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(*R*)-Eucomic acid, a leaf-opening factor of the model organism, *Lotus japonicus*

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ABSTRACT

In the post-genomic era, the biological activity of endogenous metabolites can be linked to genomic information via its target protein. Consequently, studies concerned with the identification of endogenous bioactive metabolites from model organisms should be undertaken. (*R*)-Eucomic acid (**1**) was identified as an endogenous metabolite concerning leaf movement in the model legume, *Lotus japonicus*. Absolute stereochemistry was investigated by comparison of physical characteristics of natural and synthetic enantiomers of **1**. Identification of endogenous metabolites in a model legume would be particularly advantageous for further studies on the chemical biology of this biologically intriguing phenomenon.

1. Introduction

Biological studies have become increasingly focused on model organisms, such as *Caenorhabditis elegans*,[1] *Drosophila melanogaster*,[2] *Arabidopsis thaliana*,[3] *Mus musculus*,[4] and *Homo sapiens*.[5] Additionally, *Lotus japonicus* was previously proposed as a promising model legume for studies on molecular genetics and genomics.[6–8] The use of genetic information available for these species has accelerated the pace of molecular genetic studies generally and resulted in marked advances in biology. In an attempt to increase the potential of these studies, investigations on the chemical characteristics of the endogenous metabolites of these model organisms should be undertaken.

Most legumes close their leaves in the evening, as if sleeping, and open them in the morning in what is referred to as a nyctinasty.[9] The pioneering work on plant movement was conducted by Charles Darwin in the 19th century.[10] Our previous studies showed that five pairs of endogenous chemical factors, also referred to as leaf-closing factor (LCF) and leaf-opening factor (LOF), are involved in the control of nyctinastic leaf movements.[11–13] However, the lack of genetic information on the plants used in those studies has made further studies on the chemical biology of this biological phenomenon difficult. The discovery of LOF in *L. japonicus* would therefore help us to further elucidate the chemical characteristics of nyctinasty by utilizing genomic information of the species.

2. Results and discussion

The most important aspect of isolating a biologically active endogenous chemical factor is the establishment of a bioassay with high reproducibility. In the evening, the three leaflets composing each *L. japonicus* compound leaf move upward from the horizontal to a vertical position (Fig. 1). Purification of the LOF from *L. japonicus* (Fig. 1) was conducted using a novel, highly reproducible bioassay that employs an *L. japonicus* (Gifu B-129) leaflet (Fig. 2). Briefly, the compound leaf of *L. japonicus* was removed from the stem by cutting at the petiole and the two side leaflets were cut at the base. We found that the single remaining leaflet with the petiole continued the rhythmic movement

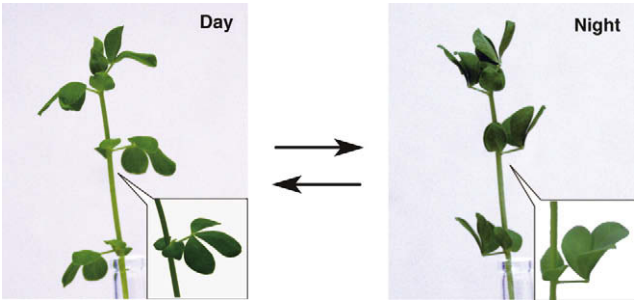

Figure 1. Circadian rhythm leaf movement of *L. japonicus* (left: in the daytime, right: at night).

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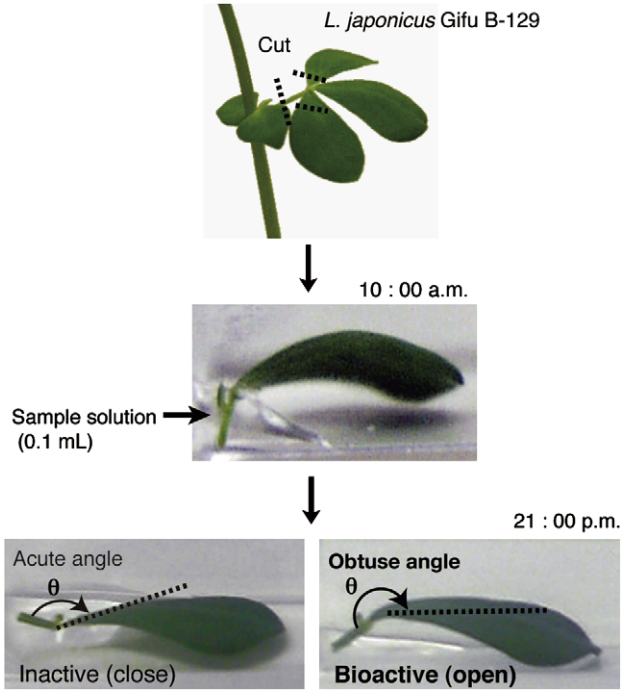


Figure 2. Bioassay using a leaflet of *L. japonicus* (top: a leaflet of *L. japonicus*, middle: a leaflet with petiole in sample solution, bottom right: a leaflet in a bioactive fraction, bottom left: biologically inactive fraction).

observed during the day on the whole plant, with the angle between the leaflet and the petiole (θ) remaining $>190^\circ$ in the daytime, becoming $<170^\circ$ at night (Fig. 2). We observed a change in the angle between the petiole and the leaflet after addition of sample. We added a sample solution to a leaflet with a petiole at 10:00. The difference in the position of the leaves observed using the bioactive and inactive fractions gradually appeared after 16:00. The bioactivity of the sample was assessed by the status of leaves at 21:00. When less than 40% of the total number of leaves had closed ($\theta<170^\circ$), the sample was judged to be bioactive.

