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TETRAHEDRON

Molecular Mechanism of the Control of Nyctinastic Leaf-movement in *Lespedeza cuneata* G. Don

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ABSTRACT: The nyctinastic leaf-movement of plants is controlled by an endogenous biological clock. In a nyctinastic plant, *Lespedeza cuneata* G. Don, this movement is controlled by the balance of the concentration between the leaf-closing and -opening substances. Quantitative HPLC analysis revealed that the change in the concentration of this leaf-opening substance is inversed through a day, and the deactivation of this substance is performed by a glucosidase whose activity is controlled by a biological clock. The leaf-movement of nyctinastic plants is caused by the balance of two leaf-movement factors , which is controlled by the enzymatic transformation.

Keywords : biologically active compounds; biosynthesis; enzymes and enzyme reaction; natural products

INTRODUCTION

Nyctinastic plants, such as *Mimosa pudica* L. and *Cassia mimosoides* L., are well known for the leaf-movement according to a circadian rhythm, which is controlled by an endogenous biological clock.[1] In 1916, Ricca suggested that the leaf-movement is controlled by some bioactive substance.[2] After that, Schildknecht *et al.*[3,4] have isolated some turgorins, chemical substances which introduce leaf-closing movement, from several nyctinastic plants, *e.g.*, *Mimosa pudica* L., *Acacia karoo*, etc., and they propounded that turgorins belong to a new class of phytohormones which controls leaf movement of all nyctinastic plants.

However, we have isolated different leaf-closing factors from each nyctinastic plant, *e. g.* , potassium chelidonate from *Cassia mimosoides* L.,[5] trigonelline from *Aeschynomene indica*,[6] and phyllanthurinolactone from *Phyllanthus urinaria* L.,[7] respectively. All of them have much stronger bioactivity than that of Schildknecht's turgorins. This result indicates that these leaf–closing substances are not common in all plants and differ in each nyctinastic plant. Moreover, we have isolated leaf–opening substances, potassium lespedezate (**2**) and potassium isolespedezate (**3**) from *Lespedeza cuneata* G. Don,[8,9] *cis-p*-coumaroylagmatine from *Albizzia julibrissin* Durazz.[10] Thus, it is deduced that the leaf movement is not controlled by the change in the concentration of the leaf-closing factor, but by the change in the balance of concentration between two bioactive substances, the leaf–closing and leaf–opening factors.[9] However, since both bioactive substances have not been

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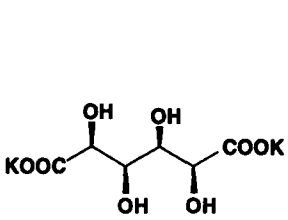
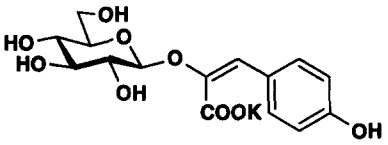
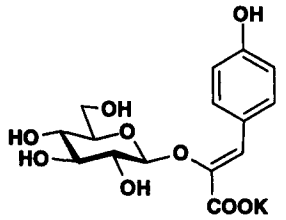
isolated from the same plant, it is unknown whether this mechanism can be adapted to actual plants. We have succeeded in the isolation of a leaf-closing substance, potassium D-idarate (**1**), from *Lespedeza cuneata* G. Don[11] using the same bioassay as in the case of **2**.



Potassium D-idarate (1)



Potassium lespedezate (2)



Potassium isolespedezate (3)

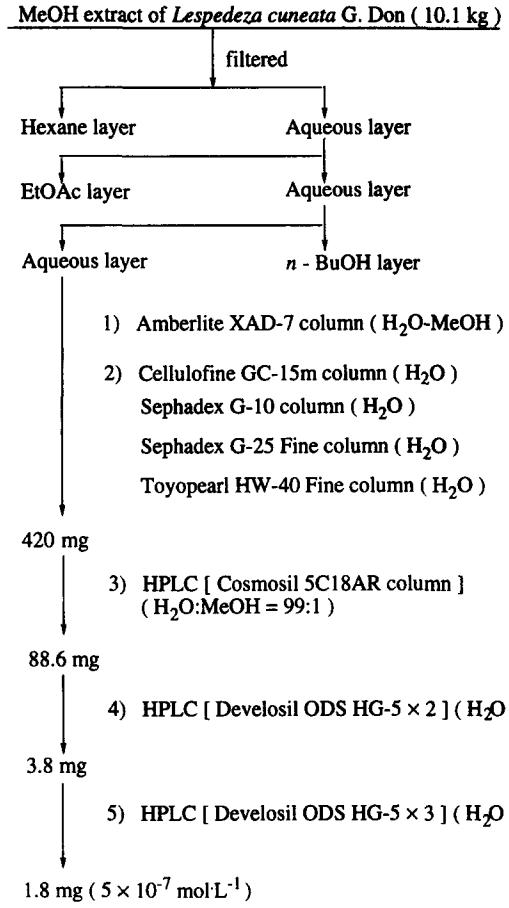
This is the first example of the isolation of both leaf-closing and -opening substances from the same nyctinastic plant. Furthermore, we have revealed that potassium lespedezate (**2**) in this plant is deactivated in the evening by its conversion into 4-hydroxy phenylpyruvic acid (**4**) by enzymatic transformation.[12] These results indicated that the leaf-movement is controlled by the change in the balance of concentration between the two bioactive substances, based on the enzymatic deactivation of the leaf-opening substance. We herein propose a new molecular mechanism of the leaf-movement in nyctinastic plants.

