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Synthetic Probe Compounds for Bioorganic Studies of Nyctinasty, Based on the Leaf-opening Substance of *Lespedeza cuneata* G. Don

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Abstract—In a previous paper, the syntheses of potassium galactolespedezate (**1**) and potassium galactoisolespedezate (**2**), artificial leaf-opening substances useful for bioorganic studies of nyctinasty were reported. The fluorescent probe compounds, fluorescence-labeled galactoisolespedezate (**3**) and iodo-one (**4**), designed on the basis of **1** and **2** were prepared. Especially, compound **3** was bioactive at 5×10^{-5} M, one-fiftieth as strong as the natural leaf-opening substance. This fluorescent probe would be useful for bioorganic studies of nyctinasty. © 2000 Elsevier Science Ltd. All rights reserved.

Most Leguminosae plants close their leaves in the evening, as if to sleep, and open them in the morning.[1] This is called nyctinasty, and such a circadian rhythmic movement has been known to be controlled by their biological clocks.[2] Recently, we have identified several bioactive substances that regulate this leaf-movement.[3–16] These bioactive substances can be used for probe compounds which would be highly useful for the purification of their receptors to enable bioorganic studies of nyctinasty.

Because of the high hydrophobicity of the leaf-movement factors, it is unlikely that these compounds pass through the plasma membrane of the plant cell and interact with target molecule in the cell. Investigation of the site where bioactive substances are perceived at the cellular level is a first step towards the bioorganic study of their receptor molecule. Various bioactive probe compounds are used for the study of their binding-site, e.g. fluorescence-labeled compounds which are widely used for the identification of receptor molecules,[17–19] and also, ^{125}I-labeled compounds which are often used for the localization of the binding site of bioactive substances because of their extremely high sensitivity for the detection of the radioisotope.[20–22]

Recently, we synthesized artificial leaf-opening substance (**1** and **2**) useful for the bioorganic studies on nyctinasty.[23,24] Based on this result, we report now the syntheses of a fluorescence-labeled leaf-movement factor (**3**) and an iodo-one (**4**) designed on the leaf-opening substance of *Lespedeza cuneata* G. Don, potassium lespedezate (**5**) and potassium isolespedezate (**6**).[4,5]

Keywords: plants; natural products; biologically active compounds; fluorescent probe.

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The most important problem involved in the design of the leaf-movement factor-based probe compounds is the unstability of **5** and **6** in the plant body, which are easily hydrolyzed by β-glucosidase. Thus, we designed some probe compounds based on the structures of the artificial leaf-opening substances, potassium galactolespedezate (**1**) and potassium galactoisolespedezate (**2**), which could not be hydrolyzed in a plant body.[23,24]

The introduction of large functional groups, such as a fluorescent FITC (fluorescein-5-isothiocyanate) group or an iodine group, to artificial leaf-opening substances requires careful consideration of the structure–activity relationship in **5** and **6**. Previous studies showed that the substituents at the phenolic hydroxyl group of **5** and **6** as well as the carboxyl group led to decrease of activity to 10^{-3} or 10^{-4} M.[5] However, considering the bioactivity of **1** and **2**, the substitution of the sugar moiety in **5** and **6**, from glucose to galactose, did not affect the bioactivity at all. From these results, it is expected that molecular recognition of **5** and **6** by its receptor molecule is attributable to the phenol moiety of these compounds. Thus, the introduction of a large functional group in the hydroxyl group at the 6′ position of the galactose moiety would not weaken the bioactivity of **1** and **2** to any extent.

Moreover, because of the resistance to the esterase in a plant body, an amide bond would be better than an ester bond to connect the FITC group with **1** and **2**. Thus, to introduce an FITC group at the 6′ position of **1** (or **2**), we should convert the hydroxyl group on the 6′ position of **1** (or **2**) into an amino group.

Compound **7**[23,24] was deprotected with sodium methoxide, and the primary alcohol in the product was tosylated. After acetylation **8** was obtained. By the deprotection of the silyl