The bioassay-guided purification of LOF was conducted as follows: methanolic extract of the terrestrial part of *L. japonicus* was concentrated in vacuo and partitioned with organic solvents. The bioactive aqueous fraction was separated using an Amberlite XAD-7 column chromatography followed by Toyopearl HW-40S gel filtration column chromatography. Following two-step purifications by MPLC and HPLC using an ODS column gave the bioactive fraction, which made the leaves of *L. japonicus* open at 1 mg/L. We established a novel robust bioassay method using sample solutions as small as 0.1 mL, which enabled bioassay-guided purification using a small amount of plant material. By employing this method, we succeeded in purifying 136 μg of LOF fraction from 41 g equiv of *L. japonicus*.

The active compound in the bioactive fraction was identified by ESI-MS and NMR. High resolution mass spectroscopy using a negative-mode FT-ICR ESI-MS produced a molecular peak at *m*/*z* 239.0561 ($[M-H]^-$), which corresponded precisely with the molecular composition of $C_{11}H_{12}O_6$ (*m*/*z* 239.0561 calcd for $C_{11}H_{11}O_6$, Δ=0.0 mmu). Determination of the LOF structure was performed by 800 MHz NMR equipped with a cold probe. Correlations in HSQC (Heteronuclear Single Quantum Coherence) and NOESY (Nuclear Overhauser Effect Spectroscopy) gave the planar structure of LOF (Fig. 3).

Existence of two isolated methylene groups was confirmed from HSQC correlations between two protons (H_{2a}, H_{2b}) and C_2, and

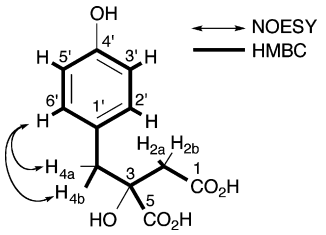


Figure 3. Structural determination of LOF **1** (arrows: NOE correlations, bold lines: HMBC correlations).

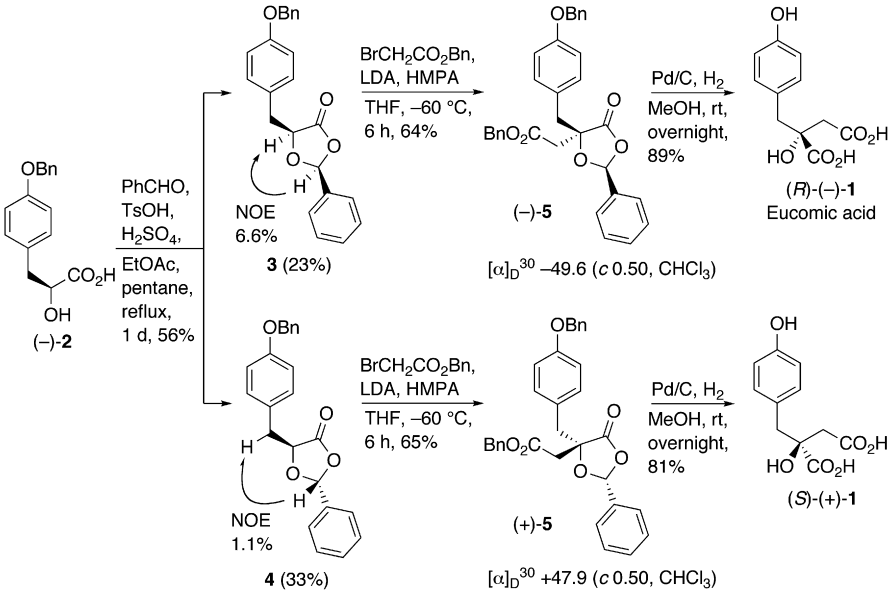
between two protons (H_{4a}, H_{4b}) and C_4, respectively. HMBC spectra also showed that the LOF had one additional quaternary carbon and two carboxylic acids by the correlations between H_{2a}–C_1, H_{2a}–C_3, H_{2a}–C_5, H_{4b}–C_3, and H_{4b}–C_5. The HMBC correlations between H_{4b}–$C_{2'}$ and $H_{2'}$–C_4, together with NOESY correlations between H_{4a}–$H_{2'}$ and H_{4b}–$H_{2'}$ gave the planar structure of LOF as **1** (Fig. 3). Compound (*R*)-(−)-**1** was a known eucomic acid isolated from bulbs of *Eucomis punctata* L'Hérit (Liliaceae) that is a weak radical scavenger.[14,15] Cation analysis of natural LOF by capillary electrophoresis revealed the presence of Na^+ (38%), Ca^{2+} (18%), and Mg^{2+} (12%) with a small amount of K^+ (1.4%). However, the Na^+ and Ca^{2+} cations in the LOF could be attributed to a contamination from environment, because the original bioactive aqueous fraction contained mainly K^+ (81.8%) and Mg^{2+} (16.2%), and a small amount of Ca^{2+}(2.0%).

