **4** from optically pure enantiomers of aglycon, (+)-**18** and (−)-**18**.

Optically pure aglycons, (+)-**18** and (−)-**18**, were prepared from commercially available (±)-methyl jasmonate ((±)-**7**) (Scheme 1). Optical resolution of racemic **7** was carried out according to the method by Kiyota.[11] Racemic **7** was reduced by using $NaBH_4$–$CeCl_3$,[15] and then one of the resulting diastereomers (**8**) was acetylated by Lipase PS with vinyl acetate to give unnatural-type acetate **10** and natural-type alcohol **11**. The resulting **10** and **11** were converted to 3,5-dinitrobenzyl (DNB) ester (**12** and **13**), respectively, and purified by recrystallization. Optical purities were determined by HPLC analyses of DNB esters **12** and **13**.[16] After deprotection, DMP oxidation of the resulting alcohols gave optically pure (−)-**7** of natural stereochemistry and (+)-**7** of unnatural stereochemistry, respectively. The resulting (−)-**7** (>99.8% ee, $[\alpha]_D$ −93.9) and (+)-**7** (>99.3% ee, $[\alpha]_D$ +91.4) were used in the following syntheses, respectively. Scheme 2 shows an example for the synthesis of natural-type aglycon (−)-**18** from (−)-**7**.[17] Ozonolysis of (−)-**7** gave aldehyde **15**. Then, the Wittig reaction of **15** with ylide from **16** gave THP-protected aglycon (**17**). Wittig reaction by using 2 equiv of Wittig reagent was carried out under salt-free condition to give the (*Z*)-isomer predominantly. The resulting **17** was deprotected by *p*-TsOH to give (−)-methyl 12-hydroxyjasmonate ((−)-**18**), which was used in the glycosidation reaction with the sugar moiety. The enantiomer, (+)-methyl 12-hydroxyjasmonate ((+)-**18**), was also synthesized in the same way.

The resulting optically pure enantiomers (−)-**18** and (+)-**18** were used for the synthesis of positive and negative probes. Positive probe **3** with natural stereochemistry was synthesized from (−)-**18** and Fmoc-protected 6′-aminogalactosyl bromide (**21**)[18] as shown in Scheme 3. Under Königs–Knorr conditions, coupling product **22** was obtained in 37% yield (Scheme 3) with 27% of acetyl (−)-**18**. Careful addition of AgOTf to keep the reaction temperature low improved the yield of glycosilation.[9] Acetyl (−)-**18** can be easily hydrolyzed by K_2CO_3 to give (−)-**18**. Then, all protective groups in **22** were deprotected simultaneously by the treatment with

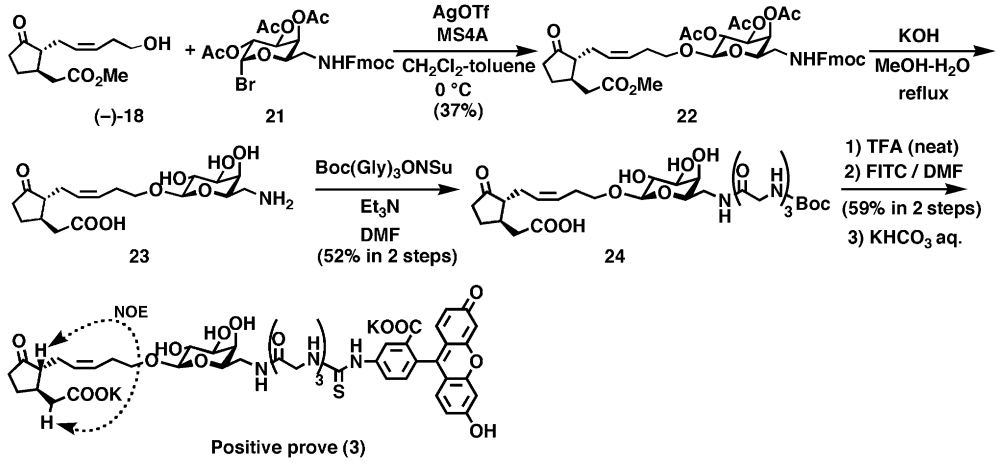


Scheme 1. Optical resolution of racemic methyl jasmonate.

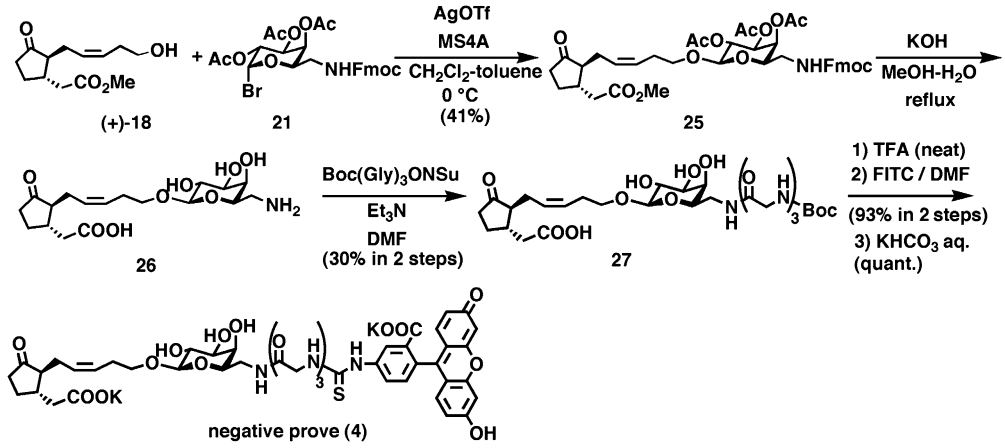


Scheme 2. Synthesis of enantiomerically pure aglycons.