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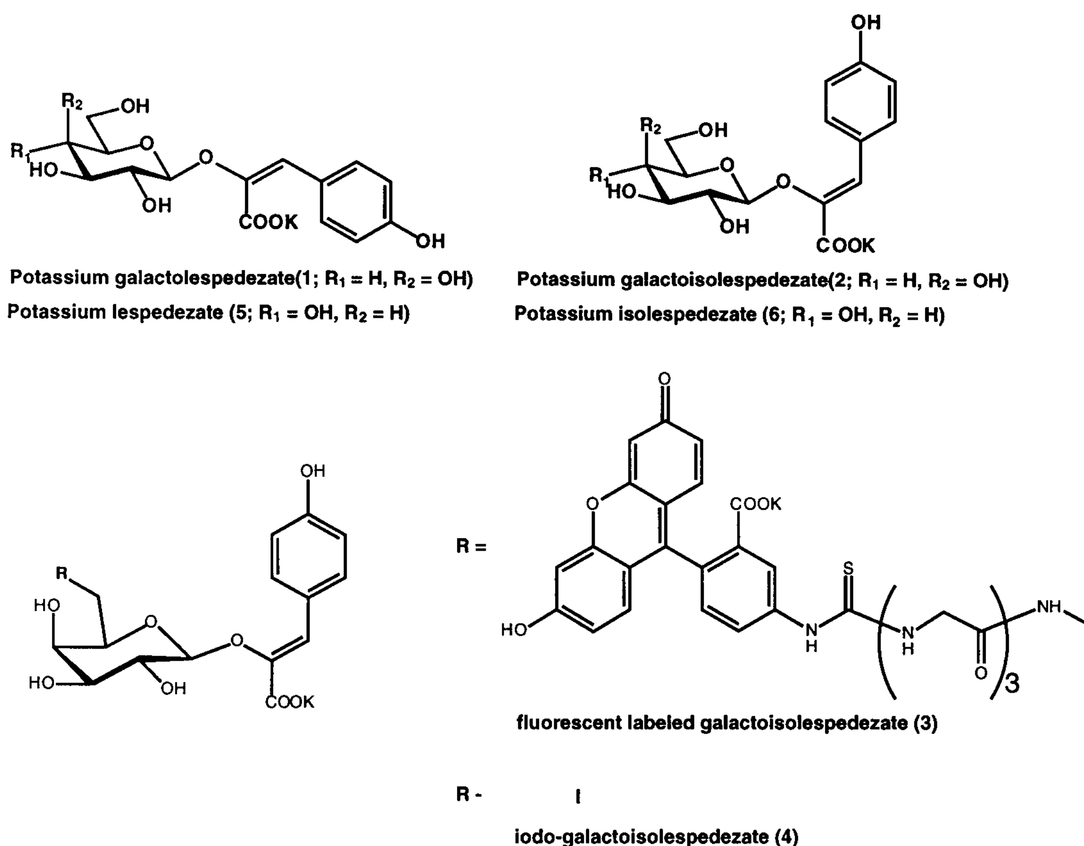
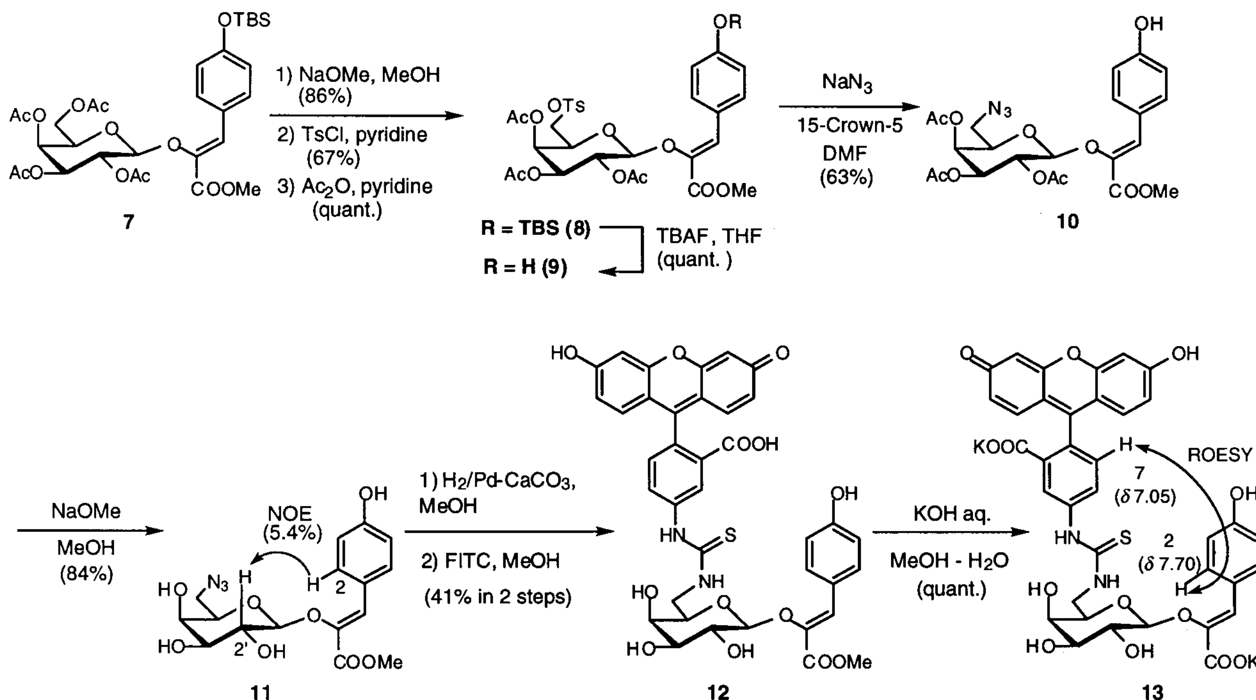
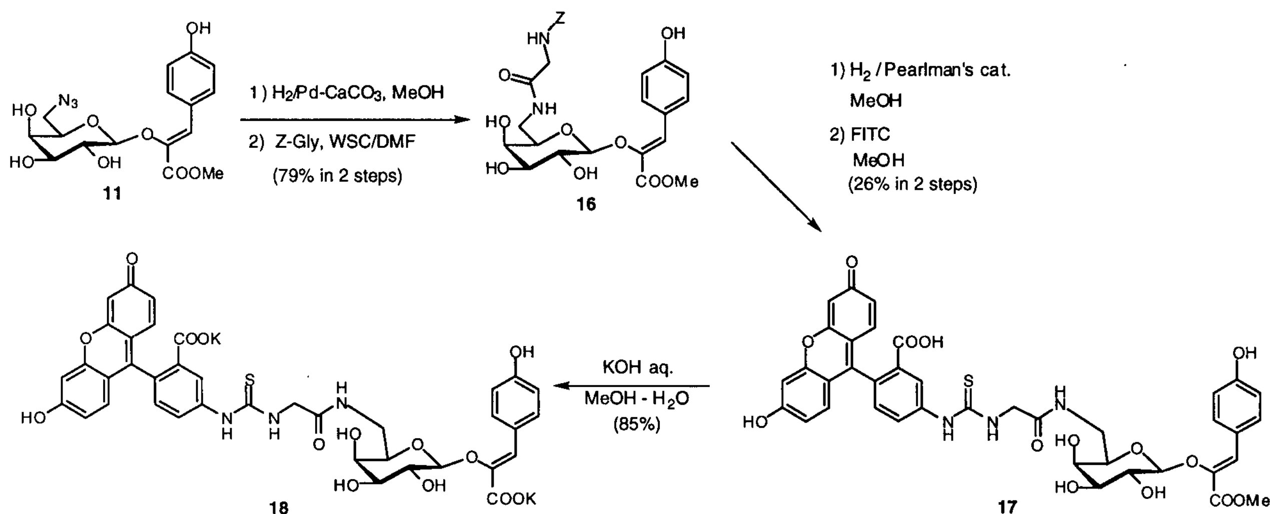


protective group using tetra-*n*-butyl ammonium fluoride, **8** was converted to tosylate **9** (Scheme 1). The resulting **9** was treated with sodium azide to give azide **10**, which was treated with sodium methoxide to give **11**. The (*Z*)-configuration of the enol double bond in **11** was determined by NOE between $H_{2'}$ and H_2. Compound **11** was then reduced by catalytic hydrogenation, and the resulting amine was coupled with FITC without purification. After deprotection of **12**, fluorescence-labeled potassium galactoisolespezate (**13**) was obtained together with a trace amount of fluorescence-labeled potassium galactolespedezate (**14**).[25] However, **13** showed leaf-opening activity



Scheme 1. Chemical synthesis of fluorescent-labeled probe compound (**13**).



Scheme 2. Chemical synthesis of fluorescent-labeled probe compound (**18**).