Syntheses of eucomic acid were previously reported by two groups.[14,16] We synthesized both enantiomers of **1** in five steps from (−)-**2**, which was easily prepared from commercially available *O*-benzyl-L-tyrosine,[17] and using both enantiomers of **1** enabled confirmation of the absolute stereochemistry of naturally occurring **1** (Scheme 1, Fig. 4). α-Hydroxyl carboxylic acid (−)-**2** was protected by using benzaldehyde to give a diastereomixture of benzylidene acetals **3** and **4**, which can be separated easily. The configuration of each diastereomer (*cis*-**3** or *trans*-**4**) was determined from NOE correlations as illustrated in Scheme 1. Treatment of **3** with LDA followed by the addition of benzyl α-bromoacetate in HMPA resulted in diastereoselective C–C bond formation to give (−)-**5** in 64% yield after recrystallization. Similarly, alkylation of **4** gave only (+)-**5** in 65% yield. Synthetic (−)-**5** and (+)-**5** exhibited highly similar optical rotation values with opposite signs. Hydrogenolysis of benzylidene acetal, benzyl ether, and benzyl ester gave (*R*)-(−)-**1** from (−)-**5** in good yields as well as (*S*)-(+)-**1** from (+)-**5**. ^1H and ^{13}C NMR data for synthetic (*R*)- or (*S*)-**1** were identical to those of natural **1**.

Successive chiral HPLC analyses on synthetic (*R*)-(−)-**1** and (*S*)-(+)-**1** were then carried out. Under optimized separation conditions, the optical purities of (*R*)-(−)-**1** and (*S*)-(+)-**1** were up to 99% ee (Fig. 4), and they can be used as good references for determining the stereochemistry of naturally occurring **1**. Under optimized conditions, the retention time of naturally occurring **1** was in good agreement with that of synthetic (*R*)-(−)-**1** (Fig. 4), indicating that natural **1** was (*R*)-(−)-**1**.

Next, the leaf-opening activities of synthetic (*R*)-(−)-**1** and (*S*)-(+)-**1** were examined using the leaf bioassay employing *L. japonicus*. We prepared a sodium salt of both enantiomers, (*R*)-(−)-**1** and (*S*)-(+)-**1**, and examined their biological activities. Leaf-opening bioactivity was assessed based on the aforementioned criteria, and the percentage of opening leaves was calculated on an hourly basis (Fig. 5).

Differences in the status of the leaves gradually appeared around 16:00 (5 h before getting dark). More than 60% of the leaves treated with 10 μM of sodium salt of (*R*)-(−)-**1** remained open at 22:00 (1 h after getting dark), and 35% of the leaflets continued to open even at



Scheme 1. Synthetic route of enantiomers of **1**.

0:00 (3 h after getting dark). And also, 100 μM solution of sodium salt of (*R*)-(−)-**1** made 60% of the leaflets open even at 0:00 (3 h after getting dark). Conversely, approximately 20% of the leaves treated using the sodium salt of (*S*)-(+)-**1** as well as H_2O remained open at 22:00. The findings of this bioassay clearly demonstrated that the sodium salt of (*R*)-(−)-**1** induced leaf opening in *L. japonicus* at 10 μM, while that of (*S*)-(+)-**1** showed no biological activity even at 100 μM, with a noticeable bioactivity observed between (*R*)-(−)-**1** and (*S*)-(+)-**1**. These results suggest the existence of a specific protein capable of recognizing the stereochemistry of **1** in *L. japonicus*, and also that our enantiodifferential approach[18,19] would be applicable for studies on the chemical biology of **1**.

Successively, we prepared a potassium salt of both enantiomers of **1** and free form of (*R*)-(−)-**1**, and examined their biological activities (Fig. 6). More than 60% of the leaves treated with 100 μM of potassium salt of (*R*)-(−)-**1** remained open at 22:00 (1 h after getting dark), but 20% of the leaves treated using the free (*R*)-(−)-**1** as well as the potassium salt of (*S*)-(+)-**1** remained open at 22:00. Thus, the potassium salt of (*R*)-(−)-**1** induced leaf opening in *L. japonicus* at 100 μM, while that of (*S*)-(+)-**1** and the free form of (*R*)-(−)-**1** showed no biological activity even at 100 μM. These results indicated that the leaf-opening activity of **1** was depended not only on the stereochemistry, but also on the counter cation.