KOH to give **23**, which was coupled with the *N*-hydroxysuccinimide ester of Boc-glycylglycylglycine. The resulting **24** was purified by HPLC under acidic condition, deprotected by TFA, and coupled with FITC to give fluorescence-labeled probe (**3**). According to the same procedure as shown in Scheme 3, negative probe **4** was synthesized from (+)-**18** (Scheme 4). The trans-relationship of the two side chains in **3** and **4** was determined by NOE experiment shown in



Scheme 3. Synthesis of positive probe **3**.



Scheme 4. Synthesis of negative probe **4**.

Scheme 3. The positive probe **3** was effective for the leaf closing of *A. saman* at 5×10^{-4} M, whereas the negative probe **4** was not effective at the same concentration.[19]

We have synthesized enantio-pair-type probes **3** and **4**. By the comparison of the results using them, we can obtain exact biological results because the enantiomer of a bioactive natural product cannot be recognized by the genuine receptor for a natural product that is involved in a biological event. For this purpose, binding experiment using plant sections to seek the target cell for **1** was carried out using a pair of probes **3** and **4**. A leaf of *A. saman* was cut to a thickness of 30 μm. Then, the sections containing a motor cell were incubated in a 0.1 M phosphate buffer (pH 7) containing 1×10^{-4} M of positive probe **3**. After staining, the stained sections were washed with 0.1 M phosphate buffer (pH 7) to remove excess fluorescence probes. Then, the stained sections were monitored by a fluorescent microscope with an appropriate filter for FITC. The use of an antifadant reagent was essential to prevent photo-bleaching (fading of fluorescence). Figure 2 shows photographs of plant pulvini, which contains a motor cell, under a fluorescence microscope. The staining pattern for the yellowish-green fluorescence of probe **3** was observed on the surface of the motor cell in the plant section (Fig. 2). No staining was observed in the competitive binding experiment using 1×10^{-4} M of probe **3** and excess amount (1×10^{-1} M) of non-labeled natural product (**1**). And when the plant section was incubated in a 0.1 M phosphate buffer (pH 7) containing 1×10^{-4} M of negative probe **4**, no staining was observed in the motor cell.

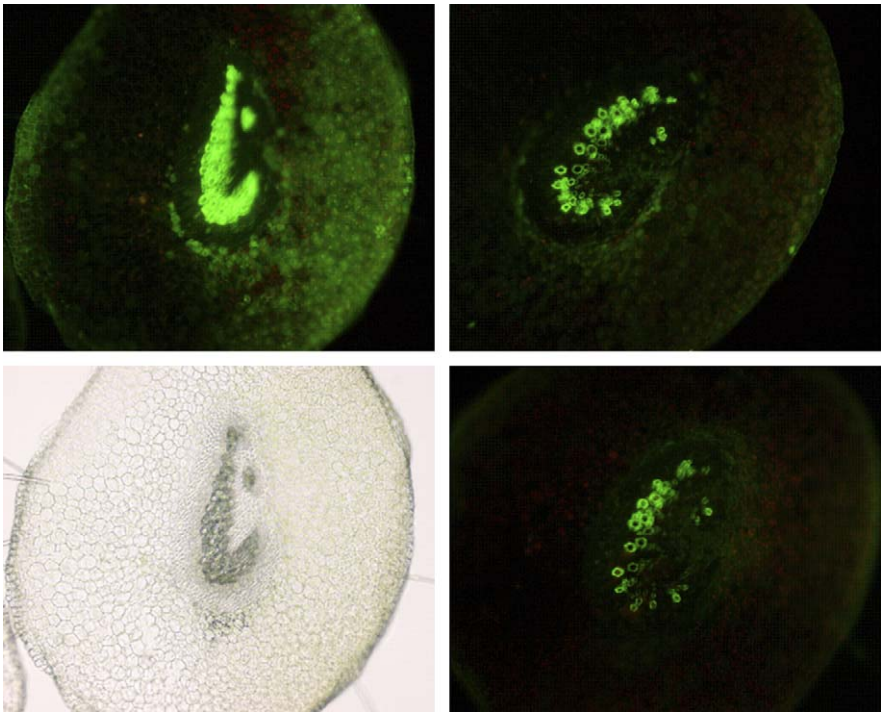


Figure 2. Binding experiment using **3** and **4** with plant section of *A. saman* containing motor cell (upper left: fluorescence image of plant section treated with 1×10^{-4} M of **3** [excitation: 450–490 nm], upper right: fluorescence image of plant section treated with 1×10^{-4} M of **4** [excitation: 450–490 nm], down left: Nomarskii image of the plant section containing motor cell, down right: fluorescence image of plant section treated with 1×10^{-4} M of **3** and 1×10^{-1} M of **1** [excitation: 450–490 nm]).

Comparing the results by probes **3** and **4**, it was clearly shown that strong fluorescence in the xylem (center of the plant section), which was observed in both cases, would be attributed to some nonspecific binding of the probes. These results strongly suggested that the genuine target cell for leaf-closing factor **1** is a motor cell, and a specific receptor for **1**, which recognizes the stereochemistry of aglycon in **1**, is involved in the motor cell of the genus *Albizzia*. The results using an enantio-pair-type probes **3** and **4** showed the effectiveness of an enantio-differential approach using molecular probes in bioorganic studies.

Next, we examined genus specificity in binding of **3** to the plant section. We revealed that **1** is a common leaf-closing substance among three *Albizzia* plants, containing *A. julibrissin* and *Albizzia lebbeck*.[6] And **1** was not effective for the plants belonging to other plant genus. These results strongly suggested that all *Albizzia* plants have specific receptor for **3**. And this indicated that genus-specific biological activity of leaf-movement factors would be attributed to the existence of a receptor for the leaf-movement factor that is common among the same genus.

