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SYNTHESES AND NOVEL BIOACTIVITIES OF ARTIFICIAL LEAF-OPENING SUBSTANCES OF *LESPEDEZA CUNEATA* G. DON, DESIGNED FOR THE BIOORGANIC STUDIES OF NYCTINASTY

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Abstract Potassium lespedezate (**1**) is the leaf-opening substance of a nyctinastic plant, *Lespedeza cuneata* G. Don. We have synthesized two sugar-derivatives of **1**, potassium galactolespedezate (**4**) and mannolespedezate (**6**). Both **4** and **6** were quite effective for the leaf–opening of *Cassia. mimosoides* at 8×10^{-7} M, as strong as **1**. However, **1** kept the leaf open only for a few days; on the other hand, **4** and **6** kept the leaf open after a week at that concentration. Additionally, remarkable difference was observed in the rate of enzymatic hydrolysis of natural and artificial leaf-opening sustance by using commercially available β-glucosidase Therefore, **4** and **6** would not be hydrolized in the plant body, and are potentially useful molecular probes for the studies of the control of the nyctinastic leaf-movement by a biological clock.

Keywords: plants; natural products; biologically active compounds; glycoside; glycosidation

INTRODUCTION

The nyctinastic leaf-movement of most leguminosae plants, has been known to be controlled by their biological clocks.[1] These plants sleep at night with their leaves closed, and awake in the morning with their leaves open, according to their circadian rhythm.[2] Recently, we have identified several bioactive substances that regulate this leaf-movement[3-15] and revealed that such a biorhythm as observed in a nyctinastic movement is introduced by the rhythmic change in the balance of concentration between leaf-closing and -opening substances.[7,8,11-15] In *Lespedeza cuneata* G. Don,[8,13,14] especially, we have identified both the leaf-opening and –closing substances and shown that a change in the balance between them is controlled by the enzymatic deactivation of the leaf-opening substances of this plant, potassium lespedezate (**1**) and isolespedezate (**2**). A biological clock controls the circadian rhythmic leaf-movement through the regulation of the activity of β-glucosidase that hydrolyzes the glycosidic bond of **1** (and **2**). Compound **1** is assumed to interact with some receptor in the plant body; thus, chemical studies on the mechanism of the action of **1** would be important for the bioorganic study of nyctinastic movement. For this reason, **1** is a potential molecular probe for this study. However, there is a severe problem inasmuch as **1** is converted into its aglycon, potassium 4-hydroxyphenylpyruvate (**3**),[13,14] by the action of β-glucosidase, and sufficient intake of

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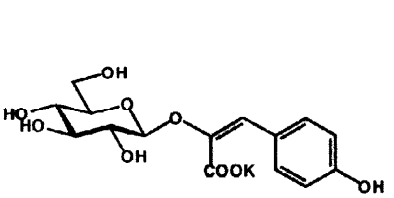
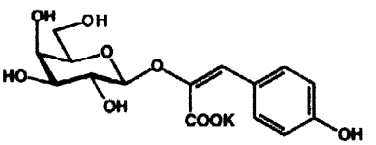
1 into the plant body could not be expected. Therefore, we tried to prepare the leaf-opening substance which could not be hydrolyzed by β-glucosidase.

RESULTS AND DISCUSSION

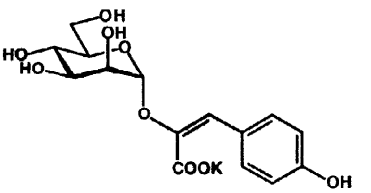
A previous report showed that the most important part of **1** for the leaf-opening activity is the *p*-hydroxyphenylpyruvate unit.[5] Shigemori *et al.* have synthesized several derivatives of **1**, and found that the α-anomer of **1** was as effectively strong as **1** in the bioassay, and even **3** showed weak leaf-opening activity.[5] The glucose unit would serve for the fixation of the enol double bond and the increase of solubility. From these results, the probe compounds were designed based on the idea that the derivatization of the sugar moiety of **1** would have little effect on the leaf-opening activity. We have, accordingly, synthesized four sugar-derivatives of **1**, that is, potassium galactolespedezate (**4**),[16] galactoisolespedezate (**5**), mannolespedezate (**6**) and mannoisolepedezate (**7**).



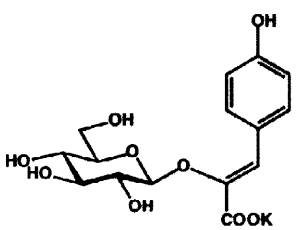
Potassium lespedezate (1)



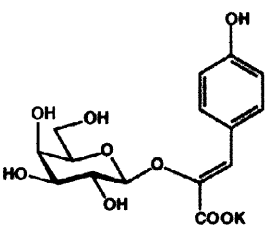
Potassium galactolespedezate (4)



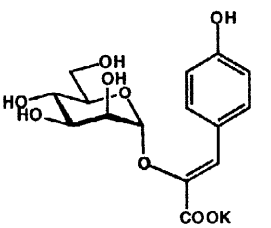
Potassium mannolespedezate (6)



Potassium isolespedezate (2)

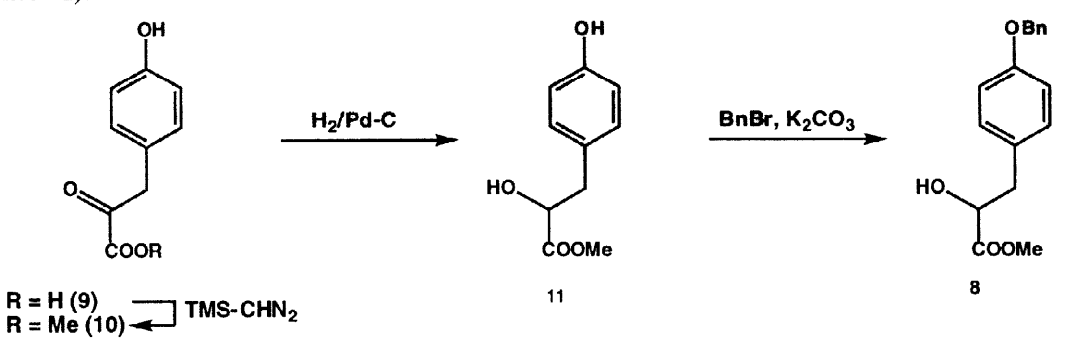


Potassium galactoisolespedezate (5)