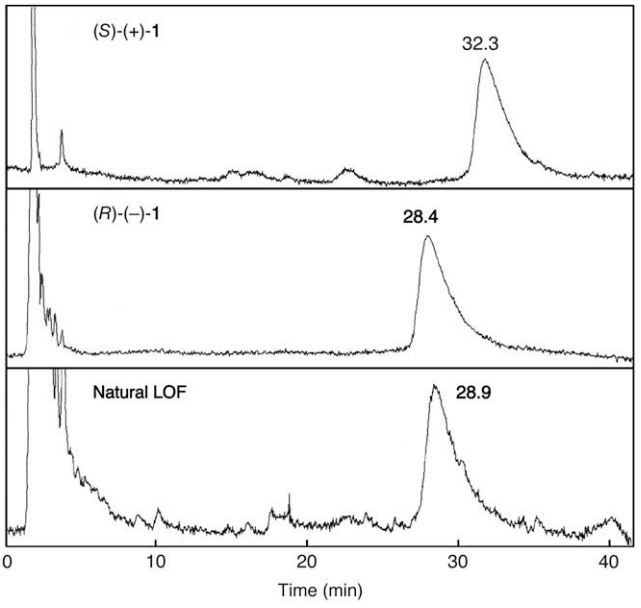


Figure 4. Chiral HPLC analyses of synthetic (*S*)-(+)-**1**, (*R*)-(−)-**1**, and naturally occurring **1**.

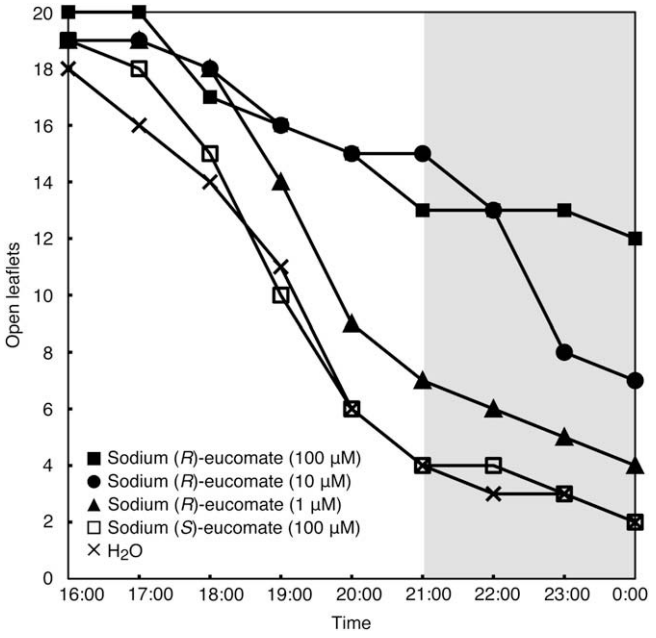


Figure 5. Time course changes in the number of opening and closing events for 20 leaves treated with sodium salt of (*R*)-(−)-**1**, sodium salt of (*S*)-(+)-**1**, and water. Lights were off after 21:00.

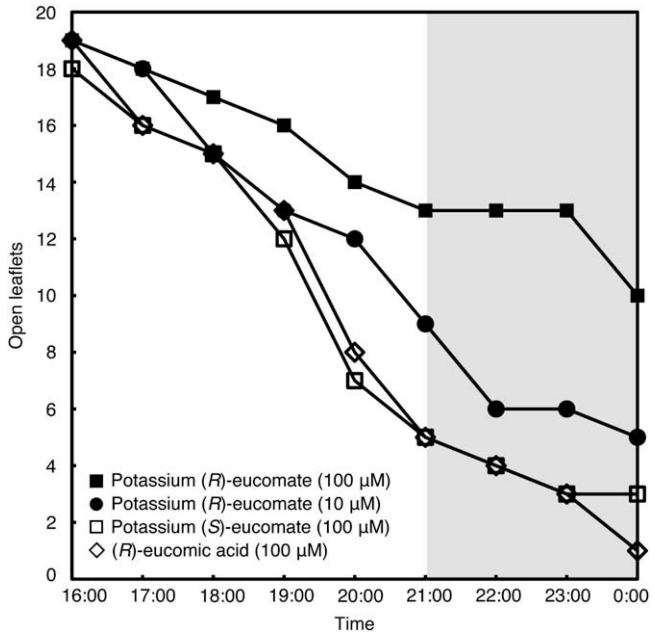


Figure 6. Time course changes in the number of opening and closing events for 20 leaves treated with potassium salt of (*R*)-(−)-**1**, potassium salt of (*S*)-(+)-**1**, and free form of (*R*)-(−)-**1**. Lights were off after 21:00.