RESULTS AND DISCUSSION

The fresh whole plant of *Lespedeza cuneata* G. Don (10.1 kg) was immersed in methanol (60 L) for three weeks and concentrated *in vacuo*. Purification of the bioactive substance was carried out with monitoring the leaf-closing activity for the leaf of *Cassia mimosoides* L. Because of the stiffness of the stem of *Lespedeza cuneata* G. Don, this leaf pumped up the sample solution poorly, and it was insufficient for use in the bioassay. Because of this experimental difficulty, we used the leaf of *Cassia mimosoides* L. for the bioassay instead of the leaf of *Lespedeza cuneata* G. Don. The leaf-opening substance of this plant, **2**, was also isolated according to the same bioassay.[8,9] The concentrated extract was partitioned with *n*-hexane, ethyl acetate and *n*-butanol. The bioactive aqueous layer was separated according to the isolation procedure cited in Scheme 1.

Because of the high polarity of **1**, the combination of three HPLC columns was very effective for the isolation. Final purification with HPLC gave **1** (1.8 mg) as a colorless powder. **1** was quite effective for the leaf-closing of *Cassia mimosoides* L. at 5×10^{-7} M in the daytime, but not effective on other plants, *Aeschynomene indica* and *Mimosa pudica* L., even at 1×10^{-4} M.

The ^{1}H-NMR spectrum of **1** gave only two very broad signals, which provided no coupling information for us. Thus, the structural determination of **1** was carried out by means of 2D-NMR and negative mode ESI MS spectroscopy. The signal at δ 180.1 ppm in ^{13}C-NMR spectrum suggested the presence of a carboxylate function. Negative mode ESI MS measurement gave the molecular ion $[M\text{-}H]^-$ at m/z 208.7, and the observation of $[M\text{-}2H]^{2-}$ at m/z 104.0, suggested that **1** was a dicarboxylic acid. Correlations in HMQC and HMBC experiments gave the structure of **1** (Fig. 1).



Scheme 1. Isolation procedure of the leaf-closing factor (**1**) from *Lespedeza cuneata* G. Don

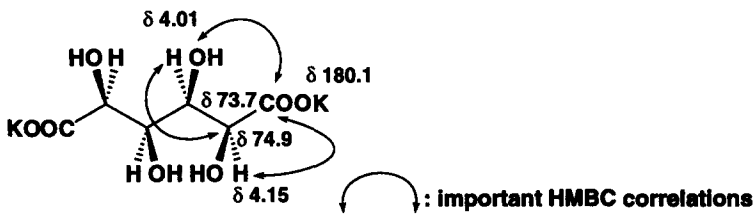


Fig. 1. Structure of the leaf-closing factor (**1**) from *Lespedeza cuneata* G. Don

Stereochemistry of **1** was determined by the comparison of spectroscopic data and bioactivity between an isolated natural product and various potassium and sodium tetrahydroxy dicarboxylates prepared from the corresponding hexoses by HNO_3 oxidation (Fig. 2).[13,14] The ^1H– and ^{13}C–NMR data of these compounds are

compiled in Table 1. Of these compounds, only potassium D-idarate gave NMR data identical with that of the natural product.