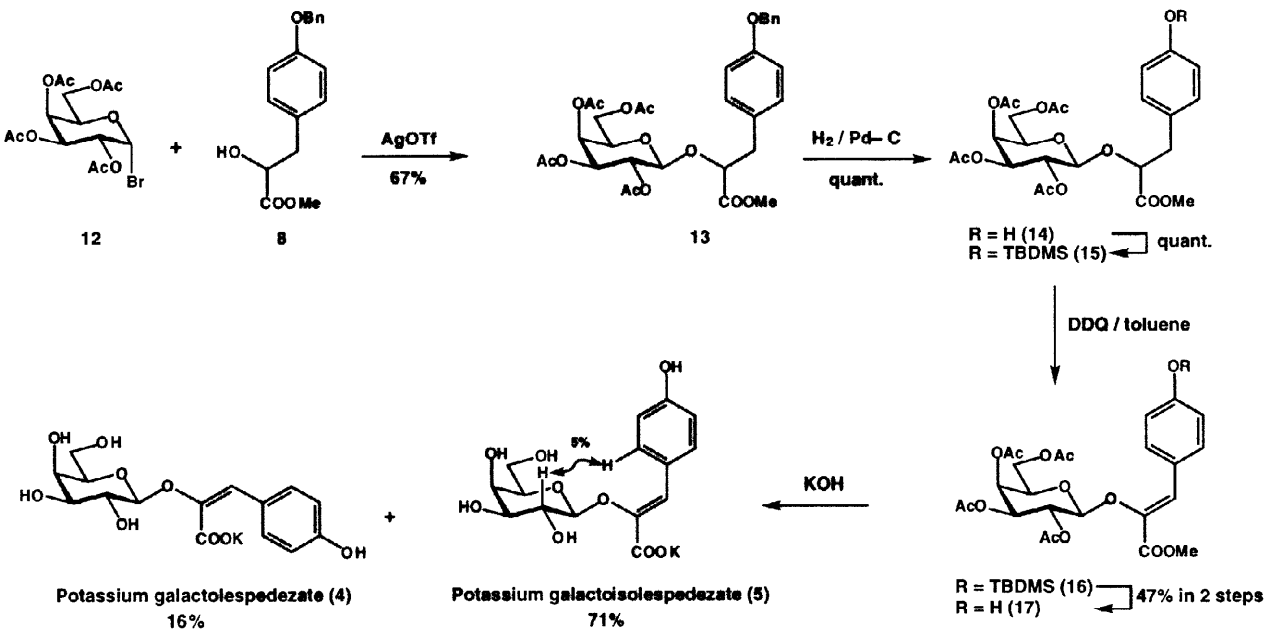
Potassium mannoisolespedezate (7)

The syntheses of **4** - **7** essentially followed the synthetic route of **1** reported by Shigemori *et al.*[17] First, we synthesized methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**) from *p*-hydroxyphenylpyruvic acid (**9**). Esterification of **9** with TMS-diazomethane gave **10**, and the following catalytic hydrogenation gave methyl 3-*p*-hydroxyphenyl-2-hydroxypropionate (**11**), which was treated with BnBr and K_2CO_3 to give **8** (**Scheme 1**).



Scheme 1. Synthesis of methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**).

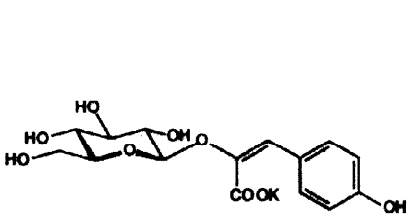
A coupling of D-acetobromogalactose (**12**) and **8** was carried out with AgOTf-Molecular Sieves 4A to give mainly β-glycoside (**13**). A racemic mixture of **8** was used in the glycosidation reaction, and the following reactions were carried out using both diastereomers without separation. A trace amount of an α-anomer was also observed in the reaction mixture; however, it was separated from **13** by silica gel column chromatography. In the glycosidation reaction, both diastereomers at the α-position of the carbonyl group were equally formed, which could not be separated by the silica gel column, and the following reactions were carried out using both diastereomers without separation. After deprotection by catalytic hydrogenation, the phenolic hydroxyl group of **14** was protected with TBDMSCl, and following DDQ oxidation of **15** using 6 eq. of DDQ for six days gave mainly one isomer, **16**, which was deprotected with nBu_4NF in THF at 0 °C to give the corresponding (*Z*)- and (*E*)-phenol (**17** and **18**) in the relative ratio 6 : 1. Finally, potassium galactolespedezate (**4**) and galactoisolespedezate (**5**) were obtained from the mixture of **17** and **18** on treatment with KOH in MeOH at 0 °C in 16 and 71% yields, respectively. Final purification of **4** and **5** was carried out by HPLC using a Cosmosil 5C18AR column with 35% MeOHaq. The synthetic route was summarized as shown in **Scheme 2**. The geometry of the double bond was confirmed by the NOE experiments: NOE (5%) was observed between $H_{2'}$ and H_2 in **5**. Compound **1** is known to isomerize easily into **2** in an aqueous solution.[5] Similarly to the case of **1** and **2**, compounds **4** and **5** were interconverted to each other, when dissolved in water.



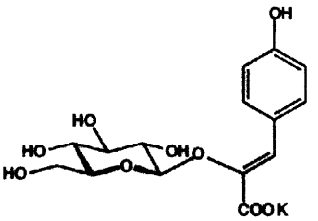
Scheme 2. Synthesis of potassium galactolespedezate (**4**) and potassium galactoisolespedezate (**5**).