Although sodium salt of (*R*)-(−)-**1** was identified as the main bioactive metabolite in the extract of *L. japonicus*, it is suspected that the genuine bioactive species of (*R*)-(−)-**1** in vivo would be a potassium salt because plants typically use potassium instead of sodium. In fact, extract of *L. japonicus* mainly contained K^+ as metal cation, as mentioned before. If our hypothesis is true, *L. japonicus* would contain enough amount of (*R*)-(−)-**1**, which operates as the potassium salt. Then, we measured an endogenous level of (*R*)-(−)-**1** in the extract of *L. japonicus* (Fig. 7). Quantitative analysis showed that the content of (*R*)-(−)-**1** in *L. japonicus* was 244 μmol per kg fresh weight. This content of (*R*)-(−)-**1** corresponded to 320 μM in concentration when the water content of the fresh plant is estimated to be 80% (data not shown). This result suggested that the genuine bioactive species of (*R*)-(−)-**1** was potassium salt.

3. Conclusions

Eucomic acid ((*R*)-(−)-**1**) was isolated from *L. japonicus* and considered as a potential LOF in this species. The identification of endogenous chemical signals within model organisms is important for combining studies of chemical and biological research. These molecules could be useful for developing biochemical reagents and methods for forward chemical genetics,[20] such as enantiodifferences[18,19] in cDNA microarray analysis, in which biologically inactive enantiomer is used as perfect control.

4. Experimental

4.1. General method

L. japonicus Gifu B-129 was grown from seed on power soil in a Biotron chamber (LPH-1000S, NK System Co., Ltd., Japan) under 16 h light at 26 °C (from 5:00 to 21:00) then 8 h dark conditions at 22 °C for bioassay.

The fresh whole *L. japonicus* (20.6 kg) plants were collected in Sendai, Japan for isolation of LOF, and only 1/20th of aqueous layer (1 kg FW equiv) was used in the next procedure.

The NMR spectra of natural LOF (0.136 mg) in 0.3 ml of D_2O using an NMR tube matched for D_2O (PS, Shigemi, Japan) were measured with an INOVA-800 NMR spectrometer system equipped with a cold probe (800 MHz for ^1H, Varian, Inc., USA). ^1H NMR, DQF-COSY, NOESY, and HSQC spectra were measured using 16 scan times, while the HMBC spectra were measured using 128 scan times. NMR spectra of synthetic compounds were recorded on a Jeol JNM-Alpha500 [^1H (500 MHz) and ^{13}C (125 MHz), Jeol Inc., Japan] using TMS in $CDCl_3$, CD_2HOD in CD_3OH (^1H; 3.33 ppm, ^{13}C; 49.8 ppm), or *t*-BuOH (^1H; 1.23 ppm, ^{13}C; 32.1 ppm) in D_2O as internal standard at various temperatures. The ESI-MS and HR ESI-MS spectra were recorded on a Bruker Esquire 4000 (Bruker Daltonics Inc., Germany) and a Bruker APEX-III (Bruker Daltonics Inc., Germany), respectively. The IR spectra were recorded on a JASCO FT/IR-410 (JASCO Inc., Japan). The specific rotations were measured by JASCO DIP-360 polarimeter (JASCO Inc., Japan). Silica gel column chromatography was performed on Silica Gel 60 N (Kanto Chemical Co. Ltd., Japan). Reversed-phase open-column chromatography was performed on Cosmosil 75C18-OPN (Nakalai Tesque Co. Ltd., Japan). TLC was performed on Silica gel F_{254}

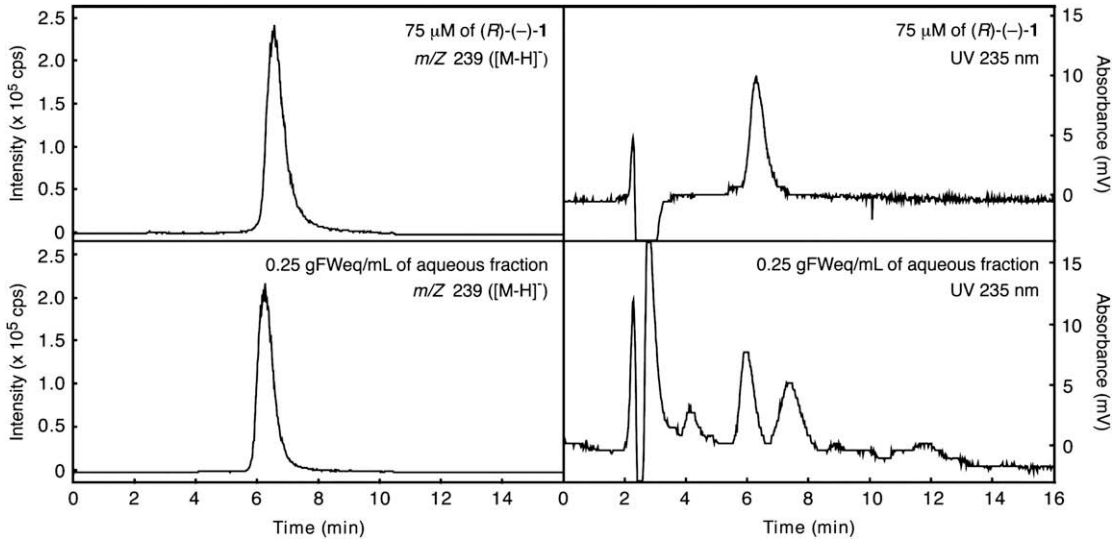


Figure 7. LC/MS analysis of the (*R*)-(−)-**1** and the aqueous fraction. LC/ESI-MS (negative) analysis of (*R*)-(−)-**1** at 75 μM and the bioactive aqueous fraction from *L. japonicus* at 0.25 g FW equiv/mL by the extracted ion chromatograms *m/z* 239 (left) and UV at 235 nm (right).