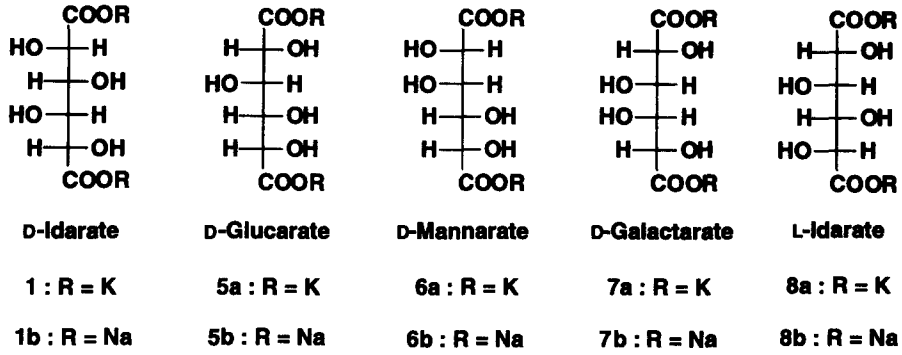


Fig. 2. Structure of tetrahydroxy dicarboxylates

Table 1.
^{1}H and ^{13}C NMR data of tetrahydroxy dicarboxylates in D_2O at 400 MHz

	^{13}C NMR (δ ppm)	^{1}H NMR (δ ppm)
natural product	180.1, 74.9, 73.7	4.15, 4,01
1 and 1b	180.1, 74.9, 73.6	4.16, 4.02
5a	178.5, 178.4, 73.7, 73.6, 71.0	4.10, 4.08, 4.03, 3.09
6a	179.1, 73.9, 71.7	4.09, 3.62
7a	181.1, 73.3, 73.0	4.14, 3.93

Table 2 shows the comparison of leaf-closing activity of these compounds. All sodium salts of these compounds were weakly bioactive, but in the potassium salts, only potassium D-idarate (**1**) showed leaf-closing activity as strong as the isolated natural product. Also, the $[\alpha]^{22}_{D}$ value of **1** was in agreement with that of the natural product. Furthermore, potassium L-idarate (**8a**), an enantiomer of **1**, showed no bioactivity. Therefore, the stereochemistry of the leaf-closing substance was elucidated as potassium D-idarate (**1**). These results indicated that both the stereochemistry and counter cation are important for the leaf-closing activity of **1**.

Table 2.
Comparison of the leaf-closing activity of various compounds

K^+ salts	Concentration [$mol \cdot L^{-1}$]	Na^+ salts	Concentration [$mol \cdot L^{-1}$]
natural product	5×10^{-7}		
1	5×10^{-7}	**1b**	5×10^{-3}
5a	1×10^{-3}	**5b**	5×10^{-3}
6a	5×10^{-4}	**6b**	$> 5 \times 10^{-3}$
7a	5×10^{-4}	**7b**	$> 5 \times 10^{-3}$
8a	5×10^{-4}	**8b**	5×10^{-4}

This is the first example of the isolation of both the leaf-closing and -opening substances from the same nyctinastic plant.

Table 3 shows the competitive interaction between **1** and **2** (or **3**)[a] When the concentration of **1** was higher than that of **2** (or **3**), the leaves were closed in the daytime. On the other hand, when the concentration of **2** (or **3**) was higher than that of **1**, the leaves were open at night. Thus, two leaf-movement factors isolated from *Lespedeza cuneata* G. Don competed with each other for the leaf of *Cassia mimosoides* L. We were able to reproduce the leaf-movement of the plant in nature by changing the balance of concentration between **1** and **2** (or **3**).

Table 3.
Competitive interaction between **1** and **2**

Concentration of **1** [$mol \cdot L^{-1}$]		Ratio of **1** and **2**					Control
		1 : 0	1 : 0.5	1 : 1	0.5 : 1	0 : 1	
Day time	1×10^{-4}	– –	– –	– –	++	++	
	1×10^{-5}	– –	– –	+ –	++	++	++
	1×10^{-6}	– –	–	+ –	++	++	
Night	1×10^{-4}	– –	– –	++	++	++	
	1×10^{-5}	– –	– –	+ –	+	++	– –
	1×10^{-6}	– –	– –	+ –	+ –	++	

++ completely open ; + nearly open ; + – at random ; – nearly closed ; – – completely closed

This result indicates that the leaf-movement of nyctinastic plants is controlled by the competitive interaction between leaf-opening and -closing substances. Therefore, we considered that the balance of concentration of these bioactive compounds would be reversed in the plant *Lespedeza cuneata* G. Don in the daytime and at night. Thus, two methanol extracts were prepared from the plants which were collected at two stages (daytime and night) and then directly used for the bioassay at such concentrations as shown in Table 4.

