Leaf-closing Substance in *Leucaena leucocephala*

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Potassium (2R,3R)-2,3,4-trihydroxy-2-methylbutanoate (1) was identified as a leaf-closing substance in the nyctinastic plant, *Leucaena leucocephala*. Compound 1 showed strong leaf-closing activity toward *L. leucocephala* and was not effective against other nyctinastic plants. The potassium ion was indispensable for the bioactivity of 1. Compound 1 gradually lost its bioactivity because of the exchange of the counter cation during isolation. A leaf-opening substance was also observed in the same plant.

Key words: nyctinasty; *Leucaena leucocephala*; leaf-closing substance; potassium (2R,3R)-2,3,4-trihydroxy-2-methylbutanoate

Most legumes close their leaves in the evening, as if to sleep, and open them in the morning. 1) This rhythmic movement of the leaves is called nyctinasty and is known to be controlled by an internal biological clock. 2) We have recently identified several bioactive substances that regulate this leaf movement, and revealed that the nyctinastic movement of a plant was controlled by the interaction between leaf-closing and -opening substances which differ among plants. 3) Moreover, we have demonstrated the importance of such leaf movement for the survival of legumes. In our previous work with an artificial leaf-opening substance, a leaf of *Cassia mimosoides* L. which had been kept open for a week was observed to wither and die. 4) This result suggests that the nyctinastic leaf movement could be involved in the survival of legumes and that it would be possible to develop a new herbicide by using the leaf-movement factor of a plant.

Nyctinasty has also been observed in *Leucaena leucocephala*, a leguminous tropical plant. *L. leucocephala* is known for its rapid growth and the secretion of allelochemicals to inhibit the growth of other plants around it which leads to rampant growth and the elimination of vicinal plants. 5) We identify in this present study the leaf-closing substance in *L. leucocephala* and also show the existence of a leaf-opening substance in an extract of the plant. We describe mainly the isolation, chemical structure, and biological activity of the leaf-closing substance in *L. leucocephala*.

Materials and Method

General notes. 2D-NMR, 1H-NMR (400 MHz), and 13C-NMR spectra (100 MHz) were recorded with a Jeol JNM-A400 spectrometer in D2O, using t-BuOH as an internal standard ([1H-NMR (δ1.23) and 13C-NMR (δ31.2)]) at various temperatures. The FAB MS spectra were measured with a Jeol JMS-700 spectrometer, using m-nitrobenzylalcohol as a matrix. HPLC analyses were carried out with a Jasco PU-960 pump equipped with a UV-970 detector to monitor the UV absorption at 220 nm. Samples were weighed by Metler Toledo MT-5 microbalance. All the solvents used for HPLC were obtained from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 mm pore size, φ47 mm) before use.

Plant material. Leaves of *L. leucocephala* were collected in August 1999 from Okinawa in Japan for subsequent extraction. *L. leucocephala* used for the bioassay was grown in a greenhouse at Keio University at 25–33°C.

Bioassay. Young leaves were detached from the stem of the *L. leucocephala* plant with a sharp razor blade and used for the bioassay. One leaf each was placed in H2O (ca 1.0 ml) in 2-ml glass tubes, in a greenhouse at 25–35°C and allowed to stand overnight. The leaves which opened again the next morn-

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ing (around 10:00 a.m.) were used for the bioassay. Each leaf was carefully transferred to a test solution in a glass tube at around 10:00 a.m. The bioactive fraction made the leaf of *L. leucocephala* close earlier than the leaf in distilled water. A 0.1 mM potassium phosphate solution (pH 5.4) instead of water was used to determine the bioactivity of 1. In this case, bioactivity was judged by earlier leaf-closing than that in a 0.1 mM potassium phosphate solution (pH 5.4).