(0.25 mm or 0.5 mm, MERCK, Germany) or RP-18F_{254S} (0.25 mm, MERCK). Elemental analyses were carried out on a CHN-corder MT-6 (Yanako Co., Ltd., Japan).

4.2. Bioassay

All bioassays were performed in a Biotron chamber (LPH-1000S, NK System Co., Ltd., Japan) under 16 h light at 26 °C (from 5:00 to 21:00) then 8 h dark at 22 °C. The leaf of a young *L. japonicus* plant, which was grown in the same chamber for 1–2 months, was removed from the stem of the plant at the petiole and the two side leaflets of the compound leaf were cut at their base. The remaining central leaflet with petiole was then floated on water overnight. In the morning (at 9:00), the leaves that had reopened and formed obtuse angles between the leaf and the petiole were selected for the bioassay.

Each leaflet with petiole was placed in a 1.5-mL plastic disposable cuvette (Kartell, Milan Co., Italy) taking care to ensure that the cut surface of the petiole was continuously immersed into the sample solution (0.1 mL), which was obtained by each step of separation procedure, with the change in the angle between petiole and leaflet observed at hourly intervals. Leaf opening was considered to have occurred when the angle between the leaflet and petiole was obtuse ($\theta>190°$), while leaf closing was considered to be when the angle was acute ($\theta<170°$). During the course of isolation, each resulting fraction was tested by this method using a set of more than nine leaflets with petioles. Leaflets with petioles placed in distilled water were used as negative controls. The bioactivity of a fraction was assessed by the status of leaves at 21:00. When less than 40% of the total number of leaves had closed ($\theta<170°$), the fraction was judged to be bioactive.

4.3. Isolation of LOF from *L. japonicus*

The fresh whole *L. japonicus* (20.6 kg) plants were collected in Sendai, Japan. Terrestrial part was placed in methanol (200 L) and extracted for 1 week and the extract was concentrated in vacuo to give an aqueous solution of (5 L, 4 kg FW equiv/L). The aqueous solution (1.0 L) was partitioned with *n*-hexane (3×500 mL), ethyl acetate (3×500 mL), and *n*-butanol (3×500 mL). 1/20th of the aqueous layer (250 mL, 1 kg FW eq) was separated by Amberlite XAD-7 column chromatography (60×600 mm, Organo Co., Ltd., Japan) by stepwise elution with 0, 10, 30, and 100% aqueous methanol to give four aqueous fractions (13, 5.5, 0.71, and 0.51 g, respectively; bioactivity, the second fraction 2 g/L, others >2 g/L). Part of the second aqueous fraction (50 mg) was further purified by MPLC using Toyopearl HW-40S (25×400 mm, Tosoh Co., Ltd., Japan) with water (flow rate: 1.0 mL/min, detection: 215 nm) to give a bioactive fraction (23.2 mg; retention time: 140–160 min, bioactivity, 1 g/L, others >1 g/L). Part of the bioactive fraction (5.0 mg) was further purified by MPLC equipped with an ODS column (24×360 mm, Lop-ODS, Nomura Chemical Co., Ltd., Japan) with a stepwise elution using 5, 20, 50, and 100% aqueous methanol (flow rate: 8.0 mL/min, detection: 215 nm) to give a bioactive 20% eluate (259 μg; bioactivity, 0.05 g/L, others >1 g/L). This process was repeated and collected 20% eluate (600 μg) was purified by HPLC on an ODS column (Develosil ODS HG-5, 4.6×250 mm, Nomura Chemical Co., Ltd.) with 2% aqueous CH_3CN (flow rate: 1.0 ml/min, detection: 215 nm) to give a bioactive fraction (136 μg; retention time: 10–12 min; bioactivity, 1 mg/L, others >10 mg/L). The fraction was concentrated in vacuo to remove CH_3CN and freeze-dried several times to give LOF as a white powder.

Eucomic acid (*R*)-(−)-**1**: ^{1}H NMR (800 MHz, D_2O) δ 7.17 (d, J=8.2 Hz, 2H, $H_{2'}$ and $H_{6'}$), 6.84 (d, J=8.2 Hz, 2H, $H_{3'}$ and $H_{5'}$), 3.01 (d, J=13.7 Hz, 1H, H_{4a}), 2.85 (d, J=15.6 Hz, 1H, H_{2b}), 2.83 (d, J=13.7 Hz, 1H, H_{4b}), 2.56 (d, J=15.6 Hz, 1H, H_{2a}); ^{13}C signals were determined by HSQC and HMBC correlations, δ 181.7, 179.1, 155.0, 131.3, 128.2, 115.0, 78.1, 45.5, 43.3; FT-ICR ESI-HRMS: m/z found 239.0561 ($[M-H]^-$), calcd for $C_{11}H_{11}O_6$ 239.0561.

