Visualization of the Precise Structure Recognition of Leaf-opening Substance by Using Biologically Inactive Probe Compounds: Fluorescence Studies of Nyctinasty in Legumes 2

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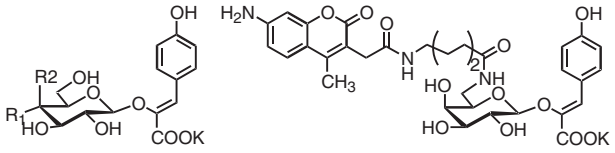
(Received January 21, 2002; CL-020068)

Intimate correlation between bioactivity and binding activity was shown using biologically active and inactive fluorescence-labeled probe compounds for nyctinasty. This results suggested the existence of a receptor which recognizes the aglycon of a leaf-opening substance on the motor cell.

Most legumes close their leaves in the evening, as if to sleep, and open them in the morning (nyctinasty).[1,2] We have isolated bioactive substances that regulate nyctinasty from several nyctinastic plants.[3] Recently, we reported that potassium lespedezate (**1**),[4] a leaf-opening substance, directly binds with a motor cell existing in the pulvini of *Cassia mimosoides* L. by using a biologically active fluorescence-labeled probe compound (**3**).[5] However, to prove the existence of a receptor molecule, it is also essential to demonstrate that biologically inactive probe compounds cannot bind to the motor cell. In this paper, we report the design and syntheses of the biologically inactive fluorescence-labeled probe compounds (**4** and **5**) and binding studies using them.



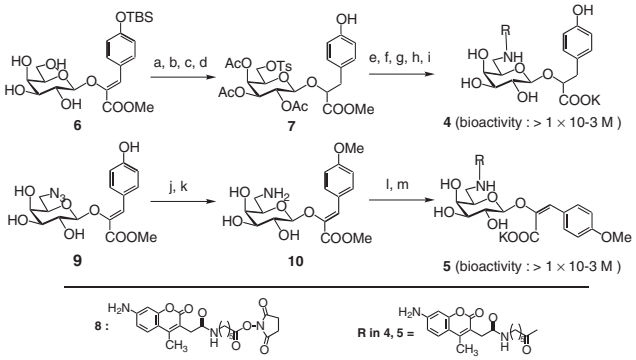
1 (R_1=OH, R_2=H)
2 (R_1=H, R_2=OH)

3

Molecular designs **4** and **5** were based on the structure-activity relationship of **1**. The structure modification in the glucose moiety of **1** did not affect its bioactivity,[6] on the other hand, modification in the aglycon of **1**, such as the reduction of the double bond, protection of the carboxylate or phenolic hydroxyl group, greatly diminished the bioactivity.[7] Thus, the biologically inactive probe compounds were designed on inactive analogs of **1** with modification in its aglycon moiety. The sugar part of the probe was changed to galactose, which prevents decomposition of the probe compounds by β-glucosidase in plant body.[5] The introduction of a fluorescence dye was carried out at the 6-position of the galactose moiety through the amide bond.[5] We chose 6-[(7-amino-4-methylcoumarin-3-acetyl) amino]-hexanoy (AMCA) groups[8] as a fluorescent dye which is the same as that contained in **3**.

We synthesized two biologically inactive probe compounds (**4** and **5**). For the synthesis of a probe compound with a reduced double bond (**4**), **6**[5] was protected and reduced with catalytic hydrogenation with Pd-C to give **7**. Resulting two epimers on the α-carbon of carboxylate (3 : 2) were separated and major product was used in the next step. Compound **7** was derivatised to an aminosugar and then coupled with **8**. After alkaline hydrolysis, **4**[9] was obtained. The absolute stereochemistry of **4** was determined by the PGME method[10] to be (*R*) by using the pentaacetate of final product **4**.

For the synthesis of a probe compound with methyl ether (**5**), **9**[5] was treated with diazomethane to give **10**, which was coupled with **8** and hydrolyzed to give **5**.[11] The geometry of the double bond in **5** changed to (*E*)-form. However, it was already shown that the geometry of the double bond in **1** easily isomerized in aqueous solution to give a mixture of both geometrical isomers which are equally effective against plant leaf.[6] The resulting **4** and **5** showed no leaf-opening activity against *C. mimosoides* even at 1×10^{-3} M. The bioactivities of **4** and **5** were no more than one-thousandth of that of probe **3**.



