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Potassium 2,3,4-trihydroxy-2-methylbutanoate, a leaf-closing substance of *Leucaena leucocephalam*

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Abstract—Potassium 2,3,4-trihydroxy-2-methylbutanoate (**1**) was idetified as a leaf-closing substance of a nyctinastic plant, *Leucaena leucocephalam*. Compound **1** was quite effective for the leaf-closing of *L. leucocephalam* at 1×10^{-6} M.

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, which has been known to be controlled by an internal biological clock.[2] Recently, we have identified several bioactive substances that regulate this leaf-movement, and revealed that nyctinastic movement of the plants is controlled by the interaction between leaf-closing and -opening substances, which are different among the plants.[3] Moreover, we have demonstrated the importance of leaf-movement for the survival of legumes. In our previous work with artificial leaf-opening substance the leaf of *Cassia mimosoides* L. which was kept open for a week was observed to wither and die.[4] This result showed that the nyctinastic leaf-movement is essential for the survival of legumes. From these findings, we envisioned that plant-specific leaf-movement factors could be useful as a herbicide. When we use such leaf-movement factor as a herbicide, it would be effective only for the legume from which it was isolated, and have no effect on other vicinal plants.

Nyctinasty is also observed in *Leucaena leucocephalam* (gin-nemu in Japanese), a leguminous tropical plant. *L. leucocephalam* is known for its rapid growth and secretion of allelochemicals to inhibit the growth of other plants around them, which leads to a grove of that plant and elimination of vicinal plants.[5] Thus, the disruption of an ecosystem by *L. leucocephalam* is a serious problem. We have tried to develop a herbicide, which is specifically effective for *L. leucocephalam*, by using the leaf-movement factor of this plant. This new type of highly ecological herbicide will exterminate only *L. leucocephalam* and, on the other hand, have no effect on other plants. Thus, we tried to identify the leaf-movement factors of *L. leucocephalam*. In this paper, we describe the isolation, chemical structure, and biological activity of the leaf-closing substance of *L. leucocephalam*.

Isolation of the leaf-closing substance was carried out based on a bioassay using a leaf of *L. leucocephalam*. The bioactive fraction closed a leaf within a few hours after the addition of a solution containing the bioactive substance.

The fresh whole plant of *L. leucocephalam* (13.0 kg) which was collected in Okinawa, Japan, was extracted with MeOH (72 L) for 1 week and concentrated in vacuo. The concentrated extract was partitioned with *n*-hexane, ethyl acetate, then with *n*-butanol. The bioactive aqueous layer was carefully separated by Amberlite XAD-7 column chromatography eluted with MeOH–H_2O (0:100, 10:90, 30:70, 50: 50, and 100:0), and the H_2O fraction showed weak leaf-opening activity. This H_2O fraction was further purified with column chromatography using TSK G3000S gel with 30% EtOH aq. In this step, we were able to separate the fraction with leaf-opening activity and another one with leaf-closing activity. The fraction with leaf-closing activity was purified by HPLC using a preparative

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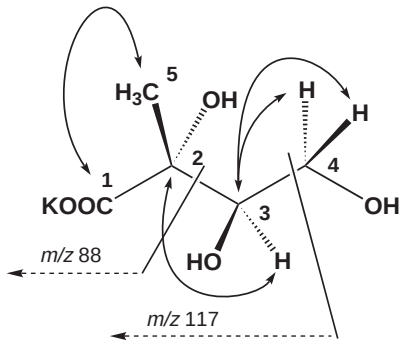

Figure 1. HMBC correlations and daughter ions observed in a linked-scan experiment of potassium 2,3,4-trihydroxy-2-methylbutanoate (**1**).

Develosil ODS-HG-5 column with 10% MeOH aq., and then Develosil ODS-HG-5 with 5% MeOH aq. to give a mixture (1.7 mg) of potassium lactate and potassium 2,3,4-trihydroxy-2-methylbutanoate (**1**) in the ratio of 1:2. We carried out further purification of **1** with HPLC with plural ODS columns, which also gave a mixture of the same compounds. Then, it was found that compound **1** was unstable around neutral or basic pH, and gradually decomposed to give lactate, which was identified by negative-mode FAB MS and ^{1}H NMR spectra. Then, we tried to isolate **1** by HPLC using a combination of two Develosil ODS-SR-5 columns under acidic conditions (5% MeOH aq. containing 0.5% TFA). Under this condition, we obtained **1** as a free acid form and the corresponding γ-lactone, 2,3-dihydroxy-2-methyl-4-butanolide (**2**), which was produced from **1** under acidic condition, as a mixture of 1:1. Despite many trials, further purification of **1** was unsuccessful.[6] However, isolated potassium lactate showed no leaf-closing activity against a leaf of *L. leucocephalam*; thus, co-existing **1** was assumed to be the genuine leaf-closing substance.