Table 1. Bioactivities of fluorescence-labeled probe compounds (**3**, **13**, **18**)

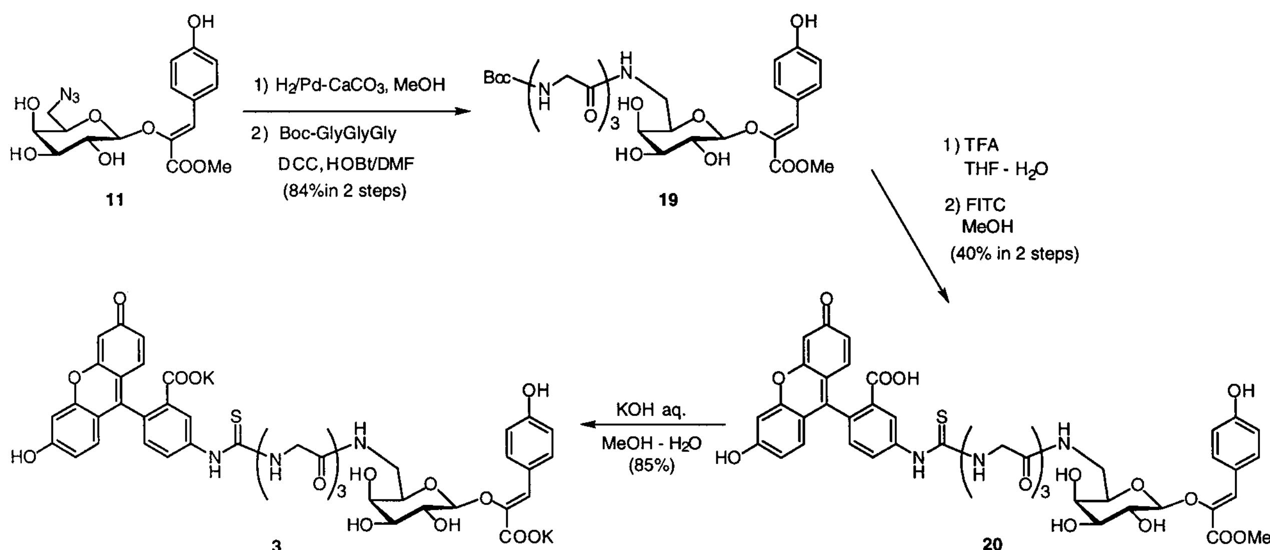
Compound	Bioactivity (M)
6	1×10^{-6}
13	5×10^{-4}
18	1×10^{-4}
3	5×10^{-5}

at 5×10^{-4} M, which is one-five hundredth as strong as **1** and **2**. The decrease of bioactivity could be due to the steric hindrance of the large fluorescent functional group with *p*-coumaroyl group; thus, the insertion of some linker between the FITC group and the active site of the artificial leaf-opening substance would be effective for the improvement of bioactivity. We used glycine and triglycine as a linker for the straightness of the peptide chain instead of long-chain carboxylic acid which is usually used as a linker.[26]

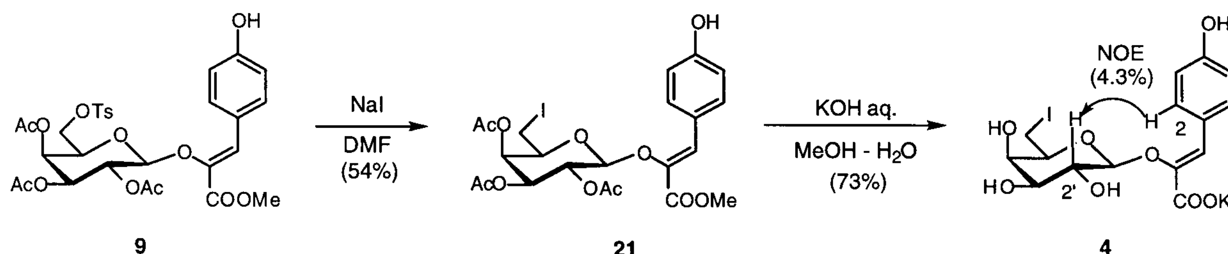
Scheme 2 shows the synthetic route of fluorescence-labeled potassium galactoisolespedezate (**18**). Azide **11** was reduced by catalytic hydrogenation, and then coupled with Z-Gly using WSC in DMF to give **16**. Compound **16** was deprotected with Pearlman's catalyst. When Pd–C was used as a catalyst instead of Pearlman's catalyst, overreduction of the enol double bond occurred. The resulting amine was coupled with the FITC reagent in situ to give **17**, which was further treated with KOH to give **18**.[25]

The bioactivity of **18** was improved up to five times the strength of **13**, thus providing the effectiveness of the spacer (Table 1).

Finally, we synthesized the third fluorescent probe with a linker of glycylglycylglycine (Scheme 3). According to the same procedures as those of **18**, using Z-Gly-Gly-Gly instead of Z-Gly, **3** could not be obtained because of the



Scheme 3. Chemical synthesis of fluorescent-labeled probe compound (**3**).



Scheme 4. Chemical synthesis of iodo-probe compound (**4**).

reduction of the enol double bond in catalytic hydrogenation for removal of the protective group. Thus, we changed the protective group of amine from a benzyloxycarbonyl to a *tert*-butoxycarbonyl group. Glycylglycylglycine was treated with $(Boc)_2O$ and the resulting protected tripeptide was coupled with the amine derived in situ from **11** using the DCC-HOBt method to give **19**. Compound **19** was then coupled with FITC to give **20**, which was further deprotected to give **3**.[25] The leaf-opening activity of fluorescent probe compound **3** was improved up to 5×10^{-5} M. The bioactivity of **3** was one-fiftieth as strong as that of the natural product; thus, **3** would be useful as a molecular probe for the bioorganic studies of nyctinasty.

The bioactivity of probe compounds (**13**, **18**, **3**) became higher with the increase of the length of the linker moiety (Table 1). In the ROESY experiments using **13**, **18**, and **3**, the correlation between the fluorescein moiety (H_7; δ 7.05) and the phenol moiety (H_2; δ 7.70) was observed only in **13** (Scheme 1); on the other hand, no correlation between the two parts was observed in **18** and **3**. Thus, the fluorescein group exists near the phenol moiety in **13**. This result suggests that the large fluorescein moiety in **13** blocks the binding of the phenol moiety to the receptor molecule to weaken the bioactivity.