Table 4.
Bioactivity of the extracts collected in the daytime and at night

Time of collection	Concentration [$g \cdot L^{-1}$]	Daytime	Night
Daytime	10	++	++
	1	++	+
	1×10^{-1}	++	+
Night	10	++	++
	1	–	+ –
	1×10^{-1}	–	– –

++ completely open ; + nearly open ; + – at random ; – nearly closed ; – – completely closed

These two crude extracts showed quite opposite activities for the leaf of *Cassia mimosoides* L. at 1 to 10^{-1} g/L.: The extract collected in the daytime showed the leaf-opening activity, and the one collected at night

[a] Each of these two compounds (**2** and **3**) afforded a mixture of two compounds when allowed to stand at room temperature

showed the leaf-closing activity. Although both extracts showed the leaf-opening activity at high concentration, 10 g/L, this result would be attributed to the bioactivity of IAA, which has been known to have weak leaf-opening activity. For example, IAA is effective for the leaf-opening of the leaf of *Cassia mimosoides* L. at 10^{-3} M [9], and induces the leaf-opening movement at night to *Mimosa pudica* [15,16] and other nyctinastic plants at 10^{-3} to 10^{-4} M.[9] Tryptophan, which is known as a precursor of IAA, is also effective for leaf-opening at high concentration (unpublished result). However, the leaf-opening activity of these IAA related compounds are very weak for the leaf of a nyctinastic plant. Thus, bioactivities of the two crude extracts, observed when they were diluted, should be attributed to the two leaf-movement factors with strong bioactivity, and we have obtained the proof that the balance of concentration between **1** and **2** (or **3**) was actually inversed in a plant through a day.

To confirm our new model of the chemical control of nyctinastic leaf-movement, we performed the quantitative analysis of the leaf-opening factors, **2** and **3**, which were more easily detected on the HPLC analysis than **1** because of their good separation from other peaks. Table 5 presents changes in the concentrations of **2**, **3** and 4-hydroxy phenylpyruvic acid (**4**) through a day.

Table 5.
Quantitative HPLC analysis of the concentration of **2**, **3**, and **4** collected in the daytime and night

Time of collection	Concentration [mol L^{-1}]		
	2	**3**	**4**
Daytime	2.7×10^{-5}	4.1×10^{-5}	2.5×10^{-5}
Night	2.2×10^{-5}	1.8×10^{-5}	1.2×10^{-4}

The quantitative analyses of these compounds were carried out by HPLC equipped with a photodiodearray detector using 0.1% TFA methanolic solution as a mobile phase. This condition gave good separation of these compounds. The extract collected in the daytime contained twofold as much **2** and **3** as the extract collected at night. These differences in the concentration of leaf-opening substances (**2** and **3**) correspond with the bioactivities of the two extracts depicted above because a twofold amount of **2** (or **3**) is sufficient to inverse the bioactivity of plant extracts (Table 3). From these results, it is obvious that the concentration of leaf-opening substances (**2** and **3**) in nyctinastic plants actually changes through a day. Interestingly, the concentration of **4** varied in a different way from that of **2** and **3**. Thus, when the concentrations of **2** and **3** increased in the daytime, that of **4** decreased, and vice versa. As shown in Table 5, quantitatively, the extract collected in the daytime contained fivefold as much **4** as the one collected at night. Presumably, **4** is one of the most important intermediates in the biosynthesis of amino acids, and all of **4** in plants would not be used for the synthesis of the leaf-opening factors **2** and **3**; therefore, it is reasonable that the amount of the change of **4** did not correspond with that of **2** and **3**.

The potassium salt of **4** is effective for the leaf-opening of the leaf of *Cassia mimosoides* L. at 5×10^{-5} M.[9] On the other hand, **2** and **3** are effective at 8×10^{-7} M [8, 9]. Thus, it is presumed that the leaf-opening substances (**2** and **3**) were deactivated in the evening by the transformation into **4**. This deactivation should be catalyzed by a β–glucosidase in *Lespedeza cuneata*. G. Don. Therefore, the differences in the concentration of

2, **3** and **4** in this plant between those in the daytime and at night would be attributable to 1) the activated biosynthesis of **2** and **3** in the morning, 2) the deactivation of **2** and **3** into **4** by β–glucosidase in the evening.