Isolation of potassium 2,3,4-trihydroxy-2-methylbutanoate (1). Fresh whole leaves of *L. leucocephala* (13.0 kg) were extracted with methanol (72 l) for one week and then concentrated in vacuo. Purification of the bioactive substance was carried out by monitoring the leaf-closing activity toward leaves of *L. leucocephala*. One-third of the concentrated extract was partitioned with *n*-hexane (300 ml × 3), ethyl acetate (300 ml × 3), and then *n*-butanol (300 ml × 3). The resulting aqueous layer was concentrated to 200 ml in vacuo. The bioactive aqueous layer was carefully separated by Amberlite XAD-7 column chromatography (955 × 470 mm, Organo Co.) using stepwise elution with MeOH-H₂O (0:10, 1:9, 3:7, 1:1 and 10:0 × 21 each). The H₂O eluate was further purified by repeated TSKgel G-3000S column chromatography (φ30 × 290 mm, Tosoh Co.) with 30% aq. EtOH. In this step, separation of the bioactive fraction with leaf-closing activity (734.5 mg, bioactive at 10 g/l) and the one with leaf-opening activity (39.7 mg, bioactive at 10 g/l) was achieved. The leaf-closing fraction was further purified by HPLC in the Develosil HG-5 packed column (φ20 × 250 mm, Nomura Chemicals Co.) with 10% aq. EtOH (4.0 ml/min flow rate, detection at 220 nm) and then by analytical HPLC in the Develosil ODS-HG-5 column with 5% aq. MeOH (1.0 ml/min flow rate, detection at 220 nm) to give a mixture (0.73 mg) of 1 and lactate. By analytical HPLC in the Develosil ODS-HG-5 column with 5% aq. MeOH (1.0 ml/min flow rate, detection at 220 nm) to give pure lactate (0.23 mg).

Purification of the 3:1 mixture (1.2 mg) of 1 and lactate was also carried out by HPLC with a combination of the two Develosil ODS-SR-5 columns, using an acidic mobile phase (5% aq. MeOH containing 0.5% TFA). Under these conditions, a 1:1 mixture (0.93 mg) of 1 in the free acid form and 2,3-dihydroxy-2-methyl-4-butanolide (2) was obtained, and no pure 1 could be isolated. The ratio was determined by the integrated values of the methyl signals in ¹H-NMR spectrum.

Potassium (2R,3R)-2,3,4-trihydroxy-2-methylbutanoate (1): ¹H-NMR (400 MHz, D₂O, rt) δ: 3.80 (1H, t, J = 5.5 Hz, H-3), 3.57 (2H, d, J = 5.5 Hz, H-4), 1.34 (3H, s, H-5) ppm; ¹³C-NMR (100 MHz, D₂O, rt): 183.0 (C-1), 80.0 (C-2), 78.6 (C-3), 65.0 (C-4), 25.0 (C-5); FAB-MS (negative): m/z 149 [M − H]⁻; FAB-MS (positive): m/z 189 [M + H]⁺.

The physical data for 2 are described next.

Preparation of 2,3-dihydroxy-2-methyl-4-butanolide (2) from potassium 2,3,4-trihydroxy-2-methylbutanoate (1). A crude mixture (0.93 mg) containing potassium lactate and potassium 2,3,4-trihydroxy-2-methylbutanoate (1) in the ratio of 1:3 was dissolved in D₂O (475 µl) containing 36% DCl (25 µl), and the solution allowed to stand at rt, the reaction being monitored by ¹H-NMR. After 24 hours, the reaction mixture was dried in vacuo and purified by Amberlite IRA-68 (Organo Co.) column chromatography with H₂O to give crude 2,3-dihydroxy-2-methyl-4-butanolide (2, 0.89 mg). The purity of 2 was estimated to be over 90% from its ¹H-NMR spectrum.

2,3-Dihydroxy-2-methyl-4-butanolide (2): ¹H-NMR (400 MHz, D₂O, rt) δ: 4.47 (1H, dd, J = 3.7, 11.0 Hz, H-4a), 4.19 (1H, d, J = 11.0 Hz, H-4b), 4.13 (1H, d, J = 3.7 Hz, H-3), 1.34 (3H, s, H-5). [α]D² = −21° (c 0.68, H₂O).

Metal ion analysis by capillary electrophoresis. Metal ion analyses were carried out on samples 1–4. Sample 1 was the bioactive fraction after TSKgel G-3000 column chromatography, which showed leaf-closing activity at 10000 ppm. Sample 2 was prepared from sample 1 by further purification with HPLC, using Develosil ODS-HG-5 columns (φ20 × 250 mm, Nomura Chemicals Co.) with 10% aq. MeOH (4.0 ml/min flow rate, detection at 220 nm). The resulting bioactive fraction was further purified by HPLC in the Develosil ODS-HG-5 column (φ4.6 × 250 mm, Nomura Chemicals Co.) with 5% aq. MeOH (1.0 ml/min flow rate, detection at 220 nm) to give 1.7 mg of a mixture of 1 and lactate in the ratio of 3:1 (sample 2) which was bioactive at 1000 ppm. The ratio of 1 to lactate was determined by the integrated values of the methyl signals in the ¹H-NMR spectrum.