Moreover, we have synthesized an iodo-probe compound (Scheme 4). To use the ^{125}I-labeled compound, we synthesized the probe compound containing a cold iodine atom at the 6′ position for the study of its bioactivity and stability under the conditions of the bioassay. Compound **9** was treated with sodium iodide to give **21**. Compound **21** was deprotected by 3.0 M KOHaq., and then the product was purified by ODS TLC to give **4**. The (*Z*)-configuration of the enol double bond in **4** was determined by NOE between $H_{2'}$ and H_2. The bioactivity of the resulting **4** and **22** was as strong as that of the original leaf-opening substance **5** and **6**. Also, its stability in the aqueous solution under the conditions of the bioassay is sufficient for usage as a molecular probe. Iodo-galactoisolespedezate (**4**) suffered no decomposition after one week. Thus, the radioactive ^{125}I-derivative of **4** would be used as a highly sensitive probe compound for the bioorganic study of receptors with respect to nyctinasty.

Here, we have succeeded in the chemical synthesis of several probe compounds for the bioorganic study of nyctinasty. Bioorganic studies using these probe compounds are now in progress.

Material and Methods

General procedures

^{1}H NMR (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on a Jeol GX400 spectrometer, using TMS in $CDCl_3$ or *t*-BuOH (^{1}H; 1.23 ppm, ^{13}C; 32.1 ppm) in D_2O as internal standards at various temperatures. ROESY experiment was carried out in CD_3OD (**12**) or CD_3OD/D_2O=8:2 (**3**, **17**) at 40°C. The FAB-MS and HR FAB-MS spectra were measured on a Jeol JMS-700 spectrometer, using glycerol as a matrix. The IR spectra were measured by JASCO FT/IR-410. The HPLC purification was carried out with a Shimadzu LC-6A pump equipped with a SPD-6A detector using Cosmosil 5C18AR column (ϕ 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 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 Co. Ltd). Reversed-phase open-column chromatography was performed on Cosmosil 75C18-OPN (Nakalai tesque 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).

Synthesis of methyl (*Z*)-2-(6′-*O*-*p*-toluenesulfonyl-2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-*t*-butyldimethylsilyloxyphenyl-acrylate (8). Compound **7** (1.75 g, 2.74 mmol) was dissolved in methanol (30 mL), and then sodium methoxide (583 mg, 11.0 mmol) was added to the solution at −50°C. The reaction mixture was stirred for 3 h, neutralized by Amberlite IR-120B (H^+), and then filtered. After evaporation, the resulting residue was purified by silica gel column chromatography ($CHCl_3$:MeOH=5:1) to give the corresponding alcohol (1.11 g, 86%).

The resulting alcohol (600 mg, 1.28 mmol) was dissolved in pyridine (5 mL), and then *p*-toluene sulfonyl chloride (293 mg, 1.54 mmol) was added to this solution at 0°C. The reaction mixture was allowed to stand at rt for 5 h, with stirring under argon atmosphere, and was evaporated with the toluene azeotrope. The residue was chromatographed on silica gel ($CHCl_3$:MeOH=9:1) to give deprotected **7** (221 mg, 28%), along with the recovered alcohol (401 mg, 67%). Deprotected **7** (566 mg, 0.882 mmol) was dissolved in pyridine (5 mL), and acetic anhydride (1 mL, 1.06 mmol) was added to this solution with stirring

overnight at rt. The reaction mixture was evaporated as the toluene azeotrope, and the residue was chromatographed on silica gel (*n*-hexane:EtOAc=2:1) to give **8** (627 mg, quant.).

^{1}H NMR (400 MHz, CD_3OD, rt): 7.68 (2H, d, *J*=8.8 Hz), 7.67 (2H, d, *J*=8.8 Hz), 7.28 (2H, d, *J*=8.8 Hz), 7.00 (1H, s), 6.81 (2H, d, *J*=8.8 Hz), 5.41 (1H, dd, *J*=10.3, 8.3 Hz), 5.36 (1H, d, *J*=3.4 Hz), 5.31 (1H, d, *J*=8.3 Hz), 5.05 (1H, dd, *J*=10.3, 3.4 Hz), 3.89 (3H, m), 3.84 (3H, s), 2.42 (3H, s), 2.06 (3H, s), 2.04 (3H, s), 1.98 (3H, s), 099 (9H, s), 0.22 (6H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, rt): 169.7, 169.5, 163.9, 145.0, 137.9, 132.4, 132.0, 129.8, 127.9, 126.1, 125.9, 119.9, 99.2, 70.8, 70.6, 68.9, 66.7, 65.6, 52.3, 25.8, 25.7, 25.5, 21.7, 20.9, 20.6, 19.3, −4.21 ppm; HR FAB MS (positive): $[M+H]^+$ Found *m/z* 751.2418, $C_{35}H_{47}O_{14}SiS$ requires *m/z* 751.2436; IR (film) ν: 1755, 1716, 1639, 1601, 1508 cm$^{-1;}$ $[\alpha]_D^{19}$=+2.86°(*c*=0.93, $CHCl_3$).

Synthesis of methyl (*Z*)-2-(6′-*O*-*p*-toluenesulfonyl-2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-acrylate (9). The resulting tosylate (569 mg, 0.759 mmol) was dissolved in THF (7 mL), and tetra-*n*-butyl ammonium fluoride (1.0 M in THF, 1.0 mL, 1.0 mmol) was added to this solution at 0°C with stirring for 5 min. Water (10 mL) was added to this solution for the extraction with EtOAc. The resulting organic layer was washed with brine, dried over anhydrous Na_2SO_4, and then evaporated. The residue was chromatographed on silica gel ($CHCl_3$:EtOAc=2:1) to give **9** (520 mg, quant.).

^{1}H NMR (400 MHz, CD_3OD, rt): 7.69 (2H, d, *J*=8.3 Hz), 7.68 (2H, d, *J*=8.3 Hz), 7.29 (2H, d, *J*=8.3 Hz), 7.01 (1H, s), 6.81 (2H, d, *J*=8.3 Hz), 5.52 (1H, s), 5.40 (1H, dd, *J*=10.3, 7.8 Hz), 5.36 (1H, d, *J*=3.4 Hz), 5.29 (1H, d, *J*=δ 7.8 Hz), 5.04 (1H, dd, *J*=10.3, 3.4 Hz), 3.90 (3H, m), 3.84 (3H, s), 2.42 (3H, s), 2.06 (3H, s), 2.05 (3H, s), 1.99 (3H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, rt.): 169.8, 163.9, 156.6, 145.1, 137.9, 132.7, 131.9, 129.8, 127.8, 126.1, 125.3, 115.2, 99.3, 70.8, 70.6, 69.0, 66.7, 65.6, 52.3, 21.7, 20.9, 20.7 ppm; HR FAB MS (positive): $[M+H]^+$ Found *m/z* 637.1558, $C_{29}H_{33}O_{14}S$ requires *m/z* 637.1591; IR (film) ν: 3437, 1754, 1715, 1638, 1606, 1585, 1514 cm$^-$1; $[\alpha]_D^{19}$=+2.13° (*c* 0.44, $CHCl_3$).