First, we examined the specificity in the bioactivity of probe **3**. The probe **3** did not show leaf-closing activity against leaves of *C. mimosoides* L., *P. urinaria*, and *Leucaena leucocephala* at 5×10^{-4} M, whereas it was effective at the same concentration for the leaf closing of two *Albizzia* plants: at 1×10^{-4} M for *A. julibrissin* and at 5×10^{-4} M for *A. saman*. From these results, the binding of **3** is expected to be specific to the section of plants belonging to the genus *Albizzia*, and no binding would be observed in the experiment using the section of other plants. Then, we used probe **3** for the binding experiment with the sections of *C. mimosoides*, *P. urinaria*, and *L. leucocephala* together with those of *A. saman* and *A. julibrissin*. The binding experiments were carried out according to the same procedure used in the precedent using *A. saman*. Thus, it was revealed that the sections of *A. juribrissin* and *A. saman* gave a fluorescence image resulting from **3** and no other sections gave the image (Fig. 3). Red stains seen in the fluorescence images are due to the porphyrin in the plant tissue. It was already revealed that the binding of probe **3** to the motor cell of *A. saman* is closely related to the recognition of stereochemistry in probe **3**. Thus, these results showed that the binding of probe **3** with a motor cell is specific to the genus *Albizzia* and strongly suggested that a genus-specific receptor molecule for the genus-specific leaf-movement factor on a motor cell would be involved in nyctinasty.

From these results, we have shown that *Albizzia* plants have a receptor for **1** in the motor cells. Together with the former result of the specific binding of a leaf-opening substance to the motor cell of the genus *Albizzia*,[8] it was strongly suggested that the *Albizzia* plant would have a pair of receptors corresponding to leaf-opening and leaf-closing factors, and both of them are common among the same genus. This result showed that the genus-specific receptor for the leaf-movement factor would be involved in the nyctinasty. And, it was estimated that each plant genus would have a genus-specific combination of leaf-movement factors and receptor molecules for them. Genus-specific recognition of the ligand by a specific receptor for the leaf-movement factor strongly suggests that the membrane receptor concerning nyctinasty would be differentiated in the comparatively later process of evolution in the plant kingdom when the legumes differentiated to various genuses. Also, our results showed that an enantio-differential approach using molecular probes can distinguish the binding of some ligand to its specific receptor from the noise due to the nonspecific bindings, which is one of the most important problem in the experiment using molecular probes; thus an enantio-differential approach would be highly useful in the bioorganic studies of biological phenomena.

2. Experimental

2.1. General procedures

NMR spectra were recorded on a Jeol JNM-A600 spectrometer [^{1}H (600 MHz) and ^{13}C (150 MHz)] and JNM AL300

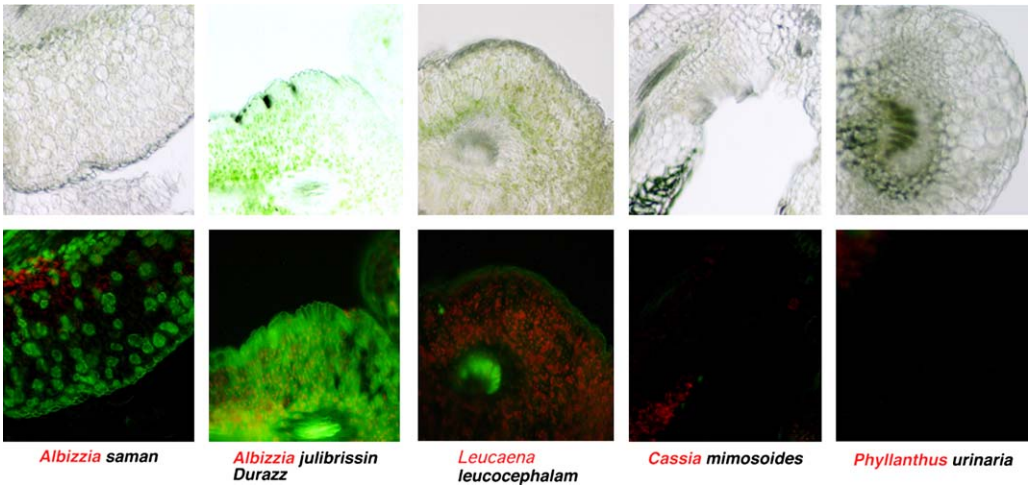


Figure 3. Photographs of plant sections in the binding experiments, which show specific binding of probe **3** with the motor cell of *Albizzia* plants (from the left, *A. saman*, *A. julibrissin*, *Leucaena leucocephala*, *Cassia mimosoides*, and *Phyllanthus urinaria*; upper: Nomarskii image of plant section, lower: fluorescent image of plant section after treatment with 1×10^{-4} M of probe **3** [excitation: 450–490 nm]).

[^{1}H (300 MHz) and ^{13}C (75 MHz)], using TMS in $CDCl_3$, CD_2HOD in CD_3OD (^{1}H; 3.33 ppm, ^{13}C; 49.8 ppm), or *t*-BuOH (^{1}H; 1.24 ppm, ^{13}C; 30.3 ppm) in D_2O as internal standards at various temperatures. The HR ESI-MS spectra were recorded on a Bruker APEX-III. The IR spectra were recorded on a 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 SPD-6A detector using COSMOCIL $5C_{18}$-AR column (∅20×250 mm) (Nakalai Tesque Co. Ltd). The solvents used for HPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 mm pore size, 47 mm dia.) before use. Silica gel column chromatography was performed on silica gel 60 K070 (Katayama Chemical Co., Ltd) or silica gel 60N (Kanto Chemical Co., Ltd). TLC was performed on silica gel F_{254} (0.25 or 0.5 mm, MERCK) or RP-$18F_{254S}$ (0.25 mm, MERCK).