Sample 3 was the bioactive fraction after TSKgel G-3000 column chromatography which showed leaf-
closing activity at 10000 ppm that was further subjected to TSK G3000 gel column chromatography after obtaining sample 1.

Sample 4 was prepared from sample 3 by further purification with HPLC in Develosil ODS-HG-5 columns (φ20 × 250 mm, Nomura Chemicals Co.) with 10% aq. EtOH (4.0 ml/min flow rate, detection at 220 nm). The resulting bioactive fraction was further purified by HPLC using Develosil ODS-HG-5 columns (φ4.6 × 250 mm, Nomura Chemicals Co.) with 5% aq. MeOH (1.0 ml/min flow rate, detection at 220 nm) to give 0.75 mg of a mixture of 1 and lactate in the ratio of 3:1 (sample 3) which was bioactive at 100–200 ppm. The ratio of 1 to lactate was determined by the integrated values of the methyl signals in the 1H NMR spectrum.

Each sample solution was dissolved in distilled water and diluted by distilled water to an appropriate concentration. These sample solutions were directly used in the metal ion analysis which was carried out by a capillary electrophoresis system (Agilent Technologies Co.) with indirect UV detection. The following experimental conditions were used: sample concentration, 100 ng/μl each; capillary, fused silica φ75 mm × 72 cm; electrolyte, 10 mM imidazole, 5 mM lactic acid, and 0.5 mM 18-crown-6 at pH 4.5 (adjusted by 0.1 M AcOH); applied potential, 20 kV; injection, 5 sec at 25 mbar; temperature, 20°C; detection, UV 280/40 nm, ref 210/10 nm.

Results and Discussion

Identification of the leaf-closing substance in L. leucocephala

Isolation of the leaf-closing substance was carried out based on a bioassay using a leaf of L. leucocephala. The bioactive fraction closed the leaf within several hours after adding a solution containing the bioactive substance.

The fresh whole plant of L. leucocephala was extracted with MeOH and concentrated in vacuo. The concentrated extract was partitioned with n-hexane, ethyl alcohol, and then with n-butanol. The bioactive aqueous layer was separated by Amberlite XAD-7 column chromatography with MeOH–H2O, and the H2O fraction showed weak leaf-opening activity. This H2O fraction was further purified by column chromatography with TSK G3000S gel. In this step, we were able to separate the fraction with leaf-opening activity and the one with leaf-closing activity. The leaf-closing fraction was repeatedly purified by HPLC to give a mixture of potassium 2,3,4-trihydroxy-2-methylbutanolate (1)⁷ and potassium lactate in the ratio of 6:1. Although detection of the free form of 1 in some plants has been reported by using GC-MS, the spectral data were insufficient.⁷ The ratio was determined from the integrated values of the methyl signals in the 1H NMR spectrum (δ1.34 for 1, δ1.31 for lactate). We carried out further purification of 1 by HPLC with a combination of multiple ODS columns to give pure lactate. We found that 1 was unstable in the neutral to basic pH range, and decomposed in the course of repeated purification to give lactate which is identified by negative-mode FAB MS and 1H-NMR spectral data. When a 3:1 mixture of 1 and lactate, which had been separately prepared, was dissolved in distilled water and lyophilized, the ratio changed to 1:1 after evaporation; when the same cycle was repeated once more, completely pure lactate resulted. This clearly demonstrated the instability of 1 under neutral conditions. The degradation of 1 into lactate would have been via the retro-Aldol type of mechanism, because the resulting lactate was obtained in racemic form.

Moreover, it was found that the bioactivity gradually decreased during extensive purification by HPLC with an ODS column due to replacement of the counter cation from potassium with that from sodium, as mentioned later. These reasons made the isolation of 1 under neutral conditions unsuccessful.

We therefore attempted to isolate 1 by HPLC under acidic conditions and obtained 1 in the free acid form together with the corresponding γ-lactone, 2,3-dihydroxy-2-methyl-4-butanolide (2), which was produced from 1 under acidic conditions, as a 1:1 mixture. Lactone 2 has also been isolated from water-stressed chickpea.⁸ The ratio of 1 and 2 was determined from the integrated values of the methyl signals in the 1H-NMR spectrum (δ1.34 for 1, δ1.45 for 2). Despite many trials, further purification of 1 was unsuccessful, and no example of the isolation of 1 has so far been reported. However, the isolated potassium lactate showed no leaf-closing activity toward a leaf of L. leucocephala, so co-existing 1 was assumed to be the genuine leaf-closing substance.