Synthesis of methyl (*Z*)-2-(6′-azido-2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-acrylate (10). Compound **9** (520 mg, 0.818 mmol) was dissolved in DMF (4 mL). After the addition of 15-crown-5 (1.6 mL, 8.18 mmol) and NaN_3 (531 mg, 8.18 mmol), the reaction mixture was stirred at 70°C for 2 days, mixed with water (20 mL), and then extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. After evaporation, the residue was chromatographed on silica gel (*n*-hexane:EtOAc=1:1) to give **10** (260 mg, 63%).

^{1}H NMR (400 MHz, $CDCl_3$, rt): 7.73 (2H, d, *J*=8.8 Hz), 7.05 (1H, s), 6.81 (2H, d, *J*=8.8 Hz), 5.47 (1H, dd, *J*=10.3, 7.8 Hz), 5.38 (1H, d, *J*=7.8 Hz), 5.34 (1H, d, *J*=3.4 Hz), 5.08 (1H, dd, *J*=10.3, 3.4 Hz), 3.84 (3H, s), 3.77 (1H, m), 3.34 (1H, dd, *J*=13.2, 7.8 Hz), 3.02 (1H, dd, *J*=13.2, 4.4 Hz), 2.19 (3H, s), 2.08 (3H, s), 2.01 (3H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, rt.): 170.0, 169.9, 164.0, 156.6, 137.7, 132.8, 130.8, 126.7, 125.5, 115.1, 99.3, 73.1, 70.1, 69.2, 67.8, 52.2, 50.4, 20.9, 20.8, 20.7 ppm; HR FAB MS (positive): $[M+H]^+$ Found *m/z* 508.1615, $C_{22}H_{26}O_{11}N_3$ requires *m/z* 508.1567; IR (film) ν: 3422, 2103, 1751, 1720, 1638, 1606, 1585, 1514 cm^{-1}; $[\alpha]_D^{19}$=+14.3° (*c*=0.50, $CHCl_3$).

Synthesis of methyl (*Z*)-2-(6′-azido-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-acrylate (11). Compound **10** (260 mg, 0.513 mmol) was dissolved in MeOH, and sodium methoxide (83.1 mg, 0.513 mmol) was added to this solution at 0°C. After stirring for 30 min, Amberlite IR-120B (H^+) was added to the reaction mixture for neutralization. The reaction mixture was then filtered and evaporated, and the residue was chromatographed on silica gel ($CHCl_3$:MeOH=5:1) to give **11** (165 mg, 84%).

^{1}H NMR (400 MHz, CD_3OD, rt): 7.75 (2H, d, *J*=8.8 Hz), 7.02 (1H, s), 6.75 (2H, d, *J*=8.8 Hz), 5.07 (1H, d, *J*=7.8 Hz), 3.83 (1H, dd, *J*=9.8, 7.8 Hz), 3.80 (3H, s), 3.72 (1H, d, *J*=3.4 Hz), 3.60 (1H, m), 3.54 (1H, dd, *J*=9.8, 3.4 Hz), 3.48 (1H, dd, *J*=13.2, 8.5 Hz), 3.17 (1H, dd, *J*=13.2, 3.9 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD, rt.): 166.5, 159.7, 139.5, 133.7, 127.3, 125.7, 116.0, 103.1, 76.1, 74.6, 72.7, 70.7, 52.6, 52.4 ppm; IR (film) ν: 3362, 2103, 1703, 1639 1605, 1586, 1514 cm^{-1}; HR FAB MS (positive): $[M+Na]^+$ Found *m/z* 404.1040, $C_{16}H_{19}O_8N_3Na$ requires *m/z* 404.1070; $[\alpha]_D^{19}$=+84.2° (*c*=0.77, MeOH).

Synthesis of methyl (*Z*)-2-[6′-(5-thioureidylfluorescein)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (12). Compound **11** (3.4 mg, 8.9 μmol) was dissolved in MeOH (0.5 mL), and then Pd–$CaCO_3$ (5 mg) was added to this solution. After stirring for 4 h under hydrogen atmosphere, the reaction mixture was filtered and evaporated to yield amine. Fluorescein isothiocyanate (isomer I, 4.1 mg, 10 μmol) was added to the acetone (1.0 mL) solution of the resulting crude amine, and then the solution was stirred at rt for 2 h. After evaporation, the residue was chromatographed on silica gel TLC to give coupling product **12** (2.7 mg, 41%).

^{1}H NMR (400 MHz, CD_3OD, 40°C): 8.08 (1H, br. s), 7.67 (1H, d, *J*=7.8 Hz), 7.61 (2H, d, *J*=7.8 Hz), 7.04 (1H, d, *J*=8.3 Hz), 6.84–6.92 (5H, m), 6.70 (2H, d, *J*=8.8 Hz), 6.61 (2H, d, *J*=8.3 Hz), 4.96 (1H, d, *J*=7.8 Hz), 3.76–3.71 (4H, m), 3.69 (3H, s), 3.58 (1H, m), 3.48 (1H, dd, *J*=8.8, 3.4 Hz) ppm; IR (film) ν: 3323, 1703, 1605, 1542, 1509 cm$^{-1;}$ ^{13}C NMR (100 MHz, CD_3OD, 30°C): 183.0, 174.7, 166.7, 159.9, 154.3, 140.0, 133.8, 130.4, 127.1, 126.9, 125.9, 122.8, 116.3, 114.0, 111.7, 103.6, 74.8, 72.9, 71.0, 52.7, 46.5 ppm; HR FAB MS (positive): $[M-H]^-$ Found *m/z* 743.1573, $C_{37}H_{31}O_{13}N_2S$ requires *m/z* 743.1547; $[\alpha]_D^{19}$=+93.1° (*c*=1.0, MeOH).