Scheme 1. The preparation of fluorescence labeled probe compounds (**4** and **5**): reagents: (a) TsCl, pyr; (b) Ac_2O, pyr; (c) TBAF, THF; (d) H_2/Pd-C, EtOAc; (e) NaN_3, 15-Crown-5, DMF; (f) MeONa, MeOH; (g) H_2/Pd-C, MeOH; (h) **8**, DMF; (i) KOH, MeOH-H_2O; (j) CH_2N_2, MeOH; (k) H_2/Pd-$CaCO_3$, MeOH; (l) 8, DMF; (m) KOH, DMF-H_2O.

We carried out fluorescence studies of the interaction between biologically inactive probe (**4** and **5**) and the plant motor cell using plant sections. A leaf of *C. mimosoides* was cut by a microslicer 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 **4** and **5**. After staining, the stained section was incubated with washing buffer to remove excess probe compounds. The same experiment was carried out by using **3** as a control experiment.

Figure 1 shows photographs of sections of plant pulvini, which contains a motor cell, under a fluorescence microscope. A staining pattern for the fluorescence of probe compound (**3**) was observed on the motor cell (Figure 1). On the other hand, no stain was observed in the section treated with (*R*)-**4** and **5** (Figure 1).

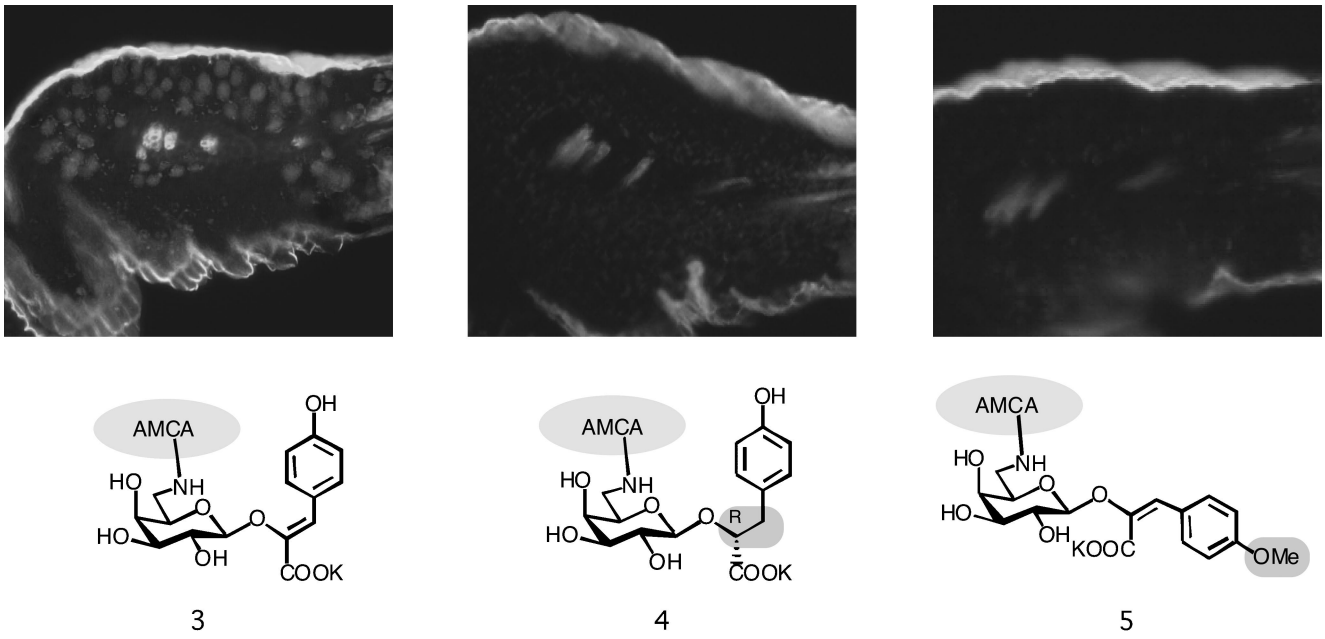


Figure 1. Fluorescence staining of pulvini containing motor cell; sections treated with; left: 3, center: 4, right: 5.

Thus, it was proved that biologically inactive probe compounds cannot bind to the plant motor cell. And also, binding of probe **3** was inhibited by the coexistence of 1000-fold concentration of non-labeled **2**. When the section was treated by 5×10^{-5} M of **3** together with 5×10^{-2} M of **2**, no staining was observed in the plant section. Results from the structure activity relationship were consistent with the results from binding experiments. Also, it was clearly shown that the binding of biologically active AMCA-labeled probe (**3**) with a motor cell is due to the specific binding of the aglycon moiety which is the active site of this molecule, and is not a nonspecific binding due to the hydrophobic AMCA group.