This new model for the chemical control of leaf-movement was strongly supported by the measurement of β–glucosidase activities using the crude enzyme prepared from *Lespedeza cuneata* G. Don collected in the daytime (11:00 AM) and in the evening (5:00 PM). Since the leaves of this plant begin to close around 5:00 PM, we considered that the activity of β-glucosidase, which catalyzes the transformation of **2** (or **3**) into **4**, would be most enhanced around this time. The crude enzyme was prepared according to the method by Watanabe *et al.* [17] The fresh whole plant of *Lespedeza cuneata* G. Don collected at the two stages was divided into the leaves and the stems immediately; they were then, frozen in liq. N_2 and powdered in acetone, separately. These acetone powders were directly used as crude enzymes. Sample solutions containing **2** were treated with crude enzymes under the following conditions: a) aetone powder (from leaves at 11:00 AM) + **2**, b) acetone powder (from stem at 11:00 AM) + **2**, c) acetone powder (from leaves at 5:00 PM) + **2**, d) acetone powder (from stem at 5:00 PM) + **2**, e) acetone powder (from leaves at 11:00 AM), f) **2**, [a) to f) were dissolved in citrate buffer (pH 5.0)]. These mixtures were subjected to HPLC analysis after filtration with a membrane filter. The β–glucosidase activity of the crude enzymes was determined by the quantitative HPLC analysis of the produced **4** in these reactions. The analytical conditions were the same as for the quantitative analysis of **2**, **3** and **4** described above. Table 6 shows the changes in the concentration of **2** and **4**.

The β–glucosidase activity was observed only with the acetone powder prepared from the leaves of plants in the evening. In contrast, the activity of the other acetone powders was almost as low as that of the blank sample. The enzyme activity was also observed in a 0.1 M citrate buffer (pH 5.0) extract of the acetone powder. Thus, the enzyme was easily extracted with this buffer solution. These results indicated that 1) the leaf-opening factor (**2**) is transformed into the weakly active compound **4** in the evening by the enzymatic deactivation, and the activity of the enzyme should be controlled by a biological clock; 2) the enzymatic deactivation can only be carried out in the leaf of the plant in the evening. Generally, many phenolic glucosides, such as the glucosides of *p*-hydroxybenzoic acid and cinnamic acid, are synthesized by glucosyltrasferase and decomposed by β-glucosidase.[18, 19] Therefore, we considered that the biosynthesis of **2** or **3** would be alsoperformed by some glucosyltransferase, and attempted to detect the glucosyltransferase activity by using **4** and a glucosyl donor as substrates. Since the leaf of *Lespedeza cuneaeata* G. Don begins to open in the morning, the glucosyltransferase, catalyzing the transfer of D-glucose from UDP-glucose to **4**, would be activated in the morning contrary to the β-glucosidase activity. The test solutions containing **4** and UDP-glucose were treated with the acetone powder prepared from plants collected in the daytime (11:00 AM) and in the morning (5:00 AM), then the products in these reaction mixtures were analyzed by HPLC according to the same procedure as in the case of β-glucosidase. However, there were no marked differences between the blank and the reaction mixtures treated with any crude enzyme under several conditions. These results indicated that **4** and UDP-glucose would be unsuitable substrates for this glucosyltransferase reaction. Further studies on this subject are in progress, and these results will be reported in due course.

Table 6.
Transformation from 2 into 4 by the action of crude enzyme

Conditons	Concentration [mol L^{-1}]	
	2	**4**
a) acetone powder (from leaves in the daytime) + **2**	1.0×10^{-3}	ND
b) acetone powder (from stems in the daytime) + **2**	1.0×10^{-3}	ND
c) acetone powder (from leaves in the evening) + **2**	1.0×10^{-3}	2.2×10^{-5}
d) acetone powder (from stem in the daytime) + **2**	1.0×10^{-3}	ND
e) acetone powder (from leaves in the daytime) + buffer	ND	ND
f) buffer + **2**	1.0×10^{-3}	ND

CONCLUSION

In 1980's, Schildknecht proposed his phytohormone-theory that the leaf-movement of nyctinastic plants are dependent on only the change in the concentration of one leaf-closing factor, turgorin, which exists in all nyctinastic plants.[3,4] However, as described in the present paper, we have revealed that the leaf-movement of plants is caused by the balance of two leaf-movement factors, the leaf-opening and -closing substances, which are different in each nyctinastic plant. From these results, we propose a new mechanism of leaf-movements in a nyctinastic plant, *Lespedeza cuneata* G. Don. (Fig. 3).[12]