Synthesis of potassium (*Z*)-2-[6′-(5-thioureidylfluorescein)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (13). Compound **12** (2.7 mg, 3.6 μmol) was dissolved in MeOH:H_2O=3:1 (0.5 mL), and 3.0 M KOHaq. (12 μL, 36 μmol) was added to this solution at 0°C. After stirring for 3 h, Amberlite IR-120B (H^+) was added to the solution. The solution was filtered and evaporated. The residue was dissolved in water (0.2 mL),

neutralized with 10% K_2CO_3aq., and then evaporated. Finally, the residue was chromatographed on an ODS TLC to give **13** (2.3 mg, 79%).

^{1}H NMR (400 MHz, CD_3OD, 30°C): 8.09 (1H, d, J=2.0 Hz), 7.70 (2H, d, J=8.8 Hz), 7.69 (1H, dd, J=8.3, 2.0 Hz), 7.05 (1H, d, J=8.3 Hz), 6.85 (1H, s), 6.78 (2H, d, J=8.8 Hz), 6.64–6.69 (4H, m), 6.51–6.55 (2H, m), 5.02 (1H, d, J=7.8 Hz), 4.06 (1H, m), 3.77–3.84 (3H, m), 3.63 (1H, dd, J=14.2, 8.4 Hz), 3.58 (1H, dd, J=9.7, 3.4 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 182.8, 171.0, 161.4, 158.9, 154.0, 142.2, 133.1, 131.5, 130.2, 128.9, 126.5, 125.5, 123.7, 116.0, 113.6, 111.5, 103.9, 103.4, 75.1, 74.9, 72.9, 70.1, 46.6 ppm; HR FAB MS (negative): $[M-H]^-$ Found m/z 805.0540, $C_{36}H_{27}O_{13}N_2SK_2$ requires m/z 805.0508; IR (film) ν: 3290, 1634, 1575, 1510 cm^{-1}; $[\alpha]_D^{20}$=+98.4° (c=1.0, MeOH).

Synthesis of methyl (*Z*)-2-[6′-(*N*-benzyloxycarbonyl-glycylamino)-β-D-galactopyranosyloxy]-3-*p*-hydroxy-phenyl-acrylate (16). Compound **11** (9.2 mg, 24 μmol) was dissolved in MeOH (0.5 mL), and the solution was stirred for 4 h under hydrogen atmosphere after the addition of Pd–$CaCO_3$ (8 mg). The reaction mixture was filtered with celite, and evaporated to yield amine. The resulting crude amine was dissolved in DMF (0.5 mL). To this solution was added Z-Gly (7.5 mg, 35 μmol), and then WSC (5.5 mg, 29 μmol) at 0°C. This reaction mixture was allowed to stand at rt overnight under argon atmosphere, and evaporated. The residue was chromatographed on silica gel to give **16** (10.3 mg, 79%).

^{1}H NMR (400 MHz, CD_3OD, 30°C): 7.75 (2H, d, J=8.8 Hz), 7.3 (5H, m), 6.99 (1H, s), 6.76 (2H, d, J=8.8 Hz), 5.08 (2H, s), 4.92 (1H, d, J=7.8 Hz), 3.80 (3H, s), 3.64–3.77 (4H, m), 3.44–3.51 (3H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 172.3, 166.7, 159.7, 139.9, 133.7, 131.5, 129.3, 128.9, 128.7, 127.0, 125.7, 116.1, 115.9, 103.7, 74.6, 74.5, 72.7, 70.2, 67.9, 52.7, 44.9, 40.8 ppm; HR FAB MS (positive): $[M+Na]^+$ Found m/z 569.1747, $C_{26}H_{30}O_{11}N_2Na$ requires m/z 569.1747; IR (film) ν: 3364, 1704, 1660, 1606, 1585, 1540, 1514 cm^{-1} $[\alpha]_D^{20}$=+83.9°(c=1.0, MeOH).

Synthesis of methyl (*Z*)-2-[6′-(glycyl 5-thioureidylfluor-escein)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (17). Compound **16** (10.3 mg, 18.9 μmol) was dissolved in MeOH (0.5 mL). After the addition of Pearlman's catalyst (8 mg), the solution was stirred for 2 h under argon atmosphere. The reaction mixture was then filtered with celite and evaporated to yield amine. FITC (isomer I, 8.8 mg, 23 μmol) was added to the crude amine dissolved in MeOH (0.5 mL), and then allowed to stand for 1 h. The reaction mixture was then evaporated, and the residue was chromatographed on ODS to give **17** (3.9 mg, 26%).

^{1}H NMR (400 MHz, CD_3OD, 30°C): 8.18 (1H, d, J=2.0 Hz), 7.80 (1H, dd, J=8.3, 2.0 Hz), 7.75 (2H, d, J=8.8 Hz), 7.15 (1H, d, J=8.3 Hz), 6.98 (1H, s), 6.75 (2H, d, J=8.8 Hz), 6.69 (2H, d, J=8.8 Hz), 6.67 (2H, d, J=2.4 Hz), 6.54 (2H, dd, J=8.8, 2.4 Hz), 4.95 (1H, d, J=7.8 Hz), 4.22 (1H, d, J=16.6 Hz), 4.14 (1H, d, J=16.6 Hz), 3.78–3.82 (2H, m), 3.80 (3H, s), 3.49–3.58 (3H, m) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 190.6, 183.5, 171.8, 170.9, 166.7, 159.7, 154.1, 142.1, 139.9, 133.8, 131.7, 130.2, 127.1, 125.7, 120.0, 116.1, 113.7, 111.5, 103.7, 103.5, 74.7, 74.6, 72.8, 70.4, 52.8, 40.9 ppm; HR FAB MS (positive): $[M+H]^+$ Found m/z 802.1940, $C_{39}H_{36}O_{14}N_3S$ requires m/z 802.1918; IR (film) ν: 3272, 1699, 1638, 1604, 1511 cm^{-1}; $[\alpha]_D^{20}$=+80.6° (c=0.94, MeOH).

Synthesis of potassium (*Z*)-2-[6′-(glycyl 5-thioureidyl-fluorescein)-β-D-galactopyranosyloxy]-3-*p*-hydroxy-phenyl-acrylate (18). Compound **17** (3.9 mg, 4.9 μmol) was dissolved in MeOH:H_2O=3:1 (0.5 mL), and 1.0 M KOHaq. (23.5 μL, 23.5 μmol) was added to this solution at 0°C. After stirring for 6 h, the solution was acidified with Amberlite IR-120B (H^+), filtered, and then evaporated. The residue was dissolved in MeOH:H_2O=1:1 (0.2 mL), neutralized with 10% K_2CO_3aq., and then evaporated. Finally, the residue was chromatographed on ODS TLC to give **18** (3.6 mg, 85%).