2.1.1. Synthesis of positive probe 3.

2.1.1.1. Methyl (1*S*,2*S*)-3-oxo-2-(formylmethyl)cyclopentaneacetate (−)-15. Ozone was bubbled through a solution of (−)-**7** (325 mg, 1.45 mmol) in CH_2Cl_2 (10 mL) and MeOH (10 mL) at −78 °C for more than 50 min. The excess amount of ozone was removed by Ar gas, then dimethyl sulfide (210 μL) was added dropwise at rt. The mixture was stirred overnight and concentrated in vacuo. The residue was chromatographed on silica gel (*n*-hexane/EtOAc=2:1) to afford (−)-**15** (281 mg, 1.42 mmol, 98%) as a colorless oil: $[\alpha]_D^{23}$ −86.8 (*c* 0.67, $CHCl_3$). IR (film) *ν*: 3422, 2856, 1732, 1717, 1437, 1387, 1340, 1212, 1167 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 9.73 (1H, s), 3.66 (3H, s), 2.89 (1H, dd, *J*=3.9, 18.9 Hz), 2.70 (1H, dd, *J*=3.9, 18.9 Hz), 2.58–2.18 (7H, m), 1.55 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 217.6, 199.7, 172.5, 51.6, 49.1, 42.3, 38.6, 38.3, 36.9, 27.6; ESI-HRMS (positive): [M+Na]$^+$ found *m*/*z* 221.0784, $C_{10}H_{14}O_4Na$ requires *m*/*z* 221.0784.

2.1.1.2. Methyl (1*S*,2*R*,2′*Z*)-3-oxo-2-[5′-(tetrahydropyran-2″-yl)oxy-2′-pentenyl]-cyclopentaneacetate (−)-17. To a mixture of 3-tetrahydropyranyloxypropyltriphenylphosphonium bromide **16** (240 mg, 0.495 mmol) and 18-crown-6 (130 mg, 0.495 mmol) in THF (5 mL) potassium hexamethyldisilazide (0.482 mmol, 0.96 mL as 0.5 mol/L solution of toluene) was slowly added at rt under Ar atmosphere. The mixture was stirred for 10 min at rt. During this period, the mixture developed a bright orange color indicating formation of the ylide. To a solution of **15** (49 mg, 0.247 mmol) in THF (0.8 mL) was slowly added a solution of the ylide (4 mL) at −81 °C for 21 h. The mixture was stirred for 1 h at −78 °C, and then allowed to stand to rt and stirred overnight whereby the orange color disappeared and a whitish suspension was formed. The mixture was concentrated and the residue was chromatographed on silica gel (*n*-hexane/EtOAc=4:1) to afford (−)-**17** (37 mg, 0.114 mmol, 46%) as a colorless oil: $[\alpha]_D^{23}$ −44.9 (*c* 2.0, MeOH). IR (film) *ν*: 2949, 2870, 1740, 1439, 1200, 1032 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, 23 °C) *δ* 5.48 (1H, dt, *J*=10.8, 7.2 Hz), 5.37 (1H, dt, *J*=10.8, 7.2 Hz), 4.56 (1H, dd, *J*=2.4, 4.2 Hz), 3.81 (1H, ddd, *J*=11.4, 7.2, 3.6 Hz), 3.75–3.69 (4H, m), 3.47 (1H, dt, *J*=11.4, 4.8 Hz), 3.42–3.33 (1H, m), 2.68 (1H, dd, *J*=19.2, 8.4 Hz), 2.38–2.17 (8H, m), 2.08 (1H, ddd, *J*=18.6, 11.1, 8.4 Hz), 1.92–1.85 (1H, m), 1.83–1.75 (1H, m), 1.69 (1H, dt, *J*=12.3, 2.7 Hz), 1.57–1.40 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 218.7, 172.4, 128.2, 127.6, 98.7, 66.8, 62.2, 53.8, 51.5, 38.7, 37.9, 37.6, 30.6, 28.0, 27.1, 25.6, 25.4, 19.5; ESI-HRMS (positive): [M+Na]$^+$ found *m*/*z* 347.1829, $C_{18}H_{28}O_5Na$ requires *m*/*z* 347.1829.

2.1.1.3. Methyl (1*S*,2*R*,2′*Z*)-2-(5-hydroxy-2-pentenyl)-3-oxo-cyclopentaneacetate ((−)methyl 12-hydroxyjasmonate) (−)-18. To a solution of (−)-**17** (40 mg, 0.12 mmol) in MeOH (1.0 mL) was added catalytic amount of *p*-TsOH·H_2O at 0 °C under Ar atmosphere. After stirring for 1 h at rt, the reaction mixture was diluted with satd $NaHCO_3$ aq and concentrated in vacuo. The residue was extracted with EtOAc, washed with brine, and dried over Na_2SO_4. Purification by silica gel column chromatography (*n*-hexane/EtOAc=2:1) gave (−)-**18** (24 mg, 102 μmol, 85%) as a colorless oil: $[\alpha]_D^{24}$ −67.9 (*c* 0.5, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 5.50 (1H, dt, *J*=10.8, 6.3 Hz), 5.46 (1H, dt, *J*=10.8, 6.3 Hz), 3.70 (3H, s), 3.66 (t, *J*=6.3 Hz), 2.70 (1H, dd, *J*=8.1, 18.9 Hz), 2.48–1.90 (10H, m), 1.50 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 219.1, 172.7, 128.5, 128.3, 62.0, 53.9, 51.7, 38.8, 37.8, 37.7, 30.9, 27.2, 25.3; ESI-HRMS (positive): [M+Na]$^+$ found *m*/*z* 263.1255, $C_{13}H_{18}O_4Na$ requires *m*/*z* 263.1254.

