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Direct observation of the target cell for leaf-movement factor using novel fluorescence-labeled probe compounds: fluorescence studies of nyctinasty in legumes. Part 1

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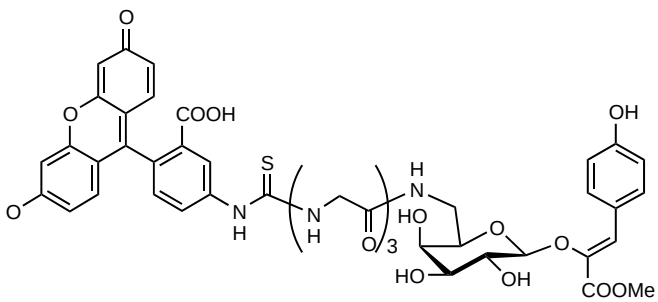
Abstract—We synthesized fluorescence-labeled probe compounds bearing AMCA (**1**), NBD (**2**), and dansyl (**3**) groups as the fluorescent functionality. In these probe compounds, NBD-type probe, **2**, showed leaf-opening activity at 5×10^{-6} M. The bioactivity of **2** is one-fifth as strong as that of the natural product, potassium isolespedezate (**6**). We carried out the binding experiment using **1** in a plant body. © 2001 Elsevier Science Ltd. All rights reserved.

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] and chemical study using these bioactive substances gave significant information on the mechanism for the control of nyctinasty.[3] The next issue is to determine how these compounds induce leaf movement. Because of the high hydrophobicity of the leaf-movement factors, it is assumed that some receptors would exist on a plasma membrane of the plant motor cell, which is essential for nyctinastic leaf movement. The bioactive substances concerning leaf movement can be used as probe compounds that are highly useful for the identification of their receptors in the plant body, which leads to bioorganic studies of nyctinasty. Investigation of the site where bioactive substances are perceived at the cellular level is the first step towards the bioorganic study of their receptor molecule. Here, we report chemical synthesis of novel fluorescence-labeled probe compounds bearing AMCA (**1**), NBD (**2**), and dansyl (**3**) groups as the fluorescent functionality, and a fluorescence study on the nyctinastic movement using these probe compounds.



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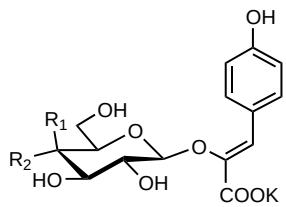

Potassium galactoisolespedezate (5, R_1=OH, R_2=H)
Potassium isolespedezate (6, R =H, R =OH)

Keywords: nyctinasty; leaf-opening substance; fluorescence; probe compound; motor cell.
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Recently, we synthesized FITC-labeled potassium galactoisolespedezate (**4**), based on the artificial leaf-opening substance **5**, which showed leaf-opening activity against the leaf of *Cassia mimosoides* L.[4–6] 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. The decrease of bioactivity in **4** would be attributed to the largeness of the FITC functionality. Based on this result, we chose AMCA [6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoyl], NBD [6-*N*-(7-nitrobenz - 2 - oxa - 1,3 - diazol - 4 - yl)aminohexanoyl], and dansyl [6-(4-((5-dimethylaminonaphthalene-1-sulfonyl)-amino))hexanoyl] groups as the fluorescent functionalities.[7] All these groups are much smaller than the FITC group. We synthesized probe compounds bearing those fluorescent functionalities according to the procedure previously reported in Ref. 6. As shown in Scheme 1, compound **7**, which was prepared according to the previously established procedure,[6] was coupled with succinimide-type activated esters (**8**–**10**). An aminosugar and a hexanoic acid linker in the fluorescent functionalities were connected through an amide bond, which is assumed to be stable against the hydrolysis by esterase in the plant body. After alkaline hydrolysis in the final step from ester (**11**–**13**) to potassium salt (**1**–**3**), pH of reaction mixtures were adjusted to weakly basic (pH 7–8) with monitoring pH by a pH meter to circumvent the decomposition of fluorescent group in the course of work-ups.