4.4. Synthesis of LOF (1) and its enantiomer

α-Hydroxyl carboxylic acid **2** was prepared from commercially available *O*-benzyl-L-tyrosine (Watanabe Chemical, Japan) with $NaNO_2$ in 1,4-dioxane and aqueous sulfuric acid.[17]

*4.4.1. Synthesis of benzylidene acetals **3** and **4***

To a solution of (−)-**2** (343 mg, 1.26 mmol) in ethyl acetate (13 mL) were added *n*-pentane (13 mL), benzaldehyde (115 mg, 1.08 mmol), TsOH (240 mg, 1.39 mmol), and one drop of H_2SO_4. The reaction mixture was stirred and refluxed for 1 day with azeotropic removal of water. The reaction was stopped by the addition of aqueous Na_2CO_3 to the reaction mixture. The organic layer was washed with saturated aqueous NaCl, dried over absolute Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate=30/1 to 20/1), and recrystallized from *n*-pentane/Et_2O to give *cis*-**3** (104 mg, 0.288 mmol, 23%) as a white powder and *trans*-**4** (148 mg, 0.411 mmol, 33%) as a white powder, respectively.

(2*S*,4*S*)-4-(4-Benzyloxybenzyl)-2-phenyl-5-oxodioxolan **3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.30 (m, 8H), 7.26–7.20 (m, 4H), 6.93 (d, J=7.6 Hz, 2H), 6.31 (d, J=1.2 Hz, 1H), 5.05 (s, 2H), 4.64 (ddd, J=6.6, 4.1, 1.2 Hz, 1H), 3.26 (dd, J=14.7, 4.1 Hz, 1H), 3.13 (dd, J=14.7, 6.6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 158.0, 137.0, 134.3, 130.9, 130.5, 128.6, 128.5, 127.9, 127.8, 127.4, 126.9, 114.9, 103.4, 76.3, 70.0, 35.9; $[\alpha]_D^{29}$ −74.6 (*c* 0.50, $CHCl_3$). Anal. Found: C, 76.58; H, 5.69. Calcd for $C_{23}H_{20}O_4$: C, 76.65; H, 5.59.

(2*R*,4*S*)-4-(4-Benzyloxybenzyl)-2-phenyl-5-oxodioxolan **4**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.29 (m, 10H), 7.25 (d, J=8.7 Hz, 2H), 6.95 (d, J=8.7 Hz, 2H), 6.12 (d, J=0.9 Hz, 1H), 5.06 (s, 2H), 4.72 (ddd, J=5.8, 4.5, 0.9 Hz, 1H), 3.19 (dd, J=14.6, 4.5 Hz, 1H), 3.13 (dd, J=14.6, 5.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.5, 158.1, 136.9, 135.5, 130.8, 130.2, 128.6, 128.6, 128.0, 127.5, 127.5, 126.1, 115.1, 103.9, 75.3, 70.0, 36.1; $[\alpha]_D^{29}$ +24.1 (*c* 0.50, $CHCl_3$). Anal. Found: C, 76.48; H, 5.78. Calcd for $C_{23}H_{20}O_4$: C, 76.65; H, 5.59.

*4.4.2. Synthesis of benzyl [(2S,4R)-4-(4-benzyloxybenzyl)-2-phenyl-5-oxodioxolan-4-yl]acetate (−)-**5***

To a solution of **3** (125 mg, 0.347 mmol) in THF (3.5 mL) was added freshly prepared LDA (0.2 M, 2.4 mL, 0.485 mmol) at −80 °C under argon atmosphere. After stirring for 1 h, benzyl α-bromoacetate (111 mg, 0.486 mmol) in HMPA (0.77 mL) was slowly added to the mixture. After additional stirring for 6 h at −60 °C, the reaction was quenched with the addition of 1 M aqueous HCl (10 mL). The reaction mixture was extracted with diethyl ether (3×10 mL), washed with saturated aqueous NaCl, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate=25/1 to 10/1), before being recrystallized from *n*-pentane/diethyl ether to give (−)-**5** (113 mg, 0.222 mmol, 64%) as a white powder.

Benzyl [(2*S*,4*R*)-4-(4-benzyloxybenzyl)-2-phenyl-5-oxodioxolan-4-yl]acetate (−)-**5**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.22 (m, 13H), 7.03 (d, J=8.7 Hz, 2H), 6.98 (d, J=7.3 Hz, 2H), 6.79 (d, J=8.7 Hz, 2H), 6.39 (s, 1H), 5.19 (d, J=12.0 Hz, 1H), 5.16 (d, J=12.0 Hz, 1H), 5.02 (s, 2H), 3.13 (d, J=14.1 Hz, 1H), 3.07 (d, J=17.0 Hz, 1H), 2.96 (d, J=14.1 Hz, 1H), 2.92 (d, J=17.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8, 160.5, 158.3, 140.0, 135.4, 135.1, 131.8, 129.8, 128.7, 128.6, 128.6, 128.6, 128.3, 128.0, 127.4, 126.4, 126.1, 114.7, 103.5, 80.8, 70.0, 67.2, 43.1, 41.3; $[\alpha]_D^{30}$ −49.6 (*c* 0.50, $CHCl_3$). Anal. Found: C, 75.32; H, 5.65. Calcd for $C_{32}H_{28}O_6$: C, 75.57; H, 5.55.