Potassium α-mannolespezedate (**6**) and potassium α-mannoisolespezedate (**7**) were prepared according to the same synthetic route using D-acetobromomannose (**19**). The glycosidation reaction gave mainly the α-anomer, which is confirmed by the coupling constant between $H_{1'}$ and $C_{1'}$ ($^1J_{C\text{-}H}$ = 173.8 Hz in **7**).[18] The geometry of the double bond was also confirmed by the NOE (11.3%) between $H_{1'}$ and H_2 in **6**.

We also synthesized potassium L-lespedezate (**20**) and potassium L-isolespedezate (**21**) in the same manner. These enantiomers of the natural leaf-opening substances, **20** and **21**, were as effective as the natural ones, **1** and **2**. These results indicated that the D-sugar part as stereogenic moieties would serve to improve the solubility of these compounds in water.



Potassium L-lespedezate (20)



Potassium L-isolespedezate (21)

The synthetic artificial leaf-opening substances (**4-7, 20-21**) were effective at 8×10^{-7} M, as strong as **1** and **2**, in the bioassay.[4,5] Additionally, these compounds showed very interesting bioactivity; the leaf-opening activity of **1** lasted for only two days at 3×10^{-6} M; after that, the leaves closed at night again. On the other hand, that of artificial leaf-opening substances lasted even after a week. The leaves treated with 3×10^{-6} M of artificial leaf-opening substances (**4-7, 20-21**) kept opening until the leaves withered and died after a week (**Table 1**). This result suggested that **1** would be completely hydrolyzed into **3** by β-glucosidase in a few days; on the other hand, our artificial leaf-opening substances would not be hydrolized by the enzyme in the plant body. These results supported the importance of β-glucosidase in the regulation of nyctinasty. The leaf has never closed without the deactivation of the leaf-opening substance by β-glucosidase. These artificial leaf-opening substances were effective and specific to *Cassia. mimosoides*, and not effective for other nyctinastic plants, such as *Mimosa pudica*, *Aeschynomene indica*, and *Phyllanthus urinaria*, at 3×10^{-5} M.

Table 1. Time course change of the leaf-opening activities in natural and artificial leaf-opening substance

	Status of the leaves						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
1	++	++	+–	––	––	––	––
4	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++
20	++	++	++	++	++	++	++
21	++	++	++	++	++	++	++

Movement of the leaf was represented by following marks: ++ completely open; + nearly open; +– at random; – nearly closed; –– completely closed

Generally, glycosidase enzymes exhibit very high selectivity for hydrolysis of their preferred sugars: β-glucosidases selectively hydrolyze β-D-glucopyranosides. Thus, we further treated **4** with β-glucosidase to examine the reactivity of **4** for this enzyme. The potential product of this reaction, **3**, existed mainly as an enol form in aqueous solutions: about 95% of **3** isomerized to an enol form within two hours in the reaction conditions used for enzymatic reaction. Therefore, we observed the content of an enol form of **3** by the HPLC analyses of the reaction mixture. After 2 hours of incubation in 0.1 M citrate buffer (pH 5.0) at 37 °C with commercially available β-glucosidase, 36% of **4** was hydrolyzed into its aglycon; on the other hand, 80 % of **1** was easily hydrolyzed into its aglycon under the same conditions. Therefore, remarkable difference was observed in the rate of enzymatic hydrolysis of natural and artificial leaf-opening sustance by using commercially available β-glucosidase. Much higher selectivity could be expected by using the naturally occurring β-glucosidase that exists in the plant body. The purification of this enzyme is now in progress.

In conclusion, we have succeeded in the preparation of the probe compounds for the bioorganic studies of nyctinastic movement which could not be hydrolyzed in a plant body. A previous report showed that the substituents at the phenolic hydroxyl group as well as the carboxyl group led to the decrease of activity to 10^{-3} or 10^{-4} M.[5] However, it is assumed that the introduction of a large functional group in the hydroxyl group at the 6' position of the sugar moiety does not affect the bioactivity of **4**-**7**; thus, the fluorescence-labeled probe molecule or the gel for the affinity chromatography could be prepared. These probe compounds would be highly useful for purification of the receptor for the leaf-opening substance, thus enabling the bioorganic studies of nyctinasty.

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. The FAB-MS and HR FAB-MS spectra were measured by a Jeol JMS-700 spectrometer, using glycerol as a matrix. 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 and TLC were performed on Silica Gel 60 K070 (Katayama Chemical). The β-glucosidase (from almonds) was purchased from Sigma Chemical Co., Ltd.

Synthesis of (±)-methyl 3-p-benzyloxyphenyl-2-hydroxypropionate (8). To a solution of **9** (5.19 g, 28.8 mmol) in MeOH-benzene = 3:1 (200 mL) TMS-diazomethane (17 mL, 1.2 eq.) was added and stirred at rt. After the generation of N_2 gas ceased, the reaction mixture was concentrated *in vacuo* to give a colorless oil, which was then dissolved in MeOH (200 mL). To this solution 10% Pd-C (500 mg) was added and stirred at rt under H_2 atmosphere for ten hours. The reaction mixture was filtered with celite and the eluent was

concentrated *in vacuo*, and the residue was purified with silica gel column chromatography (*n*-hexane-EtOAc = 1:1) to give **11** (5.64 g, quant). To a solution of **11** (2.83 g, 14.4 mmol) in DMF (60 mL) benzyl bromide (1.71 mL, 1.0 eq.) and K_2CO_3 (3.99 g, 2.0 eq.) were added and stirred at rt under Ar atmosphere overnight. To the reaction mixture H_2O (100 mL) was added and the product was extracted with EtOAc. The organic layer was washed with saturated NaCl aq. and dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane-EtOAc=2:1), and crystallized from *n*-hexane-EtOAc to give **8** (3.01 g, 73%).

(±)-methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.30-7.50 (5H, m), 7.12 (2 H, d, J = 8.3 Hz), 6.91 (2 H, d, J = 8.3 Hz), 5.03 (2 H, s), 4.42 (1 H, dd, J = 6.8, 4.4 Hz), 3.76 (3H, s), 3.06 (1 H, dd, J = 14.2, 6.8 Hz), 2.91 (1 H, dd, J = 14.2, 6.8 Hz), 2.71 (1 H, br.s, OH) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 174.4, 157.6, 136.9, 130.3, 128.4, 128.3, 127.8, 127.3, 114.7, 71.3, 69.7, 52.5, 39.7 ppm; IR (film) ν : 3480, 1738, 1611, 1583, 1511 cm^{-1}; Elemental analysis: Found; C, 71.11%; H, 6.28%; O, 22.50%; Calcd.; C, 71.31%; H, 6.34%; O, 22.35%.