2.1.1.4. Methyl (1*S*,2*R*,2′*Z*)-2-[5′-(2″,3″,4″-tri-*O*-acetyl-6″-(*N*-9*H*-fluoren-9-ylmethoxycarbonylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetate 22. A solution of (−)-**18** (17 mg, 70.8 μmol), **21** (63 mg, 107 μmol), and dried molecular sieves of 4 Å (300 mg) in anhydrous CH_2Cl_2 (1.0 mL) was slowly added toluene solution (1.0 mL) of AgOTf (27 mg, 106 mmol) at 0 °C under Ar atmosphere in dark. This reaction mixture was stirred overnight at 0 °C. Then, the reaction mixture was diluted with $CHCl_3$ and filtered through Celite. The filtrate was washed with brine and satd aq $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc=1:1) and preparative TLC (*n*-hexane/EtOAc=1:1) to afford **22** (19.4 mg, 26 mmol, 37%) as a colorless viscous oil: $[\alpha]_D^{18}$ −19.7 (*c* 0.5, MeOH). IR (film) *ν*: 3369, 2953, 1747, 1529, 1439, 1369, 1223, 1167, 1072, 760 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, 23 °C) *δ* 7.76 (2H, d, *J*=7.2 Hz), 7.57 (2H, d, *J*=7.2 Hz), 7.40 (2H, t, *J*=7.2 Hz), 7.31 (1H, t, *J*=7.5 Hz), 7.30 (1H, t, *J*=7.5 Hz), 5.44–5.35 (3H, m), 5.20 (1H, dd, *J*=10.5, 7.8 Hz), 5.02 (1H, dd, *J*=10.5, 3.0 Hz), 4.47 (1H, d, *J*=7.8 Hz), 4.39 (2H, d, *J*=6.6 Hz), 4.21 (1H, t, *J*=6.6 Hz), 3.88 (1H, dt, *J*=9.0, 6.9 Hz), 3.76 (1H, t, *J*=6.6 Hz), 3.68 (3H, s), 3.53–3.35 (2H, m), 3.26 (1H, dd, *J*=13.2, 6.6 Hz), 2.65 (1H, dd, *J*=18.9, 8.4 Hz), 2.42–2.09 (9H, m), 2.17 (3H, m), 1.90 (1H, m), 1.47 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 23 °C) *δ* 218.9, 172.5, 170.8, 170.0, 169.5, 156.4, 143.8, 141.3, 128.0, 127.7, 127.4, 127.0, 125.0, 120.0, 101.2, 71.5, 70.9, 69.5, 69.0, 68.1, 53.9, 51.6, 47.2, 40.6, 38.7, 37.8, 37.7, 27.8, 27.2, 25.4, 20.8, 20.7, 20.6; ESI-HRMS (positive): [M+Na]$^+$ found *m*/*z* 772.2940, $C_{40}H_{47}NO_{13}Na$ requires *m*/*z* 772.2940.

2.1.1.5. (1*S*,2*R*,2′*Z*)-2-[5′-(6″-(*N*-*tert*-Butoxycarbonylglycylglycylglycylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetic acid 24. Glycoside **22**

(17 mg, 22.7 μmol) in MeOH/H_2O=3:1 (4 mL) and 1 M KOH aq (114 μmol, 114 μL) was refluxed for 4 h at 85 °C. After the solution was neutralized with 1 N HCl aq and concentrated in vacuo, the residue was purified by ODS TLC (RP-18, H_2O/MeOH=2:3 containing 5% AcOH) to afford an acetate of the resulting amine **23**. To the solution of amine **23** in DMF (2 mL) with Et_3N (12 μL) was added *O*-Boc-glycylglycylglycyl *N*-hydroxysuccinimide (12 mg, 30 μmol). After stirring overnight at rt, the reaction mixture was evaporated to dryness. The residue was purified by ODS TLC (RP-18, H_2O/MeCN=3:1 containing 0.5% AcOH) to afford **24** (6.9 mg, 10.5 μmol, 52% in two steps) as a colorless viscous oil: $[\alpha]_D^{22}$ −13.8 (*c* 0.25, MeOH). IR (film) *ν*: 3310, 2924, 1668, 1653, 1539, 1368, 1252, 1167, 1058, 1033 cm^{-1}. ^1H NMR (300 MHz, CD_3OD, 21 °C) *δ* 5.53 (1H, dt, *J*=10.8, 6.9 Hz), 5.40 (1H, dt, *J*=10.8, 7.5 Hz), 4.21 (1H, d, *J*=6.9 Hz), 3.88–3.82 (5H, m), 3.74–3.72 (3H, m), 3.59–3.51 (3H, m), 3.48–3.39 (3H, m), 2.68 (1H, dd, *J*=8.1, 18.9 Hz), 2.44–1.98 (10H, m), 1.59–1.49 (1H, m), 1.45 (9H, s); ^{13}C NMR (75 MHz, CD_3OD, 22 °C) *δ* 221.9, 173.8, 172.2, 158.8, 129.1, 128.9, 104.8, 81.0, 74.7, 73.9, 72.4, 70.3, 70.2, 55.1, 44.9, 43.9, 43.5, 41.0, 40.0, 39.2, 38.7, 30.8, 29.1, 28.7, 28.2, 26.4; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 681.2950, $C_{29}H_{46}N_4O_{13}Na$ requires *m/z* 681.2954.