We carried out a bioassay using a mixture of **1** and lactate in a ratio of 2:1 which was dissolved in a 0.1 mM phosphate buffer solution at pH 5.4. From the content of **1** in this 2:1 mixture, **1** was estimated to be effective for the leaf-closing of *L. leucocephalam* at 1×10^{-6} M. On the other hand, corresponding γ-lactone (**2**) showed no leaf-closing activity even at 1×10^{-4} M.

The structural determination of **1** was carried out by means of NMR and FAB MS experiments.[7] HMQC and HMBC experiments gave the planar structure of **1**. A strong molecular ion corresponding to **1** was observed at *m*/*z* 189 in the positive mode FAB mass spectrum of bioactive fraction, on the other hand, *m*/*z* 149 was observed as a molecular ion in negative mode FAB MS experiment, which suggested that **1** exists as a potassium salt. Daughter ions observed in a linked-scan experiment are also shown in Fig. 1.

The relative stereochemistry of **1** was determined by using γ-lactone (**2**),[8] which was synthesized from a mixture of **1** and **2** by treatment with 0.01 M HCl (Fig. 2). 2,3-Dihydroxy-2-methyl-4-butanolides of *syn* and *anti* relative stereochemistry were synthesized by Kobayashi et al.[9] Thus, comparison of the ^{1}H NMR data of **2** with those reported by Kobayashi et al.[9] suggested the relative stereochemistry of **2** as shown in Fig. 2. And an NOE correlation observed in **2** confirmed this result (Fig. 2). Thus, the relative stereochemistry of **1** was determined as shown in Fig. 1.

We are now examining the effect of leaf-closure with **1** on the survival of the *L. leucocephalam*. On the other hand, we have observed the leaf-opening activity in a fraction separated by the column chromatography using a TSK G3000S gel, and the isolation of the leaf-opening substance is now in progress. Either (or both) of these leaf-movement factors would be a potential herbicide of highly selective action against *L. leucocephalam*.

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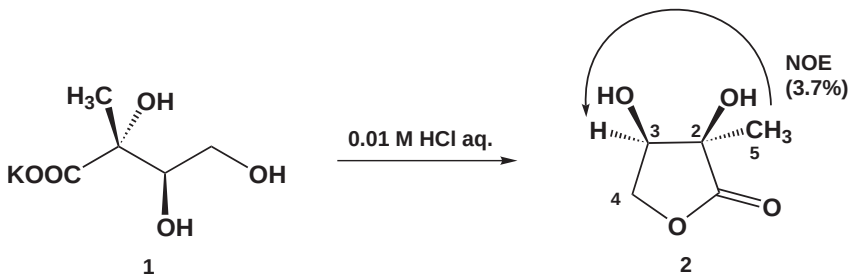

Figure 2. The preparation of γ-lactone (**2**) and the determination of stereochemistry in potassium 2,3,4-trihydroxy-2-methylbutanoate (**1**).

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6. No example of the isolation of **1** has been reported. Only the detection of **1** in plant extract using GC–MS was reported in the following reference: Kringstad, R.; Singsaas, A. O.; Rusten, G.; Baekkemoen, G.; Paulsen, B. S.; Nordal, A. *Phytochemistry*, **1980**, *19*, 543–545.
7. The NMR and MS experiments were carried out on a mixture of **1** and potassium lactate. Potassium 2,3,4-trihydroxy-2-methylbutanoate (**1**): ^{1}H NMR (400 MHz, D_2O, rt): 3.80 (1H, t, $J=5.5$ Hz, H_3), 3.57 (2H, d, $J=5.5$ Hz, H_4), 1.34 (3H, s, Me) ppm.; ^{13}C NMR (100 Mhz, D_2O, rt): 183.0 (C_1), 80.0 (C_2), 78.6 (C_3), 65.0 (C_4), 25.0 (Me) ppm.; FAB-MS (negative): $[M-H]^-$ m/z 149; FAB-MS (positive): $[M+H]^+$ m/z 189.
8. 2,3-Dihydroxy-2-methyl-4-butanolide(**2**): ^{1}H NMR (400 MHz, D_2O, rt): 4.58 (1H, dd, $J=3.7$, 11.0 Hz, H_{4a}), 4.29 (1H, d, $J=11.0$ Hz, H_{4b}), 4.23 (1H, d, $J=3.7$ Hz, H_3), 1.45 (3H, s, H_5) ppm.
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