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TRIGONELLINE, A LEAF-CLOSING FACTOR OF THE NYCTINASTIC PLANT, *AESCHYNOMENE INDICA*

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Key Word Index—*Aeschynomene indica*; Leguminosae; betaine; trigonelline; nyctinasty; leaf-closing factor.

Abstract—Trigonelline was isolated from *Aeschynomene indica* as a bioactive substance for nyctinasty. The compound was quite effective for leaf-closing of this species at 1×10^{-7} M in the daytime, but not for the nyctinastic species *Cassia mimosoides* and *Mimosa pudica*. It competed with indole-3-acetic acid (IAA) which is effective for leaf-opening. These results suggest that trigonelline may be concerned with the circadian rhythm of *A. indica*.

INTRODUCTION

Nyctinastic plants, such as *Mimosa pudica* or *Cassia mimosoides*, are well known for the movement of their leaves according to a circadian rhythm. Great efforts have been made to understand this mechanism. In 1916, Ricca [1] suggested that the movement of the plant was controlled by some bioactive substances. Subsequently, Schildknecht *et al.* isolated chemical substances which had leaf-closing activities for many nyctinastic plants [2, 3], e.g. *M. pudica* and *Acascia karoo*. He named these compounds turgorins and insisted that they were a new class of phytohormones for controlling the circadian rhythm of nyctinastic plants.

Recently, however, we have succeeded in isolating potassium chelidonate (**1**) from *C. mimosoides* and *C. occidentalis*, which is considered to be a genuine leaf-closing factor of these species [4]. Furthermore, potassium lespedezate (**2**) and potassium isolespedezate (**3**) were isolated from the nyctinastic plant, *Lespedeza cuneata*, as a leaf-opening substance. We have pursued leaf-closing and leaf-opening factors of other nyctinastic species, and the results obtained strongly suggest that distinct leaf-closing and leaf-opening substances exist in each nyctinastic species [5, 6]. Herein, we report the isolation of trigonelline (**4**) as a leaf-closing factor of a nyctinastic plant, *Aeschynomene indica*.

RESULTS AND DISCUSSION

Fresh whole plants of *A. indica* were extracted with methanol for two weeks and then carefully separated as described in the Experimental. Purification was carried out by monitoring for leaf-closing activity using two methods of bioassay. The mimosa test [6] and the *Aeschynomene* test. The isolated bioactive substance was identified as trigonelline (**4**) by comparison with an authentic sample. All the data of our isolated sample, such as ^{1}H NMR, FAB-mass spectrum, CID (collision-induced decomposition) spectrum, and retention time on HPLC analysis were in agreement with the authentic sample.

Trigonelline was quite effective for leaf-closing of the plant at 1×10^{-7} M in the daytime (Table 1). An authentic sample showed exactly the same activity. Trigonelline was effective only for leaf-closing of *A. indica* and not active on the other nyctinastic species, *C. mimosoides* and *M. pudica*. Compound **1**, the leaf-closing substance of *C. mimosoides*, was not effective on *A. indica* even at 1×10^{-2} M. As already reported [6], **1** showed no leaf-closing activity when the K^+ ion was displaced by another metal ion, such as Na^+, Mg^{2+} or Ca^{2+}. However, **4** showed no leaf-closing activity on *C. mimosoides*, regardless of the presence of K^+ ion. Thus, **4** is considered to be a new type of leaf-closing factor which does not require any K^+ ion.

Structurally related analogues of **4** were tested for leaf-closing activity. Potassium *m*-toluate (**5**), which lacks a positive charge on the nitrogen atom, and 1-methylnicotinamide chloride salt (**6**), which lacks a carboxylate anion, were ineffective for leaf-closing even at 1×10^{-2} M. Thus, the zwitterionic structure of **4** is required for its activity.

The interaction between **4** and indole-3-acetic acid (IAA), which is known to induce leaf-opening of nyctinastic plants [7–9] is shown in Table 2. When the leaves of *A. indica* were treated with IAA ($> 10^{-3}$ M), they stayed open at night even in the presence of **4**. However, when the concentration of **4** was higher than that of IAA, leaves

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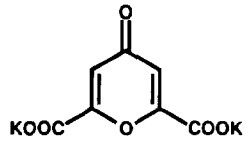
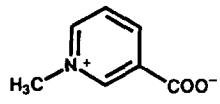
Leaf-closing substnces

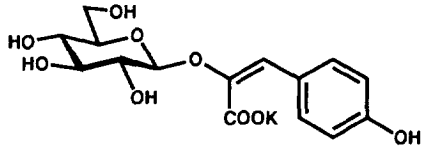


Potassium dicheridonate (1)

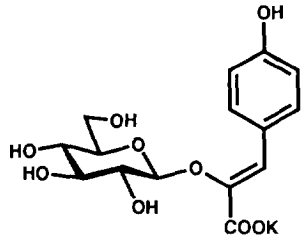


Trigonelline (4)

Leaf-opening substances

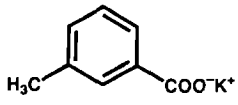


Potassium lespedezate (2)

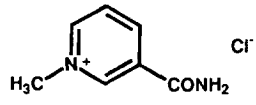


Potassium isolespedezate (3)

Analogs of trigonelline (4)



Potassium *m*-toluate (5)



1-Methylnicotinamide chloride salt(6)

Table 1. Leaf-closing activity of trigonelline (4)

Concentration (M)	Day	Night
1×10^{-3}	– –	– –
1×10^{-4}	– –	– –
1×10^{-5}	– –	– –
1×10^{-6}	– –	– –
1×10^{-7}	– –	– –
1×10^{-8}	+ +	– –

+ + Completely open; + nearly open; – nearly closed; – – completely closed.