2.1.1.6. (1*S*,2*R*,2′*Z*)-2-[5′-(6″-(*N*-Fluorescein-4-isothiocyanatoglycylglycylglycylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetic acid (positive probe) 3. Compound **24** (3.9 mg, 5.9 μmol) in TFA (0.3 mL) was mixed for 2 min at rt. The solution was evaporated to give a TFA salt of resulting amine. The TFA salt was dissolved in DMF (0.4 mL) with TEA (10 μL), and then FITC (2.8 mg, 7.2 μmol) was added to this solution at 0 °C. After stirring overnight at rt, the reaction mixture was evaporated to dryness. The residue was purified by ODS TLC (RP-18W, H_2O/MeCN=2:1 containing 0.5% TFA) to afford **3** (3.3 mg, 3.5 μmol, 59% in two steps) as yellow viscous oil. Neutralization of the resulting **3** with 0.1 mM $KHCO_3$ aq (70 μL, 7.0 μmol) gave **3** (3.6 mg as a potassium salt, quant) as an orange crystal: $[\alpha]_D^{20}$ +3.65 (*c* 0.2, MeOH). IR (film) *ν*: 3279, 1681, 1581, 1466, 1333, 1268, 1132, 915, 850, 802, 724 cm^{-1}. ^1H NMR (600 MHz, D_2O, 27 °C) *δ* 7.75 (1H, s), 7.66 (1H, s), 7.37 (1H, d, *J*=7.8 Hz), 7.25 (2H, d, *J*=9.6 Hz), 6.65–6.62 (4H, m), 5.43 (1H, dt, *J*=10.8, 6.6 Hz), 5.37 (1H, dt, *J*=10.8, 7.2 Hz), 4.32 (2H, s), 4.30 (1H, d, *J*=8.4 Hz), 4.00 (2H, s), 3.95 (1H, d, *J*=16.2 Hz), 3.91 (1H, d, *J*=16.2 Hz), 3.83–3.78 (2H, m), 3.69–3.66 (1H, m), 3.60–3.55 (2H, m), 3.49–3.38 (3H, m), 2.50 (1H, dd, *J*=4.8, 13.8 Hz), 2.34–2.20 (6H, m), 2.16–2.09 (3H, m), 1.97–1.94 (1H, m), 1.47–1.46 (1H, m); ^{13}C NMR (150 MHz, D_2O, 22 °C) *δ* 226.9, 227.4, 181.7, 180.8, 176.1, 174.8, 173.2, 163.0, 159.0, 158.8, 158.7, 131.6, 128.5, 127.6, 126.6, 124.9, 123.6, 112.6, 103.7, 102.8, 72.8, 70.8, 69.8, 68.5, 54.5, 47.7, 43.3, 42.6, 42.4, 39.9, 38.7, 38.6, 36.7, 27.4, 26.9, 25.3; ESI-HRMS (negative): $[M-2K+H]^-$ found *m/z* 946.2830, $C_{45}H_{48}N_5O_{16}S$ requires *m/z* 946.2822.

2.1.2. Synthesis of negative probe 4.

2.1.2.1. Methyl (1*R*,2*R*)-3-oxo-2-(formylmethyl)cyclopentaneacetate (+)-19. Ozone was bubbled through a solution of (+)-**7** (185 mg, 0.825 mmol) in CH_2Cl_2 (8 mL) and MeOH (8 mL) at −78 °C for more than 25 min. The excess ozone was removed by Ar gas, then dimethyl sulfide (97 μL) was added dropwise at rt. The mixture was stirred overnight and concentrated in vacuo. The residue was chromatographed on silica gel (*n*-hexane/EtOAc=2:1) to afford (+)-**19** (160 mg, 0.808 mmol, 98%) as a colorless oil: $[\alpha]_D^{22}$ +94.1 (*c* 0.67, $CHCl_3$). IR (film) *ν*: 3422, 2856, 1732, 1717, 1437, 1387, 1340, 1212, 1167 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 9.73 (1H, s), 3.66 (3H, s), 2.89 (1H, dd, *J*=3.9, 18.9 Hz), 2.70 (1H, dd, *J*=3.9, 18.9 Hz), 2.58–2.18 (7H, m), 1.55 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 217.6, 199.7, 172.5, 51.6, 49.1, 42.3, 38.6, 38.3, 36.9, 27.6; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 221.0784, $C_{10}H_{14}O_4Na$ requires *m/z* 221.0784.

2.1.2.2. Methyl (1*R*,2*S*,2′*Z*)-3-oxo-2-[5′-(tetrahydropyran-2″-yl)oxy-2′-pentenyl]-cyclopentaneacetate (+)-20. To a mixture of 3-tetrahydropyranyloxypropyltriphenylphosphonium bromide **16** (69 mg, 0.14 mmol) and 18-crown-6 (40 mg, 0.15 mmol) in THF (1.4 mL) was slowly added potassium hexamethyldisilazide (0.14 mmol, 275 μL as 0.5 mol/L solution in toluene) at rt under Ar atmosphere. The mixture was stirred for 10 min at rt. During this period, the mixture developed a bright orange color indicating the formation of the ylide. To a solution of **19** (14 mg, 0.07 mmol) in THF (0.5 mL) was added a solution of the ylide (1.5 mL) at −81 °C during 20 min. The mixture was stirred for 1 h at −78 °C, and then the mixture allowed to stand to rt and stirred overnight whereby the orange color disappeared and a whitish suspension was formed. The mixture was concentrated and the residue was chromatographed on a silica gel (hexane/EtOAc=4:1) to afford (+)-**20** (13 mg, 0.04 mmol, 57%) as a colorless oil: $[\alpha]_D^{22}$ +54.2 (*c* 2.68, MeOH). IR (film) *ν*: 2949, 2870, 1740, 1437, 1200, 1032 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 5.48 (1H, dt, *J*=10.5, 7.5 Hz), 5.37 (1H, dt, *J*=10.5, 7.5 Hz), 4.56 (1H, br s), 3.83 (1H, m), 3.75–3.66 (4H, m), 3.47 (1H, m), 3.36 (1H, dd, *J*=15.6, 7.5 Hz), 2.68 (1H, dd, *J*=18.9, 8.1 Hz), 2.37–2.02 (9H, m), 1.90–1.67 (3H, m), 1.55–1.36 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 218.8, 172.4, 128.2, 127.5, 98.7, 98.7, 66.7, 62.2, 53.8, 51.5, 38.6, 38.0, 37.9, 37.6, 30.6, 27.9, 27.1, 25.6, 25.4, 19.5; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 347.1830, $C_{18}H_{28}O_5Na$ requires *m/z* 347.1829.