Structural determination of the leaf-closing substance

The structural determination of 1 was carried out by means of NMR and FAB MS experiments (Figure 1). The strong molecular ion corresponding to 1 was observed at m/z 189 in the positive-mode FAB mass spectrum of the bioactive fraction; on the other hand, m/z 149 was observed as a molecular ion in the negative-mode FAB MS experiments, suggesting that 1 existed as a potassium salt. The product ions observed in a linked-scan experiment are also shown in Fig. 1.

The 1H-NMR spectral data suggested the existence of a methyl group (δ1.34, 3H, H-5), hydroxy methine group (δ3.80, 1H, H-3), and hydroxy methylene group (δ3.57, 2H, H-4). Vicinal coupling (J = 5.5 Hz) was observed between H-3 and H-4. The HMBC spectrum revealed connectivity between δ78.6 (C-3) and δ 3.80 (H-3), δ65.0 (C-4) and δ3.57 (H-4), and δ25.0 (C-5) and δ1.34 (H-5). The 13C-NMR signal at
\[ \delta 183.0 \] was assigned to a carbon in carboxylate group (C-1) from its chemical shift. An oxygen-connected quaternary carbon atom was observed in the \(^1\)C-NMR spectrum at \(\delta 80.0\) which showed no correlation in the HMQC spectrum. HMBC experiments gave strong correlation between the methyl signal (\(\delta 1.34\)) in the \(^1\)H-NMR spectrum and the \(^13\)C signals at \(\delta 183.0\) (C-1), 80.0 (C-2) and 78.6 (C-3). Together with the vicinal coupling observed between \(\delta 3.80\) (H-3) and \(\delta 3.57\) (H-4), the structure of 1 was determined to be 2,3,4-trihydroxy-2-methylbutanoate.\(^6\)

The relative stereochemistry of 1 was determined by using the corresponding \(\gamma\)-lactone (2). Compound 2 was prepared by the treatment with 0.01 m HCl, a 1:1 mixture of 1 and 2 which had been obtained by purification under acidic conditions (Fig. 2). NOE correlation (3.7\%) between \(\delta 1.34\) (H-5) and \(\delta 4.13\) (H-3) that was observed in 2 confirmed the relative stereochemistry of the vicinal dihydroxy group (Fig. 2). 2,3-Dihydroxy-2-methyl-4-butanolides of \(\text{syn}\) and \(\text{anti}\) relative stereochemistry have been synthesized by Kobayashi et al.\(^9\) A comparison of the \(^1\)H-NMR data for 2 with those reported by Kobayashi et al.\(^9\) supports the \(\text{syn}\) relative stereochemistry of 2 as shown in Fig. 2 (Table 1). The relative stereochemistry of 1 was thus determined to be \(\text{anti}\) as shown in Fig. 1.

The absolute stereochemistry of 1 was then determined by comparing the optical rotation value of \(\gamma\)-lactone (2), [\(\alpha\)]\(^{25}\)_D \(-21^\circ\) (c 0.68, H2O), with that of synthetic (2\(R\),3\(R\))-2,3,4-trihydroxy-2-methylbutanoate (1) (Fig. 2). Since the purity of the sample used in the [\(\alpha\)]\(^{25}\)_D measurement was estimated to be over 90% based on the \(^1\)H-NMR spectrum, the difference in the optical rotation value would mostly have been due to water that could not be completely removed from the sample by pumping due to its hygroscopic features. The modified Mosher’s method\(^10\) could not be used in this case because of the insolubility of 2 in CH\(_2\)Cl\(_2\) which was used as the solvent for esterification in the preparation of the MTPA ester. The \(\gamma\)-lactone (2) showed no leaf-closing activity even at \(1 \times 10^{-4}\) M.