*Synthesis of Methyl 2-(2', 3', 4', 6'-tetra-O-acetyl-β-D-galactopyranosyloxy)-3-p-benzyloxyphenyl-propionate (**13**).* To a suspension of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**12**) (464 mg, 1.5 eq.), methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**) (215 mg, 0.753 mmol), and dry powdered molecular sieves 4A (1.5 g) in CH_2Cl_2 (5 mL) were added silver triflate (290 mg, 1.5 eq.) at 0 °C under Ar atmosphere for one hour. The reaction mixture was filtered on celite and the insoluble solid was washed with $CHCl_3$. The filtrate was washed with $NaHCO_3$ aq. and saturated NaCl aq. successively. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane-EtOAc = 4:3) to give a mixture of diastereomers (**13**, 309 mg, 67%). From **13** (20.3 mg), each diastereomer was separated with preparative TLC (benzene-acetone= 10:1) to give **13a** (10.2 mg) and **13b** (9.3 mg).

Methyl 2-(2', 3', 4', 6'-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-benzyloxyphenyl-propionate (**13a**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.40-7.30 (5H, m), 7.09 (2 H, d, J = 8.3 Hz), 6.87 (2 H, d, J = 8.3 Hz), 5.37 (1 H, dd, J = 3.4, 1.0 Hz), 5.25 (1 H, dd, J = 10.3, 7.8 Hz), 5.04 (2H, s), 5.00 (1 H, dd, J = 10.3, 3.4 Hz), 4.57 (1 H, t, J = 5.3 Hz), 4.52 (1 H, d, J = 7.8 Hz), 4.13 (1 H, dd, J = 11.2, 6.8 Hz), 4.08 (1 H, dd, J = 11.2, 6.8 Hz), 3.83 (1 H, dt, J = 6.8, 1.0 Hz), 3.62 (3 H, s), 3.07 (1 H, dd, J = 14.0, 5.3 Hz), 3.02 (1 H, dd, J = 14.0, 5.3 Hz), 2.16 (3 H, s), 2.03 (3 H, s), 1.98 (3 H, s), 1.97 (3 H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 170.8, 170.1, 170.0, 169.9, 169.5, 157.6, 137.0, 130.6, 128.4, 128.0, 127.8, 127.3, 99.6, 76.5, 70.8, 70.6, 70.0, 68.5, 67.0, 61.2, 51.8, 38.1, 20.8, 20.7, 20.6 ppm; IR (film)ν : 1751, 1611, 1512 cm^{-1}; $[\alpha]_D^{20}$ –24.8° (c 0.66, $CHCl_3$); HR FAB MS (positive): $[M+H]^+$ Found *m/z* 616.2157, $C_{31}H_{36}O_{13}$ requires *m/z* 616.2156; (**13b**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.40-7.30 (5H, m), 7.09 (2 H, d, J = 8.3 Hz), 6.88 (2 H, d, J = 8.3 Hz), 5.33 (1 H, d, J = 3.4 Hz), 5.24 (1 H, dd, J = 10.0, 8.0 Hz), 5.04 (2H, s), 4.91 (1 H, dd, J = 10.0, 3.4 Hz), 4.43 (1 H, d, J = 8.0 Hz), 4.13 (1 H, dd, J = 8.8, 4.4 Hz), 4.09 (1 H, dd, J = 10.7, 6.0 Hz), 4.05 (1 H, dd, J = 10.7, 6.0 Hz), 3.83 (1 H, t, J = 6. 0 Hz), 3.72 (3 H, s), 2.99 (1 H, dd, J = 14.2, 8.8 Hz), 2.91 (1 H, dd, J = 14.2, 4.4

Hz), 2.14 (3 H, s), 2.05 (3 H, s), 1.95 (3 H, s), 1.75 (3 H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 171.5, 170.2, 170.0, 169.8, 168.8, 157.6, 136.9, 130.3, 128.5, 128.4, 127.9, 127.3, 114.8, 101.9, 81.7, 71.0, 70.9, 70.0, 68.5, 67.0, 61.4, 52.1, 38.5, 20.8, 20.7, 20.6 ppm; IR (film)ν : 1750, 1611, 1513 cm^{-1}; $[\alpha]_D^{22}$ –2.52° (c 0.55, $CHCl_3$); HR FAB MS (positive): $[M+H]^+$ Found *m/z* 616.2136, $C_{31}H_{36}O_{13}$ requires *m/z* 616.2156.

Synthesis of Methyl 2-(2', 3', 4', 6'-tetra-O-acetyl-β-D-galactopyranosyloxy)-3-p-hydroxyphenyl-propionate (14). To a solution of **13** (268 mg, 0.435 mmol) in EtOAc-MeOH = 1:1 (10 mL) 10% Pd-C (20 mg) was added and stirred at rt under H_2 atmosphere overnight. The reaction mixture was filtered on celite and the filtrate was concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane:EtOAc = 1:2) to give a mixture of diastereomers (**14**) (212 mg, quant.). From **14** (21. 5 mg), each diastereomer was separated with preparative TLC (benzene-acetone= 5:1) to give **14a** (9.3 mg) and **14b** (8.6 mg).