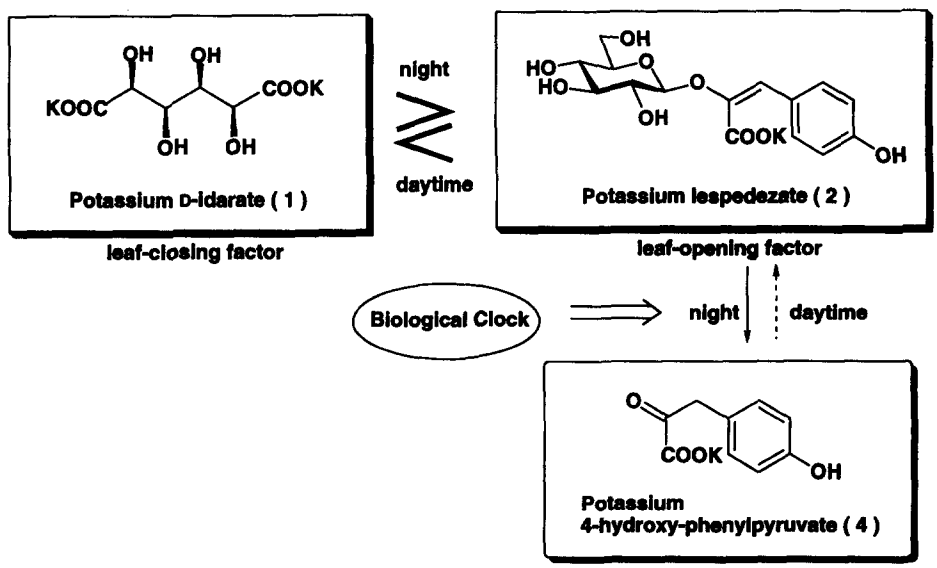


Fig. 3. The molecurar mechanism of the leaf-movement in *Lespedeza cuneata* G. Don

We have considered that the enzymatic deactivation of leaf-opening factors **2** and **3** plays a very important role in the chemical control of the leaf-movement of *Lespedeza cuneata* G. Don. By this transformation in the

evening, the balance of concentration between leaf-closing substance (**1**) and leaf-opening substance (**2**) is inversed through a day, wherein a biological clock should control the leaf-movement by activation or expression of the enzyme in this step. There are now two plausible mechanisms for the control of the balance between leaf-closing and leaf-opening substances: 1) the biosynthesis and metabolism of two bioactive compounds are under the control of a biological clock; 2) that of only the leaf-opening substance is under the control of a biological clock; Further study must be carried out on the metabolism of the leaf-closing substance of *Lespedeza cuneata* G. Don. Recently, we have identified a new set of inversely effective leaf-movement factors from another nyctinastic plant, *Cassia mimosoides* L., through bioassay using the leaves of the same plant as plant material (Fig. 4).[20]

Thus, we presume that the leaf-movement of all nyctinastic plants must be controlled by two inversely effective leaf-movement factors, as shown in this paper, contrary to the previously established theory introduced by Schildknecht.[3,4]

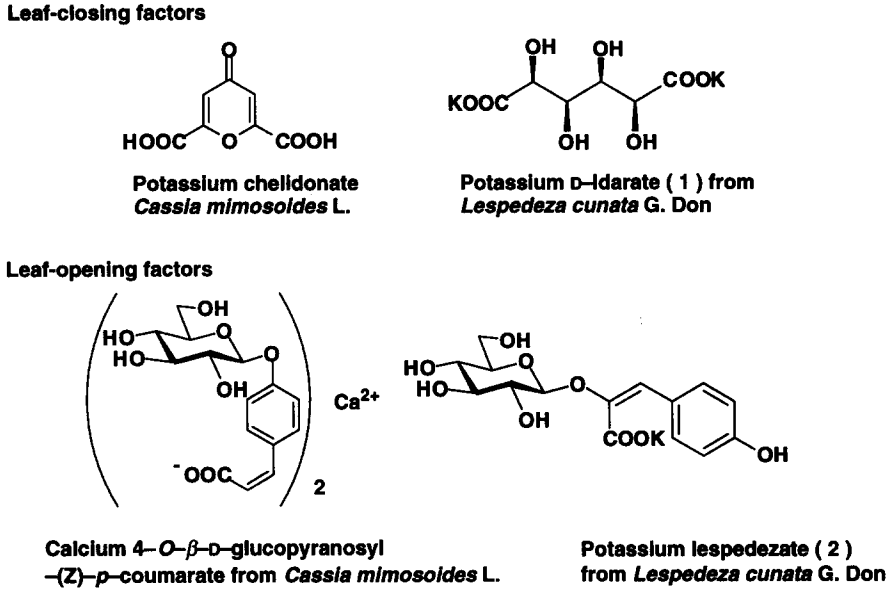


Fig. 4. The leaf-closing factor and -opening factor from *Cassia mimosoides* L. and *Lespedeza cuneata* G. Don

EXPERIMENTAL

General. The optical rotations were recorded as H_2O solutions on a Jasco DIP-360 polarimeter at room temperature. IR spectra were recorded as film on a Jasco A-202 spectrophotometer. The UV-VIS spectra were obtained as H_2O solutions on a Jasco UVIDEC-610A spectrophotometer at room temperature. 2D-NMR, ^{1}H-NMR spectra (400 MHz), and ^{13}C-NMR spectra (100 MHz) were recorded on a JEOL JMN-A400 spectrometer in D_2O using *t*-BuOH as an internal standard [^{1}H–NMR (δ 1.23 ppm) and ^{13}C–NMR (δ 31.2 ppm)] at various temperatures.