2.1.2.3. Methyl (1*R*,2*S*,2′*Z*)-2-(5-hydroxy-2-pentenyl)-3-oxo-cyclopentaneacetate ((+)-*ent*-methyl 12-hydroxyjasmonate) (+)-18. To a solution of (+)-**20** (11 mg, 33.3 μmol) in MeOH (1.0 mL) was added a catalytic amount of *p*-TsOH·H_2O at 0 °C under Ar atmosphere. After stirring for 1 h at rt, the reaction mixture was diluted with satd $NaHCO_3$ aq and concentrated in vacuo. The residue was extracted with EtOAc, washed with brine, and dried over Na_2SO_4. Purification by silica gel column chromatography (*n*-hexane/EtOAc=2:1) gave (+)-**18** (7 mg, 29.1 μmol, 87%) as a colorless oil: $[\alpha]_D^{22}$ +72.1 (*c* 0.5, MeOH). ^1H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 5.50 (1H, dt, *J*=10.8, 6.3 Hz), 5.46 (1H, dt, *J*=10.8, 6.3 Hz), 3.70 (3H, s), 3.66 (t, *J*=6.3 Hz), 2.70 (1H, dd, *J*=8.1, 18.9 Hz), 2.48–1.90 (10H, m), 1.50 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 22 °C) *δ* 219.1, 172.7, 128.5, 128.3, 62.0, 53.9, 51.7, 38.8, 37.8, 37.7, 30.9, 27.2, 25.3; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 263.1255, $C_{13}H_{18}O_4Na$ requires *m/z* 263.1254.

2.1.2.4. Methyl (1*R*,2*S*,2′*Z*)-2-[5′-(2″,3″,4″-tri-*O*-acetyl-6″-(*N*-9*H*-fluoren-9-ylmethoxycarbonylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetate 25. To a solution of (+)-**18** (40 mg, 166 μmol), **21** (150 mg, 250 μmol), and dried molecular sieves of 4 Å (300 mg) in anhydrous CH_2Cl_2 (2.2 mL) was slowly added AgOTf (64 mg, 250 mmol) in toluene (2.23 mL) at 0 °C under Ar atmosphere in the dark. This reaction mixture was stirred for 3.5 h at 0 °C. Then, the reaction mixture was diluted with $CHCl_3$ and filtered through Celite. The filtrate was washed with brine and satd $NaHCO_3$ aq. The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc=2:3) and preparative TLC (*n*-hexane/EtOAc=1:1) to afford **25** (51 mg, 68 mmol, 41%) as a colorless viscous oil: $[\alpha]_D^{21}$ +22.3 (*c* 1.46, MeOH). IR (film) *ν*: 3369, 2953, 1749, 1521, 1508, 1437, 1369, 1225, 1165, 1070, 745 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, 22 °C) *δ* 7.76 (2H, d, *J*=7.5 Hz), 7.58 (2H, d, *J*=7.5 Hz), 7.41 (2H, t, *J*=7.5 Hz), 7.31 (2H, br t, *J*=7.5 Hz), 5.47–5.35 (3H, m), 5.20 (1H, dd, *J*=10.2, 8.7 Hz), 5.02 (1H, dd, *J*=10.2, 2.7 Hz), 4.47–4.36 (3H, m), 4.22 (1H, t, *J*=6.3 Hz), 3.88 (1H, dd, *J*=15.6, 6.9 Hz), 3.78–3.69 (4H, m), 3.50 (1H, dd, *J*=15.6, 6.9 Hz), 3.40 (1H, dd, *J*=13.8, 6.6 Hz), 3.26 (1H, dt, *J*=13.8, 6.6 Hz), 2.66 (1H, dd, *J*=18.9, 8.4 Hz), 2.44–2.00 (18H, m), 1.90 (1H, m), 1.47 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3$, 23 °C) *δ* 218.8, 172.5, 170.8, 170.0, 169.4, 156.4, 143.8, 143.8, 141.3, 127.9, 127.7, 127.4, 127.0, 125.0, 120.0, 101.3, 71.6, 70.9, 69.6, 69.0, 68.1, 66.8, 53.9, 51.6, 47.1, 40.7, 38.7, 37.8, 37.7, 27.8, 27.2, 25.4, 20.8, 20.7, 20.6; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 772.2937, $C_{40}H_{47}NO_{13}Na$ requires *m/z* 772.2940.

2.1.2.5. (1*R*,2*S*,2′*Z*)-2-[5′-(6″-(*N*-*tert*-Butoxycarbonylglycylglycylglycylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetic acid 27. Glycoside **25** (46 mg, 61.3 μmol) in MeOH/H_2O=3:1 (4 mL) was refluxed with 1 M KOH aq (307 μmol, 307 μL) for 5 h at 87 °C. After, the reaction mixture was neutralized with 1 N HCl aq and concentrated in vacuo. The residue was purified by ODS TLC (RP-18, H_2O/MeOH=2:3 containing 5% AcOH) to afford an acetate of the resulting amine **26**. To a solution of amine in DMF (0.5 mL) was Et_3N (12 μL) was added *O*-Boc-glycylglycylglycyl *N*-hydroxysuccinimide (47 mg, 123 μmol). After stirring overnight at rt, the reaction mixture was evaporated to dryness. The residue was purified by ODS TLC (RP-18, H_2O/MeCN=4:1, 0.5% AcOH) and HPLC with COSMOSIL 5C_{18}-AR column (∅20.0×250 mm, H_2O/CH_3CN=3:1) to afford **27** (12 mg, 18.2 μmol, 30% in two steps) as a colorless viscous oil: $[\alpha]_D^{22}$ +33.4 (*c* 0.92, MeOH). IR (film) *ν*: 3317, 2934, 1650, 1534, 1369, 1254, 1168, 1225, 1073, 1030 cm^{-1}. ^1H NMR (300 MHz, CD_3OD, 23 °C) *δ* 5.52 (1H, dt, *J*=10.8, 6.9 Hz), 5.41 (1H, dt, *J*=10.8, 6.9 Hz), 4.20 (1H, d, *J*=7.2 Hz), 3.88–3.82 (5H, m), 3.75–3.72 (3H, m), 3.58–3.39 (6H, m), 2.66 (1H, dd, *J*=8.4, 19.5 Hz), 2.44–1.90 (10H, m), 1.49 (1H, m), 1.44 (9H, m); ^{13}C NMR (75 MHz, CD_3OD, 22 °C) *δ* 222.0, 173.8, 172.3, 172.1, 158.8, 129.1, 128.9, 104.9, 81.0, 74.7, 73.9, 72.4, 70.3, 70.2, 55.2, 44.9, 43.9, 43.5, 41.0, 40.6, 39.4, 38.7, 29.1, 28.7, 28.3, 26.4; ESI-HRMS (positive): $[M+Na]^+$ found *m/z* 681.2950, $C_{29}H_{46}N_4O_{13}Na$ requires *m/z* 681.2954.