These results strongly suggested that some receptor for the **1** exists on the motor cell. Along with the previous result,[5] some properties were revealed on a receptor molecule of **1**, that is, this receptor recognizes the precise structure of aglycon moiety; on the other hand, it does not recognize the sugar moiety precisely.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (No. 12045259 and No. 12680598), Pioneering Research Project in Biotechnology given by the Ministry of Agriculture, Forestry and Fisheries, Inamori Foundation, Mitsubishi Chemical Corporation Fund, Naito Foundation for financial support.

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9 Properties of **4**: ^{1}H NMR (400 MHz, CD_3OD, RT): δ 7.48 (1H, d, $J = 8.8$ Hz), 7.11 (2H, d, $J = 8.6$ Hz), 6.66 (2H, d, 8.6 Hz), 6.64 (1H, dd, $J = 8.8$, 2.2 Hz), 6.50 (1H, d, $J = 2.2$ Hz), 4.21 (1H, dd, $J = 5.4$, 6.4 Hz), 4.18 (1H, d, $J = 7.6$ Hz), 3.66 (1H, d, $J = 3.4$ Hz), 3.48–3.57 (4H, m), 3.43 (1H, dd, $J = 8.6$, 4.6), 3.34 (1H, dd, $J = 3.4$, 10.0 Hz), 3.20 (1H, dd, $J = 8.6$, 13.7 Hz), 3.17 (2H, t, $J = 6.8$ Hz), 2.97 (1H, dd, $J = 13.9$, 5.4 Hz), 2.94 (1H, dd, $J = 13.9$, 6.4 Hz), 2.36 (3H, s), 2.20 (2H, t, $J = 7.3$ Hz), 1.45–1.63 (4H, m), 1.32 (2H, m); ^{13}C NMR (100 MHz, D_2O, 30 °C): δ 181.3, 179.5, 175.1, 156.6, 156.0, 155.8, 153.7, 133.1, 131.6, 129.0, 117.6, 115.6, 115.5, 113.9, 104.9, 103.1, 83.2, 75.5, 75.0, 73.1, 71.7, 42.3, 41.8, 40.0, 38.1, 36.4, 30.6, 28.0, 27.5, 17.1; HRMS-FAB (negative); $[M\text{-}K]^-$ found *m/z* 670.2597, calcd for $C_{33}H_{40}O_{12}N_3$, 670.2612; IR (film) ν: 3344, 1635, 1603, 1557 cm^{-1}; $[\alpha]_D^{21}$ +20.0° (c 0.14, H_2O).
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11 Properties of **5**: ^{1}H NMR (400 MHz, D_2O, 30 °C): δ 7.76 (2H, d, $J = 9.0$ Hz), 7.57 (1H, d, $J = 8.5$ Hz), 6.89 (2H, d, $J = 9.0$ Hz), 6.82 (1H, s), 6.80 (1H, dd, $J = 2.2$, 8.5 Hz), 6.64 (1H, d, $J = 2.2$ Hz), 4.88 (1H, d, $J = 7.8$ Hz), 3.87 (1H, d, $J = 3.4$ Hz), 3.82 (1H, dd, $J = 7.8$, 9.8 Hz), 3.78 (3 H, s), 3.71 (1H, dd, $J = 13.9$, 3.4 Hz), 3.68 (1H, dd, $J = 9.8$, 3.7 Hz), 3.55 (2H, s), 3.45 (1H, dd, $J = 13.9$, 3.7 Hz), 3.28 (1H, dd, $J = 13.9$, 9.8 Hz), 3.15 (2H, dt, $J = 2.9$, 5.6 Hz), 2.40 (3H, s), 2.04 (2H, t, $J = 7.3$ Hz), 1.10–1.44 (6 H, m); ^{13}C NMR (100 MHz, D_2O, 30 °C): δ 174.5, 173.2, 166.1, 160.6, 155.3, 155.2, 153.1, 147.2, 133.4, 132.5, 128.4, 128.2, 122.8, 115.4, 115.0, 114.7, 113.3, 104.6, 102.5, 75.0, 74.9, 73.0, 70.9, 57.0, 41.7, 41.0, 37.2, 35.7, 29.6, 27.2, 26.5, 16.5; HRMS-FAB (negative); $[M\text{-}K]^-$ found *m/z* 682.2628, calcd for $C_{33}H_{40}O_{12}N_3$, 682.2612; IR (film) ν: 3351, 1601, 1556, 1516 cm^{-1}; $[\alpha]_D^{22}$ +50.5° (c 0.20, H_2O).