^{1}H NMR (400 MHz, D_2O, 30°C): 7.80 (1H, d, J=2.0 Hz), 7.78 (2H, d, J=8.8 Hz), 7.62 (1H, dd, J=8.3, 2.0 Hz), 7.32 (2H, dd, J=8.3, 2.9 Hz), 7.23 (1H, d, J=8.3 Hz), 6.78–6.82 (6H, m), 6.72 (1H, s), 4.95 (1H, d, J=7.8 Hz), 4.25 (1H, d, J=17.1 Hz), 4.13 (1H, d, J=17.1 Hz), 3.94 (1H, d, J=3.4 Hz), 3.80 (1H, m), 3.60–3.73 (2H, d, m), 3.56 (1H, d, J=14.2, 3.9 Hz), 3.42 (1H, d, J=14.2, 9.3 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD:D_2O=4:1, 30°C): 183.0, 172.3, 172.1, 157.2, 156.5, 146.4, 140.9, 132.6, 131.6, 129.4, 129.1, 128.7, 126.9, 123.6, 121.1, 118.3, 115.9, 114.0, 103.7, 103.4, 74.5, 74.4, 72.5, 70.2, 49.8, 40.8 ppm; HR FAB MS (negative): $[M-K]^-$ Found m/z 824.1158, $C_{38}H_{31}O_{14}N_3SK$ requires m/z 824.1163; IR (film) ν: 3290, 1638, 1587, 1510 cm^{-1}; $[\alpha]_D^{22}$=+43.0° (c=0.48, H_2O).

Synthesis of methyl (*Z*)-2-[6′-(*N*-*t*-butoxycarbonylglycyl-glycylglycylamino)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (19). Compound **11** (57.0 mg, 150 μmol) was dissolved in MeOH (1.0 mL). After the addition of Pd–$CaCO_3$ (15 mg), the reaction mixture was stirred for 2 h under hydrogen atmosphere. After filtration with celite and then evaporation, amine was obtained. The resulting crude amine was dissolved in DMF (0.5 mL), and then Boc-GlyGlyGly (52.0 mg, 180 μmol) and 1-hydroxy-benzotriazole (26.8 mg, 225 μmol), DCC(46.4 mg, 225 μmol) were added to this solution at 0°C. The reaction mixture was stirred overnight under argon atmosphere, and then evaporated. The residue was suspended in MeOH:H_2O=3:2, and precipitated DCUrea was filtered out. After evaporation, the resulting residue was chromatographed on Cosmosil 75C_{18}-OPN (MeOH:H_2O=1:2) to yield **19** (78.6 mg, 84%).

^{1}H NMR (400 MHz, CD_3OD, 30): 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, $CDCl_3$, 30°C): 173.5, 171.9, 171.7, 166.7, 159.7, 139.9, 133.7, 127.1, 125.7, 116.1, 103.7, 81.0, 74.6, 74.3, 72.8, 69.9, 52.7, 49.8, 44.9, 43.8, 43.5, 40.6, 28.7 ppm; HR FAB MS (positive): $[M+H]^+$ Found

m/*z* 627.2517, $C_{27}H_{39}O_{13}N_4$ requires *m*/*z* 627.2514; IR (film) ν: 3323, 1691, 1681, 1667, 1605, 1583, 1538, 1514 cm^{-1}; $[\alpha]_D^{18}=+78.8°$ (c=1.0, MeOH).

Synthesis of methyl (*Z*)-2-[6′-(glycylglycylglycyl 5-thioureidylfluorescein)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (20). Compound **19** (24.0 mg, 38.3 μmol) was dissolved in THF-H_2O=30:1 (0.3 mL). To this solution was added TFA (0.3 mL) at 0°C. After stirring for 1 h, the reaction mixture was evaporated to dryness as the water azeotrope to yield amine as TFA salt. The resulting crude amine was dissolved in MeOH (1 mL), and then trimethyamine (5.3 μL, 38.3 μmol) and FITC (isomer I, 17.9 mg, 46.0 μmol) were added to this solution at 0°C. After stirring for 2 h, the reaction mixture was evaporated and the residue was purified by ODS TLC (MeOH:H_2O=2:1) to give **20** (14.1 mg, 40%).

^{1}H NMR (400 MHz, CD_3OD, 30°C): 8.25 (1H, d, J=2.0 Hz), 7.81 (1H, dd, J=8.3, 2.0 Hz), 7.73 (2H, d, J=8.8 Hz), 7.11 (1H, d, J=8.3 Hz), 6.97 (1H, s), 6.73 (2H, d, J=8.8 Hz), 6.67 (4H, m), 6.54 (2H, dd, J=9.3, 2.2 Hz), 4.93 (1H, d, J=7.8 Hz), 4.35 (1H, d, J=16.6 Hz), 4.28(1H, d, J=16.6 Hz), 3.90 (2H, s), 3.77–3.93 (7H, m), 3.51–3.55 (2H, m), 3.37 (1H, dd, J=13.2, 6.9 Hz), 3.26 (1H, m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, 30°C): 202.4, 189.4, 183.8, 173.0, 172.1, 171.7, 166.8, 159.7, 154.2, 142.0, 139.8, 133.7, 131.6, 130.3, 127.2, 125.9, 125.7, 118.3, 116.1, 114.0, 111.6, 103.7, 103.5, 74.6, 74.3, 73.7, 72.8, 69.8, 47.2, 44.1, 43.4, 40.4 ppm; HR FAB MS (negative): $[M-H]^-$ Found *m*/*z* 914.2238, $C_{43}H_{40}O_{16}N_5S$ requires *m*/*z* 914.2191; IR (film) ν: 3357, 1654, 1648, 1641, 1606, 1590, 1510 cm^{-1}; $[\alpha]_D^{19}=+39.4°$ (c=1.0, MeOH).