HPLC-system. The HPLC analysis was carried out using Shimadzu LC-6A pump system equipped with SPD-6A detector (Shimadzu Co., Ltd.) and Jasco PU-960 pump system equipped with UV-970 detector (Jasco Co., Ltd.). Jasco PU-980 pump system equipped with MD-910 photodiodearray detector (Jasco Co., Ltd.) using the BORWIN software for data processing were used for a quantitative analysis. All solvents used for HPLC were obtained from Kanto Chemical Co., Ltd., and a filtrate with a membrane filter (Cellulose Acetate, pore size: 0.45 μm, 47 mm, Toyo Roshi Kaisha, Ltd.) before used.

Plant Materials. Lespedeza cuneata G. Don used for both the isolation of the leaf-closing factor and the preparation of acetone powders were collected on the banks of the Tama River. *Cassia mimosoides* L. used for the bioassay were grown in a greenhouse of Keio University at 25 to 33 °C for several months.

Bioassay. Young leaves separated from the stem of plants, *Cassia mimosoides* L., with a sharp razor blade were used for the bioassay. Two leaves were immersed in distilled water in a 20-mL glass tube in the greenhouse around 11:00 AM to allow them to recover from the cutting injury. Each test solution was poured into the test tubes by a microsyringe around 11:00 AM. In the bioassay of the leaf-closing factor, the reaction time depends on the concentration of the active substance, the minimum amount of which was judged by leaf-closing in few hours.

Isolation of Potassium D-Idarate (1) from Lespedeza cuneata G. Don. The fresh whole plants (10.1 kg) were extracted with methanol (60 L) for three weeks. The extract was filtered and evaporated to 700 mL under reduced pressure. The aqueous residue was partitioned with *n*-hexane (1 L × 3), EtOAc (1 L × 3) and *n*-BuOH (1 L × 3). The residue was chromatographed on an Amberlite XAD-7 column (ϕ 5.4 × 54 cm, Organo Co., Ltd.) eluted with MeOH-H_2O (0 : 10, 1 : 9, 3 : 7, 5 : 5, and 10 : 0). The bioactive H_2O eluate was chromatographed repeatedly on Sephadex G-10 (ϕ 3.8 × 48 cm, Pharmacia Biotech Co., Ltd.), G-25 Fine (ϕ 3.8 × 47 cm, Pharmacia Biotech Co., Ltd.), Celullofine GC-15m (ϕ 3.8 × 40 cm, Seikagaku Co., Ltd.), and Toyopearl HW-40 Fine (ϕ 3.0 × 40 cm, Tosoh Co., Ltd.) column eluted with H_2O. The bioactive fraction was separated by HPLC using preparative Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co., Ltd., mobile phase; H_2O, flow rate; 4.5 mL/min, detected at 215 nm), and then a combination of two analytical Develosil ODS HG-5 columns (ϕ 4.6 × 250 mm, Nomura Chemical Co., Ltd., mobile phase; H_2O, flow rate; 0.3 mL/min, detected at 215 nm). Finally, the active fraction was purified by HPLC using a combination of three Develosil ODS HG-5 columns (mobile phase, H_2O; flow rate, 0.5 mL/min; detected at 215 nm) to give potassium D-idarate (**1**) as colorless powder (1.8 mg). **1** : ^{1}H-NMR (400 MHz, D_2O) δ 4.15 (2 H, br.s, H_1 & H_4), 4.01 (2 H, br.s, H_2 & H_3).; ^{13}C-NMR (100 MHz, D_2O, 35 °C) δ 180.1, 74.9, 73.7.; ESI-MS (negative): [M-H]$^-$ m/z 208.7, [M-2H]$^{2-}$ m/z 104.0. $[\alpha]^D_{22}$ + 5.3 ° (c = 0.1, H_2O).