Methyl 2-(2', 3', 4', 6'-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-propionate (**14a**): ^1H NMR (400 MHz, $CDCl_3$): 7.05 (2 H, d, *J* = 8.3 Hz), 6.75 (2 H, d, *J* = 8.3 Hz), 5.34 (1 H, d, *J* = 3.4 Hz), 5.25 (1 H, dd, *J* = 10.3, 8.0 Hz), 5.02 (1H, br.s, OH), 4.91 (1 H, dd, *J* = 10.3, 3.4 Hz), 4.43 (1 H, d, *J* = 8.0 Hz), 4.13 (1 H, dd, *J* = 9.3, 4.4 Hz), 4.09 (1 H, dd, *J* = 12.0, 6.0 Hz), 4.05 (1 H, dd, *J* = 12.0, 6.0 Hz), 3.84 (1 H, t, *J* = 6. 0 Hz), 3.73 (3 H, s), 2.98 (1 H, dd, *J* = 14.3, 9.3 Hz), 2.90 (1 H, dd, *J* = 14.3, 4.4 Hz), 2.14 (3 H, s), 2.06 (3 H, s), 1.96 (3 H, s), 1.81 (3 H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 171.6, 170.3, 170.1, 169.9, 169.0, 154.4, 130.5, 128.2, 115.2, 101.9, 81.7, 70.9, 70.9, 68.4, 66.9, 61.4, 52.2, 38.0, 20.8, 20.7 ppm; IR (film)ν : 3446, 1750, 1616, 1596, 1517 cm^{-1}; $[\alpha]_D^{21}$ +4.48° (c 0.46, $CHCl_3$); HR FAB MS (positive): $[M+H]^+$ Found *m/z* 526.1748, $C_{24}H_{30}O_{13}$ requires *m/z* 526.1686; (**14b**): ^1H NMR (400 MHz, $CDCl_3$): 7.04 (2 H, d, *J* = 8.3 Hz), 6.72 (2 H, d, *J* = 8.3 Hz), 5.38 (1 H, d, *J* = 3.4 Hz), 5.26 (1 H, dd, *J* = 10.0, 7.8 Hz), 5.12 (1H, br.s, OH), 5.00 (1 H, dd, *J* = 10.0, 3.4 Hz), 4.56 (1 H, t, *J* = 6.0 Hz), 4.51 (1 H, d, *J* = 7.8 Hz), 4.13 (1 H, dd, *J* = 12.3, 6.5 Hz), 4.07 (1 H, dd, *J* = 12.3, 6.5 Hz), 3.84 (1 H, t, *J* = 6. 5 Hz), 3.64 (3 H, s), 3.07 (1 H, dd, *J* = 14.2, 6.0 Hz), 3.01 (1 H, dd, *J* = 14.2, 6.0 Hz), 2.17 (3 H, s), 2.05 (3 H, s), 2.00 (3 H, s), 1.99 (3 H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 170.8, 170.3, 170.1, 170.0, 169.7, 154.4, 130.8, 127.7, 114.9, 99.6, 76.5, 70.7, 70.6, 68.4, 66.9, 61.2, 51.9, 38.0, 20.9, 20.8, 20.7 ppm; IR (film)ν: 3446, 1750, 1616, 1596, 1517 cm^{-1}; $[\alpha]_D^{20}$ –24.0° (c 0.52, $CHCl_3$); HR FAB MS (positive): $[M+H]^+$ Found *m/z* 526.1689, $C_{24}H_{30}O_{13}$ requires *m/z* 526.1686.

Synthesis of Methyl 2-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyloxy)-3-p-t-butyldimethylsilyloxy-phenylpropionate (15). To a solution of **14** (163 mg, 0.310 mmol) in DMF (4 mL) TBDMSCl (93.7 mg, 2.0 eq.), imidazole (84.4 mg, 4.0 eq.), and DMAP (5 mg) were added at rt under Ar atmosphere overnight. After the addition of water (30 mL), the reaction mixture was partitioned with EtOAc. The organic layer was washed with $NaHCO_3$ aq. and saturated NaCl aq. successively. The organic layer was washed with saturated NaCl aq. and dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane-EtOAc = 1:1) to give a mixture of diastereomers

(**15**) (191 mg, quant.). From **15** (23.1 mg), each diastereomer was separated with preparative TLC (benzene-acetone= 10:1) to give **15a** (12.1 mg) and **15b** (9.6 mg).

Methyl 2-(2', 3', 4', 6'-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-*t*-butyldimethylsilyloxyphenyl-propionate (**15a**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.03 (2 H, d, J= 8.3 Hz), 6.74 (2 H, d, J = 8.3 Hz), 5.34 (1 H, d, J = 3.4 Hz), 5.26 (1 H, dd, J = 10.7, 8.0 Hz), 4.91 (1 H, dd, J = 10.7, 3.4 Hz), 4.42 (1 H, d, J = 8.3 Hz), 4.13 (1 H, dd, J = 8.8, 4.4 Hz), 4.09 (1 H, dd, J = 11.2, 6.4 Hz), 4.05 (1 H, dd, J = 11.2, 6.4 Hz), 3.84 (1 H, t, J = 6. 4 Hz), 3.71 (3 H, s), 2.98 (1 H, dd, J = 14.2, 8.8 Hz), 2.90 (1 H, dd, J = 14.2, 4.4 Hz), 2.15 (3 H, s), 2.06 (3 H, s), 1.96 (3 H, s), 1.83 (3 H, s), 0.97 (9H, s), 0.17 (6H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 171.6, 170.3, 170.1, 169.9, 168.9, 154.4, 130.2, 128.7, 119.9, 101.9, 81.7, 70.9, 68.4, 66.9, 61.3, 52.1, 38.1, 32.6, 25.6, 20.8, 20.7, 18.3, -4.25 ppm; IR (film)ν: 1750, 1609, 1511 cm^{-1}; $[\alpha]_D^{19}$ +0.11° (c 0.48, $CHCl_3$); HR FAB MS (positive): [M+ Na]$^+$ Found *m/z* 663.2469, $C_{30}H_{44}O_{13}SiNa$ requires *m/z* 663.2448; (**15b**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.03 (2 H, d, J = 8.8 Hz), 6.72 (2 H, d, J = 8.8 Hz), 5.38 (1 H, d, J = 3.4 Hz), 5.26 (1 H, dd, J = 10.3, 8.0 Hz), 5.01 (1 H, dd, J = 10.3, 3.4 Hz), 4.58 (1 H, t, J = 5.9 Hz), 4.51 (1h, d, J = 8.0 Hz), 4.14 (1 H, dd, J = 11.3, 6.8 Hz), 4.09 (1 H, dd, J = 11.3, 6.8 Hz), 3.84 (1 H, t, J = 6.8 Hz), 3.60 (3 H, s), 3.06 (1 H, dd, J = 14.2, 5.9 Hz), 3.02 (1 H, dd, J = 14.2, 5.9 Hz), 2.17 (3 H, s), 2.05 (3 H, s), 2.00 (3 H, s), 1.99 (3 H, s), 0.98 (9 H, s), 0.17 (6H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 170.8, 170.2, 170.1, 169.9, 169.6, 154.3, 130.5, 128.2, 119.6, 99.6, 76.5, 70.8, 70.6, 68.4, 66.9, 61.1, 51.8, 38.1, 25.8, 20.9, 20.8, 20.7, 18.3, -4.31 ppm; IR (film)ν: 1752, 1609, 1511 cm^{-1}; $[\alpha]_D^{20}$ –23.0° (c 0.61, $CHCl_3$); HR FAB MS (positive): [M+ Na]$^+$ Found *m/z* 663.2441, $C_{30}H_{44}O_{13}SiNa$ requires *m/z* 663.2448.