Table 2. Interaction between trigonelline (4) and IAA

Trigonelline (M)	IAA (M)	Daytime	Night
—	1×10^{-3}	+ +	+ +
—	1×10^{-4}	+ +	+
—	1×10^{-5}	+ +	– –
3×10^{-3}	1×10^{-3}	+ +	– –
1×10^{-3}	1×10^{-3}	+ +	+
3×10^{-4}	3×10^{-4}	–	– –
3×10^{-4}	1×10^{-4}	– –	– –
1×10^{-4}	1×10^{-3}	+ +	+
1×10^{-4}	3×10^{-4}	+ +	– –
3×10^{-5}	1×10^{-5}	– –	– –
1×10^{-5}	1×10^{-4}	–	– –
1×10^{-5}	3×10^{-5}	–	– –
1×10^{-5}	1×10^{-5}	–	– –

+ + Completely open; + nearly open; – nearly closed; – – completely closed.

remained completely closed in the daytime. It is possible that **4** controls the nyctinasty of *A. indica*, together with some leaf-opening factor competing with **4**, as seen in the case of **1** [6], which competes with IAA, as well as with **2** and its geometrical isomer (**3**).

Two different leaf-closing factors from two nyctinastic species *C. mimosoides* and *A. indica* have now been isolated. This strongly suggests that various leaf-closing substances exist individually in each nyctinastic species. However, leaf-closing substances should be identified from various nyctinastic plants together with leaf-opening substances in order to confirm this theory. In addition, we suggest that circadian rhythms are at-

tributable to a balance between a leaf-closing substance and leaf-opening one, controlled by an internal clock.

EXPERIMENTAL

General. HPLC was carried out with UV, monitoring at 260 nm; sizes of the columns were ϕ 20 × 250 mm (Cosmosil 5C18AR), ϕ 4.6 × 250 mm (Develosil ODS HG5), and ϕ 6.0 × 250 mm (Inertsil ODS-2). ^{1}H NMR (400 MHz) were recorded in D_2O with *t*-BuOH as int. standard (1.23 ppm). FAB-MS and CID-MS were recorded using glycerol as matrix. CID-MS was performed with Ar as collision gas at − 29 eV.

Plant material. The extracted plant, *A. indica*, was collected at Toyota, Japan in August 1993. The plant used for bioassay of leaf-closing was grown in the greenhouse at Keio University at 23 to 33° for several months.

Bioassay. Young leaves were detached from the stem of *A. indica* with a razor blade. Single leaves were immersed in distilled H_2O (*ca* 1 ml) in a 20-ml glass tube in the greenhouse and allowed to stand overnight. Leaves which opened again the next morning (*ca* 10:00) were used for the bioassay. Each test soln (*ca* 100 ml) was carefully poured into the test tubes by a microsyringe around 11:00. The reaction time depended on the concn of active substance, the minimum amount of which was judged by leaf-closing in a few hr.

Extraction and isolation. Fr. whole plants (7.2 kg), were extracted with MeOH (30 l) at room temp. for *ca* 2 weeks. The extract was condensed and centrifuged (2800 rpm, 30 min). The supernatant was partitioned with EtOAc (2.5 l) and then with *n*-BuOH (3 l). The aq. layer possessed leaf-closing activity for *A. indica*. Bioassay of each fr. was carried out using the mimosa and *Aeschynomene* tests. The aq. layer was chromatographed on an Amberlite XAD-7 column (ϕ 5.5 × 63 cm), eluted with H_2O–MeOH (10:0, 9:1, 8:2 and 1:1); the H_2O fr. exhibited leaf-closing activity. The bioactive fr. was sepd by gel filtration using Cellulofine GC-15m column (ϕ 5.5 × 65 cm) eluted with H_2O–MeOH (10:0, 9:1, 8:2 and 1:1); the H_2O fr. exhibited leaf-closing activity. The bioactive fr. was sepd by gel filtration using Cellulofine GC-15m column (ϕ 5.5 × 65 cm) eluted with H_2O, and then HPLC using a Cosmosil 5C18AR column eluted with 5% aq. MeOH (flow rate 4 ml min^{-1}). Further purification by Sephadex G-10 CC (ϕ 3.2 × 54 cm using H_2O as eluent), followed by HPLC on Cosmosil 5C18AR column eluting with 3% aq. MeOH gave the active fr. (87 mg). This was purified further by HPLC on a Cosmosil 5C18AR column eluting with 1% aq. MeOH to give another active fr. (14 mg). Final purification was carried by HPLC using a combination of three columns (Develosil ODS HG5 + Inertsil ODS-2 × 2) to give **4** (2 mg), as a white powder.

Compound **4**. ^{1}H NMR (400 MHz, D_2O): δ9.10 (*br, s*, H-2), 8.83 (*d*, J = 8 Hz, H-4), 8.80 (*d*, J = 6 Hz, H-6), 8.06 (*dd*, J = 6 and 8 Hz, H-5), 4.42 (*s*, N-Me). FAB-MS (positive *m/z*: 138.0 $[M + H]^+$. UV–VIS spectrum (H_2O) nm: λ_{max} (ε) 264 (2700), 207 (4300). Spectral data and biological activity were identical to an authentic sample.

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