Synthesis of Potassium D-Idarate (1). D-Idarate (**1**) was prepared from D-idose according to the method by Linstead *et al.*.[13] D-idose (Sigma Chemical Co., Ltd., 100 mg) was dissolved in 1.2 mL of nitric acid (*d*: 1.15) and heated on a boiling water-bath. After the initial vigorous oxidation, the solution was evaporated to small volume, and nitric acid in excess was removed as completely as possible by repeated evaporation with water. The syrup obtained was diluted and neutralized with potassium carbonate, and then evaporated under reduced pressure. The residue was purified by HPLC using preparative Cosmosil 5C18AR column (ϕ 20 × 250 mm, Nacalai Tesque Co., Ltd., mobile phase; H_2O, flow rate; 4.0 mL/min, detected at 215 nm) to afford **1** (73 mg, 46 %). Both L-idarate (**8a, 8b**) and D-mannarate (**6a, 6b**) were prepared according to the same procedure as in the case of **1**. Potassium D-idarate (**1**); ^{1}H-NMR (400 MHz, D_2O) δ 4.16 (2 H, br.s, H_1 & H_4), 4.02 (2 H, br.s, H_2 & H_3) ppm.; ^{13}C-NMR (100 MHz, D_2O, 35 °C) δ 180.1, 74.9, 73.6.; $[\alpha]^D_{22}$ + 6.0 ° (c = 0.1, H_2O). **8a**; $[\alpha]^D_{22}$ -5.6 ° (c = 0.1, H_2O), **6a**; $[\alpha]^D_{22}$ + 18.0 ° (c = 0.1, H_2O). The data of ^{1}H- and ^{13}C-NMR of the other tetrahydroxy dicarboxylates were compiled in Table 1.

Quantitative Analysis of Potassium Lespedezate (2), Potassium Isolepedezate (3) and 4-hydroxy phenylpyruvic acid (4). The fresh whole plants of *Lespedeza cuneata* G. Don collected in the daytime (10:00 AM, 3.0 kg) and at night (7:00 PM, 3.4 kg) were extracted with methanol (20 L) for two weeks, respectively. Two extracts were filtered and evaporated to 200 mL under reduced pressure. Each of the two residues was partitioned with *n*-hexane (200 mL × 3) and ethyl acetate (200 mL × 3), and then each residue was evaporated to 150 mL under reduced pressure. The amounts of **2**, **3**, and **4** in two aqueous layers were determined by HPLC analysis (for **2** and **3**: mobile phase; 10% MeOH aq. containing 0.1 % TFA, flow rate; 1.0 mL/min, detected at 300 nm, or for **4**: mobile phase; 30% MeOH aq. containing 0.1 % TFA, flow rate; 0.5 mL/min, detected at 300 nm). Water content of the plant material was estimated to be 80 % of total weight in the calculation of the concentration. The identification of each peak was established by retention time confirmed by co-injection of the authentic samples.

Preparation of Acetone Powder and Crude Enzyme Extraction for β-Glucosidase Reaction. Acetone powder was prepared according to the method by Watanabe *et al.*[17] After the fresh whole plants of *Lespedeza cuneata* G. Don collected at two stages (11:00 AM and 5:00 PM) were divided into leaves and stems, each group was frozen with liq. N_2. They were homogenized with a blender in acetone (1 L), then chilled with dry ice and filtered. The debris was washed with dry ice-acetone (ca. 3 L). Drying *in vacuo* with ice-cooling gave an acetone powder. Yields were as follows : 15 g (leaves) and 5.0 g (stems) from the whole plant (100 g) collected in the daytime (11:00 AM) ; 24 g (leaves) and 10 g (stems) from the whole plant (250 g) collected in the evening (5:00 PM): The acetone powder was directly used as a crude enzyme. The buffer extracts of crude enzyme were prepared from each acetone powder. The acetone powder (3 g) was immersed in 50 mL of 0.1 mM citrate buffer (pH 5.0) containing 2 mM dithiothreitol (Junsei Chemical Co., Ltd.) and 5mM Na_2EDTA (Junsei Chemical Co., Ltd.) for 4 h at 4 °C. After filtration with a membrane filter, the enzyme activity was extracted in a buffer solution.

Crude Enzyme Reaction. The acetone powder (1.0 g) was immersed in 0.1 M citrate buffer (1 mL, pH 5.0), 1 mM potassium lespedezate (**2**) and 9 mM dithiothreitol (Junsei Chemical Co., Ltd.). After incubation of 30 min at 37 °C, the reaction was quenched by treatment with boiling water (100 °C, 5 min). The reaction mixture was filtered with a membrane filter (Cellulose Acetate, pore size: 0.45 μm, DISMIC-13CP, Toyo Roshi Kaisha, Ltd.) and then the filtrate was analyzed by HPLC. The analytical conditions on HPLC are the same as in the case of the quantitative analysis of **2**, **3** and **4**.

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