Synthesis of Methyl (Z)-2-(2', 3', 4', 6'-tetra-O-acetyl-β-D-galactopyranosyloxy)-3-p-hydroxyphenyl-2-acrylate (17). To a solution of **15** (183 mg, 0.285 mmol) in dry toluene (5 mL) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (128 mg, 2.0 eq.) and refluxed for six days under Ar atmosphere. The reaction mixture was filtered through a celite and the filtrate was washed with $NaHCO_3$aq. and then EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (toluene-acetone = 15:1) to afford a mixture of crude **16** containing **15** (86.2 mg). To a solution of crude **16** in dry THF (2 mL) tetra-*n*-butylammonium fluoride (0.2 mL, 1.5 eq.) was added with ice-cooling for 30 min. After adding water (3 mL), the reaction mixture was partitioned with EtOAc, and washed with $NaHCO_3$aq. The organic layer was evaporated under reduced pressure and the residue was purified by preparative TLC ($CHCl_3$-acetone = 10:1) to afford **17** (33.1 mg, 47% in 2 steps) and recovered **14** (20.6 mg, 14%).

Methyl (Z)-2-(2', 3', 4', 6'-tetra-*O*-acetyl-β-D-galactopyranosyloxy)-3-*p*-hydroxyphenyl-2-acrylate (**17**): ^{1}H NMR (400 MHz, $CDCl_3$): 7.70 (2 H, d, J = 8.8 Hz), 7.04 (1 H, s), 6.81 (2 H, d, J = 8.8 Hz), 6.31 (1 H, br.s, OH), 5.45 (1 H, dd, J= 10.0, 8.0 Hz), 5.40 (1 H, d, J= 3.0 Hz), 5.26 (1 H, d, J= 8.0 Hz), 5.09 (1 H, dd, J= 10.0, 3.0 Hz), 4.02 (1 H, dd, J = 11.2, 6.8 Hz), 3.98 (1 H, dd, J = 11.2, 6.8 Hz), 3.88 (1 H, t, J = 6.8 Hz), 3.84 (3 H, s), 2.17 (3 H, s), 2.06 (3 H, s), 2.01 (3 H, s), 1.97 (9H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): 170.3, 170.1, 170.0, 169.9, 164.1, 157.1, 137.8, 132.7, 126.7, 125.0, 115.2, 99.6, 70.9, 70.8, 69.3, 66.9, 60.9,

52.2, 20.9, 20.7, 20.6 ppm; IR (film)ν : 3582, 1750, 1637, 1607, 1585, 1514 cm^{-1}; $[\alpha]_D^{23}$ –6.86° (c 1.89, $CHCl_3$); HR FAB MS (positive): $[M+H]^+$ Found *m/z* 525.1627, $C_{24}H_{29}O_{13}$ requires *m/z* 525.1609.

Synthesis of Potassium galactolespedezate (4) and Potassium galactoisolespedezate (5). To a solution of a mixture of **17** and **18** (32.0 mg, 0.0611 mmol) in MeOH-H_2O = 3:1 (3 mL) 3.0 M KOHaq. (0.6 mL, 3.0 eq.) was added with ice-cooling and stirred for one hour. The reaction mixture was concentrated *in vacuo*, and acidified with Amberlite IR-120 B (H^+) column chromatography. The eluate was concentrated *in vacuo*, purified by HPLC using a Cosmosil 5C18AR column (ϕ 20 × 250 mm) with 35% MeOHaq. containing 1% AcOH, and neutralized with K_2CO_3, to give **4** (3.7 mg, 16%) and **5** (17.5 mg, 71%), respectively.
Potassium galactolespedezate (**4**): ^1H NMR (400 MHz, D_2O, 30 °C): 7.24 (2 H, d, *J* = 8.3 Hz), 6.84 (2 H, d, *J* = 8.3 Hz), 6.20 (1 H, s), 4.89 (1 H, dd, *J* = 4.4, 3.3 Hz), 3.99 (1 H, d, *J* = 1.5 Hz), 3.88-3.70 (5H, m) ppm.; ^{13}C NMR (100 MHz, D_2O, 30 °C): 172.6, 150.0, 143.4, 130.2, 127.1, 116.2, 109.1, 101.4, 76.3, 73.3, 71.3, 69.3, 61.7 ppm; IR ν: 3260, 1590, 1514 cm^{-1}; $[\alpha]_D^{28}$ –24.2° (c 0.170, H_2O); HR FAB MS (negative): $[M-K]^-$ Found *m/z* 341.0823, $C_{15}H_{17}O_9$ requires *m/z* 341.0873; Potassium galactoisolespedezate (**5**): ^1H NMR (400 MHz, D_2O, 30 °C): 7.78 (2 H, d, *J* = 8.8 Hz), 6.94 (2 H, d, *J* = 8.8 Hz), 6.75 (1 H, s), 4.99 (1 H, d, *J* = 7.8 Hz), 3.94 (1 H, d, *J* = 3.4 Hz), 3.83 (1 H, dd, *J* = 9.8, 7.8 Hz), 3.74 (1 H, dd, *J* = 11.7, 5.4 Hz), 3.71 (1 H, dd, *J* = 11.7, 4.4 Hz), 3.70 (1 H, dd, *J* = 9.8, 3.4 Hz), 3.64 (1 H, dd, *J* = 5.4, 4.4 Hz) ppm; ^{13}C NMR (100 MHz, D_2O, 30 °C): 172.6, 150.0, 143.4, 130.2, 127.1, 116.2, 109.1, 101.4, 76.3, 73.3, 71.3, 69.3, 61.7 ppm; IR ν: 3234, 1575, 1512 cm^{-1}; $[\alpha]_D^{29}$ +49.5° (c 0.715, H_2O); HR FAB MS (negative): $[M-K]^-$ Found *m/z* 341.0854, $C_{15}H_{17}O_9$ requires *m/z* 341.0873.