Synthesis of potassium (*Z*)-2-[6′-(glycylgylcylglycyl 5-thioureidylfluorescein)-β-D-galactopyranosyloxy]-3-*p*-hydroxyphenyl-acrylate (3). Compound **20** (11.5 mg, 12.5 μmol) was dissolved in MeOH:H_2O=3:1 (0.5 mL), and then 1.0 M KOH aq. (0.1 mL, 0.1 mmol) was added to this solution at 0°C. After stirring for 2 h, the reaction mixture was acidified with Amberlite IR-120B (H^+), filtered, and then evaporated. The residue was dissolved in MeOH:H_2O=1:1 (0.2 mL), and then neutralized with 10% K_2CO_3aq. After evaporation, the residue was purified by ODS TLC (MeOH:H_2O=3:2) to give **3** (6.0 mg, 49%).

^{1}H NMR (400 MHz, CD_3OD, 30°C): 8.15 (1H, d, J=2.0 Hz), 7.75 (1H, dd, J=8.3, 2.0 Hz), 7.63 (2H, d, J=8.8 Hz), 7.02 (1H, d, J=8.3 Hz), 6.81 (1H, s), 6.70–6.77 (6H, m), 6.61 (2H, dt, J=8.7, 2.0 Hz), 4.85 (1H, d, J=8.8 Hz), 4.85 (1H, d, J=17.1 Hz), 3.86 (1H, d, J=17.1 Hz), 4.30 (1H, d, J=17.1 Hz), 3.92 (1H, d, J=17.1 Hz), 3.74–3.79 (4H, m), 3.55–3.59 (2H, m), 3.40 (1H, d, J=13.7, 5.4 Hz), 3.24 (1H, dd, J=13.7, 8.3 Hz) ppm; ^{13}C NMR (100 MHz, CD_3OD, 30°C): 184.9, 173.9, 173.2, 172.8, 172.1, 162.6, 159.6, 155.1, 150.2, 143.2, 133.9, 132.8, 131.2, 130.0, 127.7, 126.5, 123.4, 121.2, 116.8, 114.7, 112.4, 104.9, 104.4, 75.9, 75.5, 73.8, 71.2, 48.7, 44.7, 44.2, 41.7 ppm; HR FAB MS (negative): $[M-2K+H]^-$ Found *m*/*z* 900.2019, $C_{42}H_{38}O_{16}N_5S$ requires *m*/*z* 900.2034; IR (film) ν: 3294, 1736, 1661, 1639, 1606, 1586, 1546, 1513 cm^{-1}; $[\alpha]_D^{20}=+58.0°$ (c=0.38, MeOH).

Synthesis of methyl (*Z*)-2-(6′-iodo-2′,3′,4′,6′-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-acrylate (21). Compound **9** (100 mg, 0.157 mmol) was dissolved in DMF (1.0 mL). After the addition of NaI (236 mg, 1.573 mmol), the solution was stirred at 80°C for 2 days. After the addition of water (5 mL), the reaction mixture was extracted with EtOAc. The resulting organic layer was washed with brine, dried over anhydrous Na_2SO_4, and then evaporated. The residue was chromatographed on silica gel (benzene:acetone=4:1) to give **21** (49.9 mg, 54%).

^{1}H NMR (400 MHz, CD_3OD, rt): 7.72 (2H, d, J=8.8 Hz), 7.04 (1H, s), 6.82 (2H, d, J=8.8 Hz), 5.55 (1H, d, J=3.4 Hz), 5.43 (1H, dd, J=10.3, 8.3 Hz), 5.40 (1H, s), 5.34 (1H, d, J=8.3 Hz), 5.09 (1H, dd, J=10.3, 3.4 Hz), 3.84 (3H, s), 3.80 (1H, t, J=7.3 Hz), 3.03 (2H, d, J=7.3 Hz), 2.18 (3H, s), 2.07 (3H, s), 2.01 (3H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$, rt.): 170.0, 169.8, 156.5, 138.0, 132.8, 130.8, 126.1, 125.5, 115.1, 114.9, 99.0, 74.1, 70.8, 68.9, 68.2, 60.4, 52.3, 21.7, 20.9, 20.8, 20.7, 14.3 ppm; HR FAB MS (positive): $[M+Na]^-$ Found *m*/*z* 615.0371, $C_{22}H_{25}O_{11}INa$ requires *m*/*z* 615.0339; IR (film) ν: 3421, 1750, 1716, 1606, 1585, 1514 cm^{-1}; $[\alpha]_D^{22}=+21.8°$ (c=0.36, $CHCl_3$).

Synthesis of potassium (*Z*)-2-(6′-iodo-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-acrylate (4). Compound **21** (38.1 mg, 69.4 μmol) was dissolved in MeOH:H_2O=3:1 (0.50 mL). To this solution was added 3.0 M KOHaq. (171 μL, 0.513 mmol) at 0°C. After stirring for 2 h, the reaction mixture was neutralized with Amberlite IR-120B(H^+). After filtration, the filtrate was evaporated, and the residue was chromatographed on ODS TLC (MeOH:H_2O=4:3) to give **4** (23.1 mg, 73%) and its geometrical isomer (0.3 mg).

^{1}H NMR (400 MHz, D_2O, rt): 7.72 (2H, d, J=8.8 Hz), 6.85 (2H, d, J=8.8 Hz), 6.79 (1H, s), 4.96 (1H, d, J=7.8 Hz), 4.06 (1H, d, J=3.4 Hz), 3.76 (1H, dd, J=10.3, 7.8 Hz), 3.73 (1H, t, J=6.8 Hz), 3.66 (1H, dd, J=10.3, 3.4 Hz), 3.27 (1H, dd, J=10.3, 6.8 Hz), 3.23 (1H, dd, J=10.3, 6.8 Hz) ppm; ^{13}C NMR (100 MHz, D_2O, 30°C): 158.9, 147.0, 143.1, 134.8, 127.4, 117.8, 116.9, 104.1, 102.9, 78.0, 75.1, 73.3, 71.9, 51.3 ppm; HR FAB MS (negative): $[M-K]^-$. Found *m*/*z* 450.9898, $C_{15}H_{16}O_8I$ requires *m*/*z* 450.9890; IR (film) ν: 3348, 1606, 1577, 1512 cm^{-1}: $[\alpha]_D^{19}=+59.9°$ (c=0.35, H_2O).

Bioassay. The young leaves detached from the stem of the plant *Cassia mimosoides* L. 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 am.) were used for the bioassay. Each test solution was carefully poured into test tubes with a microsyringe around 10:00 am. The bioactive fraction was judged by the leaf-opening after the leaf-closing of the plant leaf in the blank solution containing no sample.

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