**Biological activity of the leaf-closing substance**

We found that the leaf-closing activity of 1 was greatly affected by the co-existing counter cation. When the mixture was dissolved in distilled water, it

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**Table 1.** \(^1\)H-NMR Data for Compound 2 and 2,3-Dihydroxy-2-methyl-4-butanolides\(^7\) (in ppm)

<table>
<thead>
<tr>
<th></th>
<th>Compound 2</th>
<th>syn-2,3-Dihydroxy-2-methyl-4-butanolide</th>
<th>anti-2,3-Dihydroxy-2-methyl-4-butanolide</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H_{as})</td>
<td>4.47 (1H, dd, (J = 3.8, 11) Hz)</td>
<td>4.44 (1H, dd, (J = 3.6, 11) Hz)</td>
<td>4.44 (1H, dd, (J = 6.3, 9.6) Hz)</td>
</tr>
<tr>
<td>(H_{as})</td>
<td>4.19 (1H, d, (J = 11) Hz)</td>
<td>4.15 (1H, d, (J = 11) Hz)</td>
<td>4.27 (1H, (J = 6.3) Hz)</td>
</tr>
<tr>
<td>(H_{3})</td>
<td>4.13 (1H, d, (J = 3.8) Hz)</td>
<td>4.09 (1H, d, (J = 3.6) Hz)</td>
<td>3.92 (1H, dd, (J = 6.3, 9.6) Hz)</td>
</tr>
<tr>
<td>(H_{3})</td>
<td>1.34 (3H, s)</td>
<td>1.30 (3H, s)</td>
<td>1.24 (3H, s)</td>
</tr>
</tbody>
</table>
showed leaf-closing activity at $1 \times 10^{-3}$ m. However, when 1 was dissolved in a 0.1 mm potassium phosphate buffer solution at pH 5.4, its bioactivity was dramatically improved to $1 \times 10^{-4}$ m. The same buffer containing no leaf-closing substance was ineffective for closing *L. leucocephala* leaves. However, during the bioassay carried out in the 0.1 mm sodium phosphate buffer solution at pH 5.4, 1 did not show leaf-closing activity even at $5 \times 10^{-4}$ m. It was considered from this result that 1 needed the potassium ion as its counter cation to exhibit leaf-closing activity. The above-mentioned decrease of leaf-closing activity during the course of purification can thus be explained as being due to replacement of the counter cation during purification from the potassium cation to the sodium cation which was contained in an excess amount in the HPLC solvent. A cation analysis of the bioactive fractions obtained in each step of purification supports this explanation. Quantitative analyses of the cations contained in the bioactive fractions were carried out by capillary electrophoresis (CE) with indirect UV detection. It was thus observed that the potassium cation was gradually replaced by the sodium cation in the course of repeated purification as the activity of the bioactive fractions decreased. For example, the bioactive fraction obtained after the second step of purification (TSKgel G-3000 column chromatography) showed leaf-closing activity at 10000 ppm (sample 1), and the cation analysis of this fraction by CE gave a weight ratio of $K^+ : Na^+ = 50:37$ (13:17 in molar ratio). However, with a mixture of 1 and lactate obtained after further purification by HPLC (sample 2), a small improvement of bioactivity (1000 ppm) was observed, and CE analysis of this mixture gave a weight ratio of $K^+ : Na^+ = 18:62$ (10:61 in molar ratio) (Table 2). On the other hand, when the separation of the bioactive fractions was carried out by HPLC using aq. EtOH as the mobile phase, cation exchange during purification from the potassium cation to the sodium cation was observed, and CE analysis of this mixture gave a weight ratio of $K^+ : Na^+ = 55:41$ (14:18 in molar ratio) (Table 2). It is concluded from these results that cation exchange during purification decreased the bioactivity of the resulting fractions, indicating that there was strong correlation between the bioactivity and content of the potassium cation. The necessity for the potassium cation in the leaf-closing activity of 1 suggests strong correlation between the potassium channel and the leaf movement introduced by 1.11)

All of our leaf-movement factors previously isolated have shown specific bioactivity against the leaf of the original plant. We therefore examined the specific bioactivity of 1 by using the leaves of *Aeschynomene indica* L., *Cassia mimosoides* L., *Phyllanthus urinaria* L., and *Sasbania speciosa* Taub. The bioassay was carried out with a $5 \times 10^{-4}$ m solution of 1; 1 was not effective against these leaves except for *C. mimosoides*. This weak bioactivity of 1 toward *C. mimosoides* would have been due to the structural similarity of the leaf-closing substances between *C. mimosoides* and *L. leucocephala*. The leaf-closing substance of *C. mimosoides*, potassium idarate, has a 2,3,4-trihydroxy carboxylate moiety, which is also contained in 1, in its molecule.

We also observed leaf-opening activity in a fraction separated by column chromatography with TSK G3000S gel, and the isolation of the leaf-opening substance is now in progress.

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