*4.4.3. Synthesis of benzyl [(2R,4S)-4-(4-benzyloxybenzyl)-2-phenyl-5-oxodioxolan-4-yl]acetate (+)-**5***

According to the abovementioned procedure for (−)-**5**, **4** (92.1 mg, 0.256 mmol) gave (+)-**5** (84.0 mg, 0.165 mmol, 65%) as a white powder. $[\alpha]_D^{30}$ +47.9 (*c* 0.50, $CHCl_3$).

*4.4.4. Synthesis of (R)-(−)-**1** (eucomic acid)*

To a solution of (−)-**5** (63.1 mg, 0.124 mmol) in MeOH (5 mL) was added 5% Pd/C (60 mg). After the reaction mixture was stirred under a H_2 atmosphere overnight, the mixture was filtered through Celite and washed with 50% aqueous MeOH (3×5 mL). After evaporation, the residue was purified by HPLC on an ODS column (4.6×250 mm, Develosil ODS-HG-5, Nomura Chemical Co., Ltd.) at a flow rate of 0.5 mL/min with 15% MeOH (retention time: 22.5–23.0 min). The purified fraction was concentrated and freeze-dried to give pure (*R*)-(−)-**1** (eucomic acid) (26.1 mg, 89%) as a white powder. $[\alpha]_D^{28}$ −12.9 (*c* 0.50, MeOH); lit[14] $[\alpha]_D^{24}$ −13±2 (*c* 1.08, H_2O).

*4.4.5. Synthesis of (S)-(+)-**1***

According to the abovementioned procedure for (*R*)-(−)-**1**, (+)-**5** (36.1 mg, 71.0 μmol) gave (*S*)-(+)-**1** (13.8 mg, 57.5 μmol, 81%) as a white powder. $[\alpha]_D^{27}$ +12.3 (*c* 0.50, MeOH).

4.5. Chiral HPLC analyses of synthetic (*S*)-(+)-1, (*R*)-(−)-1, and naturally occurring 1

The purified naturally occurring **1**, synthetic (*S*)-(+)-**1**, and (*R*)-(−)-**1** were analyzed by HPLC on a chiral column (Sumichiral OA-3100, 4.6×250 mm, Sumitomo Chemical Co., Ltd., Japan) with 0.1 M NH_4OAc in MeOH and 1,4-dioxane (1:1) (flow rate: 1.5 mL/min, detection: 280 nm). These chromatograms and retention times are shown in Figure 4.

4.6. Cation analysis of naturally occurring 1

The cation's content in purified natural leaf-opening factor (*R*)-(−)-**1** was analyzed by capillary electrophoresis using an Agilent CE system and Cation Solution Kit (Agilent Co., Ltd., USA). Separations were performed on 64.5 cm long fused-silica capillaries with 50 μm I.D. Before each analysis, the capillary was rinsed for 4 min with running buffer containing 20 mM imidazole and 1.0 mM 18-crown-6, and 0.5 mM lactic acid adjusted to pH 4.5 with acetic acid. Sample solution was injected with a pressure of 50 mbar for 4.0 s. The separation run was conducted over 5 min at 20 °C at a constant voltage of +20 kV. Detection was performed by indirect UV monitoring; the signal wavelength was set at 310 nm and the reference at 215 nm. The negative UV peaks of analytes were inverted by reference to a signal wavelength of 310 nm. The content of each cation was calculated using peak area compared to internal standards.

4.7. Quantitative analysis of (*R*)-(−)-1 in the extract of *L. japonicus*

A triacontyl column (2.0×150 mm, Develosil RPAQUEOUS, Nomura Chemical, Tokyo) was attached to an LC system and 5 μL of sample solution was eluted at a flow rate of 0.2 mL/min with 35% aqueous CH_3OH with 0.1% formic acid for 16 min. LC/ESI-MS (negative) was acquired in extracted ion chromatograms scan mode of *m*/*z* 239. Calibration was established by peak areas for injection of (*R*)-(−)-**1** solution at 50, 75, and 100 μM, which were 5.99×10^6, 9.19×10^6, and 1.21×10^7 area (retention time: 6.3 min), respectively. The intensity of (*R*)-(−)-**1** was calculated to be 2.43×10^4 area/pmol from the regression curve ($R^2>0.999$). Quantitative analysis was performed by injection of the bioactive aqueous fraction at 0.25 g FW equiv/mL in the same condition. The peak area was 7.15×10^6. The content of (*R*)-(−)-**1** in *L. japonicus* was calculated to be 244 μmol/kg fresh weight.

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