Synthesis of Potassium mannolespedezate (6) and Potassium mannoisolespedezate (7). To a suspension of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**19**) (464 mg, 1.5 eq.), methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**) (214 mg, 0.748 mmol), and dry powdered molecular sieves 4A (1.5 g) in CH_2Cl_2 (5 mL) was added silver triflate (316 mg, 1.7 eq.), and the reaction mixture was stirred at 0 °C under Ar atmosphere for one hour. The reaction mixture was filtered on celite and the insoluble solid was washed with $CHCl_3$. The filtrate was washed with $NaHCO_3$ aq. and saturated NaCl aq. successively. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane-EtOAc = 5:4) to give a colorless oil (373 mg, 81%), which was deprotected by catalytic hydrogenation, and the phenolic hydroxyl group was protected with TBDMSCl. To a solution of the product (202 mg, 0.344 mmol) in dry toluene (5 mL) was added 2,3-dichloro-5, 6-dicyano-1, 4-benzoquinone (170 mg, 2.2 eq.) and refluxed for six days under Ar atmosphere. The reaction mixture was filtered through a celite and the filtrate was washed with $NaHCO_3$aq. and then saturated NaCl aq., successively. After being dried over anhydrous Na_2SO_4, the organic layer was evaporated under reduced pressure and the residue was purified by silica gel column chromatography (benzene-acetone = 10:1) to afford a mixture of manno-**16** and manno-**17** (56.4 mg, 26%). After deprotection, to a solution of a mixture of manno-**16** and manno-**17** (43.1 mg, 0.0823 mmol) in MeOH-H_2O = 3:1 (2 mL) 3.0 M KOHaq. (0.2 mL,

1.5 eq.) was added and stirred under ice-cooling for 15 min. The reaction mixture was concentrated *in vacuo*, and acidified with Amberlite IR-120 B (H^+) column chromatography. The eluate was concentrated *in vacuo*, purified by HPLC using a Cosmosil 5C18AR column (ϕ 20 × 250 mm) with 35% MeOHaq. containing 1% AcOH, and neutralized with K_2CO_3, to give **6** (3.7 mg, 12%) and **7** (25.1 mg, 80%), respectively.

Potassium mannolespedezate (**6**): ^1H NMR (400 MHz, D_2O, 30 °C): 7.24 (2 H, d, J = 8.8 Hz), 6.84 (2 H, d, J = 8.8 Hz), 6.25 (1 H, s), 5.35 (1 H, d, J = 1.5 Hz), 4.12 (1 H, dd, J = 3.4, 1.5 Hz), 3.99 (1 H, dd, J = 9.8, 3.4 Hz), 3.92 (1 H, d, J = 10 Hz), 3.82 (1 H, ddd, J = 10, 6.0, 2.0 Hz), 3.79 (1 H, dd, J = 10, 6.0 Hz), 3.73 (1 H, t, J = 10 Hz) ppm; ^{13}C NMR (100 MHz, D_2O, 30 °C): 172.6, 148.3, 141.8, 132.1, 130.1, 116.6, 110.1, 98.8, 74.3, 71.1, 70.6, 61.7, 61.2 ppm; IR ν : 3278, 1657, 1576, 1513 cm^{-1}; $[\alpha]_D^{22}$ +45.7° (c 0.24, H_2O); HR FAB MS (negative): $[M-K]^-$ Found *m/z* 341.0854, $C_{15}H_{17}O_9$ requires *m/z* 341.0873; Potassium mannoisolespedezate (**7**): ^1H NMR (400 MHz, D_2O, 30 °C): 7.58 (2 H, d, J = 8.3 Hz), 6.96 (2 H, d, J = 8.3 Hz), 6.75 (1 H, s), 5.52 (1 H, t, J = 2.0 Hz), 4.23 (1 H, dd, J = 3.4, 2.0 Hz), 4.00 (1 H, dd, J = 10, 3.4 Hz), 3.70 (1 H, t, J = 10 Hz), 3.59 (1 H, dd, J = 12, 4.4 Hz), 3.55 (1 H, dd, J = 12, 2.0 Hz), 3.36 (1 H, ddd, J = 10, 4.4, 2.0 Hz) ppm; ^{13}C NMR (100 MHz, D_2O, 30 °C): 171.7, 156.1, 146.0, 131.9, 126.9, 120.1, 116.2, 100.1, 75.2, 71.4, 70.5, 66.8, 61.3 ppm; IR ν : 3336, 1620, 1569, 1512 cm^{-1}; $[\alpha]_D^{22}$ +68.7° (c 1.26, H_2O); HR FAB MS (negative): $[M-K]^-$ Found *m/z* 341.0854, $C_{15}H_{17}O_9$ requires *m/z* 341.0873.