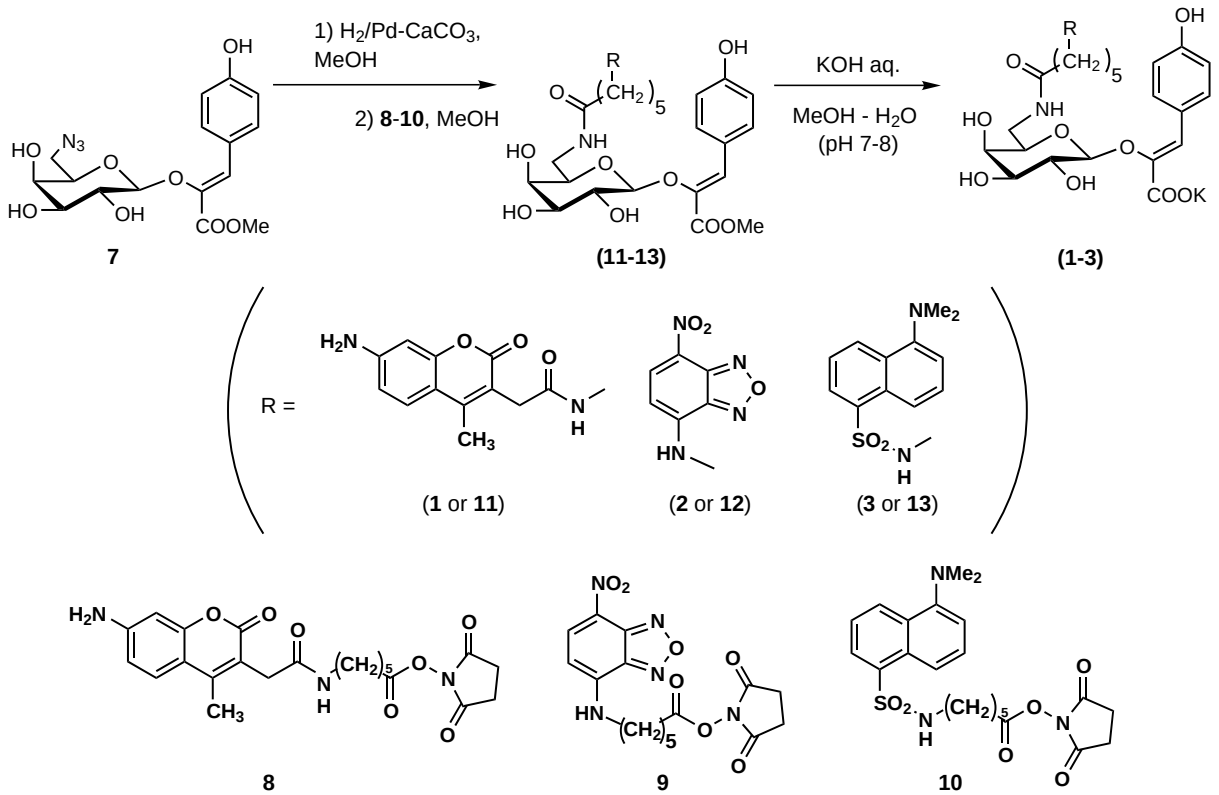
The resulting fluorescence-labeled probe compounds (**1**–**3**)[8–10] showed leaf-opening activity against the leaf of *C. mimosoides*. The bioactivities of all the probe compounds are shown in Table 1. Especially, the NBD-labeled probe compound (**2**) showed the strongest bioactivity, and was effective at 5×10^{-6} M, which was one-fifth as strong as that of the natural product (**6**).

We used probe **1** for fluorescence study of the interaction between the leaf-opening substance and the plant motor cell, which gains volume in the process of leaf-opening and loses volume in the process of leaf-closing and plays a central role in the plant leaf movement.[11] A leaf of *C. mimosoides* was cut by a microslicer (Dousaka EM Co., Ltd.) to a thickness of thirty micrometers. Then the section containing a motor cell was incubated overnight in an aqueous solution containing 5×10^{-5} M of **1**. After staining, the stained section was incubated for 30 minutes with washing buffer to remove excess fluorescent probes. Then, the stained section was monitored by using a fluorescence microscope with an appropriate filter. The use of an antifadant reagent (Slow Fade™ Antifade Kits, Molecular Probes Inc.) was essential to prevent photobleaching (fading of fluorescence). Fig. 1 shows photographs

Table 1. The leaf-opening activity of each fluorescence-labeled probe (status of leaf at 9:00 p.m.)

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

++: Completely open; +: weakly open; +–: random; –: closed.



Scheme 1. The preparation of fluorescence labeled probe compounds (**1**–**3**).