2.1.2.6. (1*R*,2*S*,2′*Z*)-2-[5′-(6″-(*N*-Fluorescein-4-isothiocyanatoglycylglycylglycylamino)-β-D-glucopyranosyloxy)-2′-pentenyl]-3-oxo-cyclopentaneacetic acid (negative probe) 4. Compound **27** (9 mg, 13.7 μmol) in TFA (0.4 mL) was mixed for 3 min at rt. This solution was evaporated to give a TFA salt of resulting amine. The TFA salt was dissolved in DMF (0.5 mL) with TEA (7 μL), and then FITC (6.4 mg, 16.4 μmol) was added to the solution at 0 °C. After stirring overnight at rt, the reaction mixture was evaporated to dryness. The residue was purified by ODS TLC (RP-18, H_2O/MeCN=4:1 containing 0.5% AcOH) to afford **4** (12 mg, 12.7 μmol, 93% in two steps) as yellow viscous oil. Neutralization with 0.1 mM $KHCO_3$ aq (254 μL, 25.4 μmol) gave **4** (13 mg as a potassium salt, quant) as an orange crystal: $[\alpha]_D^{27}$ +14.4 (*c* 0.12, MeOH). IR (film) *ν*: 3293, 2930, 1637, 1576, 1559, 1465, 1394, 1331, 1111 cm^{-1}. ^1H NMR (600 MHz, D_2O, 23 °C) *δ* 7.79 (1H, br d), 7.64 (1H, d, *J*=7.7 Hz), 7.21 (2H, m), 7.16 (1H, m), 6.67 (2H, d, *J*=7.7 Hz), 6.63 (2H, m), 5.43 (1H, dt, *J*=10.9, 6.8 Hz), 5.37 (1H, dt, *J*=10.9, 6.8 Hz), 4.30 (3H, m), 3.99 (2H, s), 3.94 (1H, d, *J*=16.8 Hz), 3.89 (1H, d, *J*=16.8 Hz), 3.83–3.79 (2H, m), 3.67 (1H, dd, *J*=5.5, 7.8 Hz), 3.59–3.55 (2H, m), 3.46–3.38 (3H, m), 2.52 (1H, dd, *J*=4.9, 13.9 Hz), 2.36–2.21 (6H, m), 2.16–2.09 (3H, m), 1.97 (1H, m), 1.48 (1H, m); ^{13}C NMR (150 MHz, D_2O, 22 °C) *δ* 227.4, 181.9, 181.7, 177.0, 173.1, 172.4, 171.5, 158.2, 140.8, 148.8, 131.7, 131.0, 129.5, 128.5, 127.7, 125.9, 124.8, 121.9, 114.2, 103.6, 102.8, 72.8, 70.8, 69.8, 69.0, 54.5, 47.7, 43.0, 42.6, 39.9, 38.7, 38.1, 27.5, 27.0, 26.8, 25.1; ESI-HRMS (negative): $[M-2K+H]^-$ found *m/z* 946.2830, $C_{45}H_{48}N_5O_{16}S$ requires *m/z* 946.2822.

2.2. Bioassay

The young leaves of the plant *A. saman*, which was grown in the biotron (Nippon Medical & Chemical Instrumentals Co., Ltd) of Tohoku University, were used for bioassay. The young leaves were detached from the stem of *A. saman* with a sharp razor blade. One leaf was placed in H_2O (ca. 300 μL) using a 20-mL glass tube in the biotron kept at 32 °C and allowed to stand overnight. The leaves which opened again in the morning were used for the bioassay. Each test solution was carefully poured into test tubes with a micropipette around 10:30 a.m. Then, the leaves immersed in the sample solution was put in a bell funnel, and decompressed by an aspirator. The sample solution was pumped up through the vessel to the stroma by compulsion. The bioactive fraction made the leaves close within a few minutes in this bioassay. Other nyctinastic plants, *A. julibrissin*, *C. mimosoides*, *P. urinaria*, and *Leucaena leucocephala*, which were used in the bioassay, were also grown in the biotron of Tohoku University.

2.3. Fluorescence study using a fluorescence microscope

The leaf of *A. saman* opening in the morning 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 Co., Ltd) to a thickness of 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 fluorescence-labeled probe

compounds **3** and **4**, respectively, and allowed to stand for staining under shielded condition at rt for 4.5 h. After staining, the sections were washed by incubation with 0.1 M phosphate buffer (pH 7) for 10 min. This section was placed on a slide glass and covered by a cover glass after adding a drop of antifade reagent (Slow Fade™ Gold Antifade Reagent, Molecular Probes Inc.). The observation of these sections was carried out by using ECLIPSE E800 microscope (Nikon, Co., Ltd) equipped with VFM fluorescence instrument. The B-2A filter (Nikon CO., Ltd; excitation wavelength 450–490 nm) was used for FITC. The plant sections of other nyctinastic plants *A. julibrissin*, *C. mimosoides*, *P. urinaria*, and *L. leucocephala* were prepared similarly and treated with fluorescence-labeled probe compounds in the same procedure.

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19. Natural leaf-closing factor **1** was effective at 1×10^{-5} M for *A. julibrissin*, and at 4×10^{-5} M for *A. saman* and *A. lebbeck*. This would be because the leaves of *A. saman* and *A. lebbeck* are larger than those of *A. julibrissin*.