Synthesis of Potassium L-*lespedezate* (**20**) *and Potassium* L-*isolespedezate* (**21**). To a suspension of 2, 3, 4, 6-tetra-*O*-acetyl-α-L-glucopyranosyl bromide (493 mg, 1.20 eq.), methyl 3-*p*-benzyloxyphenyl-2-hydroxypropionate (**8**) (396 mg, 1.38 mmol), and dry powdered molecular sieves 4A (1.5 g) in CH_2Cl_2 (5 mL) was added silver triflate (540 mg, 1.50 eq.), and the reaction mixture was stirred at 0 °C under Ar atmosphere for one hour. The reaction mixture was filtered on celite and the insoluble solid was washed with $CHCl_3$. The filtrate was washed with $NaHCO_3$ aq. and saturated NaCl aq. successively. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified with silica gel column chromatography (*n*-hexane-EtOAc = 1:1) to give a colorless oil (307 mg, 42%), which was deprotected by catalytic hydrogenation, and the phenolic hydroxyl group was protected with TBDMSCl. To a solution of the product (228 mg, 0.357 mmol) in dry toluene (4 mL) was added 2,3-dichloro-5, 6-dicyano-1, 4-benzoquinone (162 mg, 2.0 eq.) and refluxed for six days under Ar atmosphere. The reaction mixture was filtered through a celite and the filtrate was washed with $NaHCO_3$ aq. and then saturated NaCl aq. After being dried over anhydrous Na_2SO_4, the organic layer was evaporated under reduced pressure and the residue was deprotected, and purified by silica gel column chromatography (benzene-acetone = 10:1) to afford L-gluco-**16** (84.6 mg, 45% in 2 steps) and L-gluco-**17** (26.6 mg, 14% in 2 steps). To a solution of a mixture of L-gluco-**16** and L-gluco-**17** (72.0 mg, 0.137 mmol) in MeOH-H_2O = 3:1 (6 mL) 3.0 M KOHaq. (0.5 mL, 2.0 eq.) was added and stirred under ice-cooling for 40 min. The reaction mixture was concentrated *in vacuo*, and acidified with Amberlite IR-120 B (H^+) column chromatography. The eluate was concentrated *in vacuo*, purified by HPLC using a Cosmosil 5C18AR column (ϕ 20 × 250 mm) with 35% MeOH aq.

containing 1% AcOH, and neutralized with K_2CO_3, to give **20** (6.3 mg, 12%) and **21** (38.8 mg, 75%), respectively.

Potassium L-lespedezate (**20**): ^{1}H NMR (400 MHz, D_2O, 30 °C): 7.18 (2 H, d, J = 8.8 Hz), 6.79 (2 H, d, J = 8.8 Hz), 6.12 (1 H, s), 4.88 (1 H, d, J = 7.8 Hz), 3.89 (1 H, dd, J = 12.7, 2.0 Hz), 3.84 (1 H, dd, J = 12.7, 5.9 Hz), 3.54 (1 H, dd, J = 9.8, 9.3 Hz), 3.50 (1 H, m), 3.46 (1 H, dd, J = 9.3, 7.8 Hz), 3.41 (1 H, t, J = 9.8 Hz) ppm; ^{13}C NMR (100 MHz, D_2O, 30 °C): 172.4, 155.6, 150.7, 130.6, 127.9, 116.4, 110.0, 101.5, 77.3, 76.7, 74.1, 70.7, 61.9 ppm; IR ν: 3400, 1630, 1600, 1580, 1510 cm^{-1}; HR FAB MS (negative): [M–K]$^-$ Found m/z 341.0823, $C_{15}H_{17}O_9$ requires m/z 341.0873; $[\alpha]_D^{21}$ +40.9° (c 0.57, H_2O)[19]; Potassium L-isolespedezate (**21**): ^{1}H NMR (400 MHz, D_2O, 30 °C): 7.69 (2 H, d, J = 8.8 Hz), 6.87 (2 H, d, J = 8.8 Hz), 6.73 (1 H, s), 5.01 (1 H, d, J = 7.3 Hz), 3.75 (1 H, dd, J = 12.5, 1.7 Hz), 3.64 (1 H, dd, J = 12.5, 5.1 Hz), 3.55 (1 H, dd, J = 9.3, 7.3 Hz), 3.52 (1 H, t, J = 9.3 Hz), 3.42 (1 H, t, J = 9.3 Hz), 3.33 (1 H, m) ppm; ^{13}C NMR (100 MHz, D_2O, 30 °C): 172.6, 156.9, 146.2, 132.8, 127.0, 121.4, 116.5, 102.2, 77.4, 76.9, 74.8, 70.4, 61.5 ppm; IR ν: 3400, 1605, 1570, 1510 cm^{-1}; HR FAB MS (negative): [M–K]$^-$ Found m/z 341.0823, $C_{15}H_{17}O_9$ requires m/z 341.0873; $[\alpha]_D^{20}$ -50.7° (c 1.00, H_2O).[19]

Bioassays. Young leaves separated from the stems of *Cassia mimosoides* L. with a sharp razor blade were used for the bioassay. The leaves were immersed in distilled water (*ca.* 1.0 ml) in a 5–mL glass tube in a greenhouse and allowed to stand overnight. Leaves which opened again the next morning (at around 10 a.m.) were used for the bioassay. Each test solution dissolved in water was poured into test tubes with a microsyringe at around 11 a.m., and the bioactivity was judged by leaf-opening until 9:00 p.m. On the other hand, the control leaf immersed in distilled water as a control closed at 5:30 p.m.

Enzymatic reaction. A reaction mixture (1.0 mL) containing 1.0 mM of substrate, 0.1 M citrate buffer (pH 5.0), and β-glucosidase (10 unit) was incubated at 37 °C for 2 hours. After the reaction was stopped by treatment with boiling water, the reaction mixture was analyzed by HPLC equipped with a photodiode array detector with 20% MeOH containing 0.1% TFA (flow rate: 1.0 mL/min, detection: 220 nm) using Develosil ODS HG-5 column (ϕ 4.6 x 250 mm). The time course of reaction was checked by the observation of the enol (Rt = 7.5 min) and keto (Rt = 21.1 min) form of **3**.

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19. Potassium lespedezate (**1**): $[\alpha]_D^{22}$ –40.0° (c 0.57, H_2O).; Potassium isolespedezate (**2**): $[\alpha]_D^{21}$ +50.9° (c 1.00, H_2O).