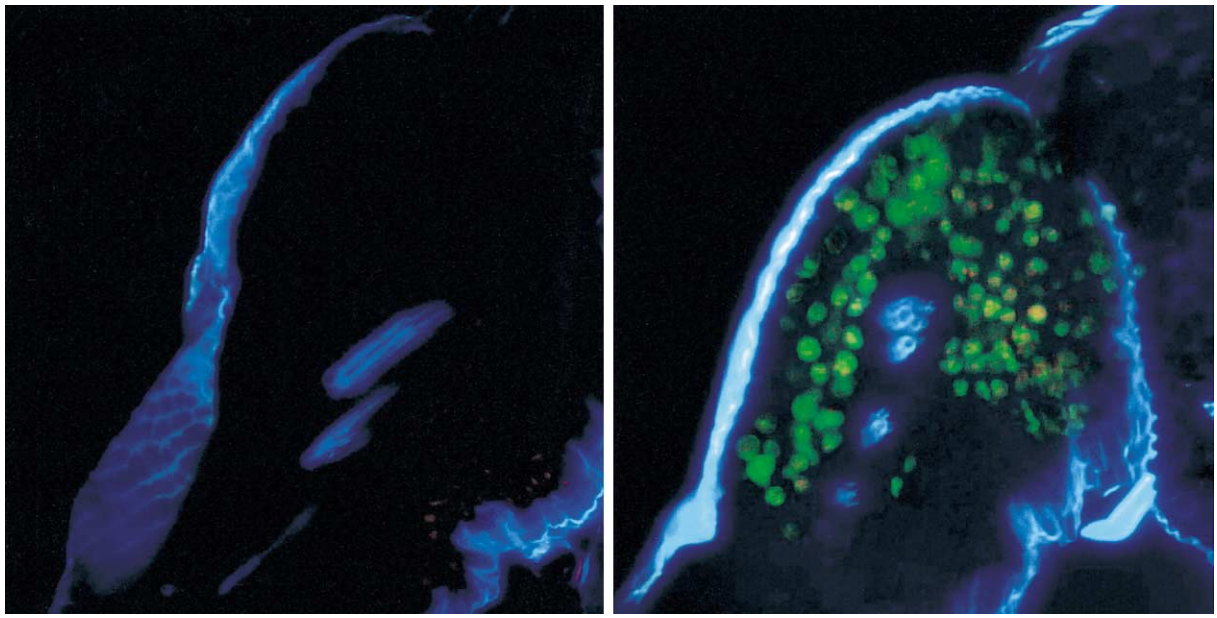


Figure 1. Fluorescence study of pant pulvini containing motor cells using a probe compound (**1**); left: control, right: a section treated with **1**.

of plant pulvini, which contains motor cell, under a fluorescence microscope. The staining pattern for the fluorescence of probe compound (**1**) was observed on the surface of motor cell (Fig. 1). No stain was observed in the control section, which was treated with a solution containing no **1** (Fig. 1). Probe **2**, the fluorescence of which is similar to the background fluorescence (autofluorescence) of the plant tissue under a fluorescence microscope, was inappropriate for the fluorescence study. In conclusion, we have succeeded in visualization of the binding site of the leaf-opening substance in the plant body. These results suggest that the specific binding site for **1** (or **5**) should exist on the plasma membrane of the motor cell.

Acknowledgements

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8. Potassium (*Z*)-2-[6′-(6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenylacrylate (**1**): ^{1}H 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, $J=8.7$ Hz), 6.67 (1H, dd, $J=8.7$, 2.1 Hz), 6.52 (1H, d, $J=2.1$ Hz), 3.82 (1H, t, $J=8.9$ 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): $[M\text{-}K]^-$. Found m/z 668.2471, $C_{33}H_{38}N_3O_{12}$ requires m/z 668.2455; IR (film) ν: 3341, 1677, 1605, 1557, 1515 cm^{-1}; $[\alpha]_D^{22}$ +60.0°(*c* 0.14, MeOH).
9. Potassium (*Z*)-2-[6′-(6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanamide)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenylacrylate (**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): $[M\text{-}K]^-$. Found m/z 616.1937, $C_{27}H_{30}N_5O_{12}$ requires m/z 616.1891; IR (film) v: 3341, 1584, 1510 cm^{-1}; $[\alpha]_D^{19}$ +62.5° (c 0.26, MeOH).

10. Potassium (*Z*)-2-[6′-(6-(4-((5-dimethylaminonaphthalene-1 - sulfonyl)amino))hexanamide) - β - D - galactopyranosyloxy]-3-*p*-hydroxyphenylacrylate (**3**): ^1H 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, $J=8.7$ 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): $[M\text{-}K]^-$. Found m/z 686.2334, $C_{33}H_{40}N_3O_{11}S$ requires m/z 686.2384; IR (film) v: 3307, 1651, 1607, 1575, 1514 cm^{-1}; $[\alpha]_D^{20}$ +54.9° (c 0.11, MeOH).
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