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Note

Synthesis of Anti-Tumor Dimeric Indole Alkaloids in *Catharanthus roseus* Was Promoted by Irradiation with Near-Ultraviolet Light at Low Temperature

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We have found that coupling between catharanthine and vindoline occurs non-enzymatically in the presence of flavin mononucleotide and manganese ions with near-ultraviolet light irradiation *in vitro*. The present study found that the concentrations of catharanthine and vindoline in *Catharanthus roseus* decreased and those of dimeric indole alkaloids increased under near-ultraviolet light at 4 °C. It indicates that this coupling reaction at 4 °C occurs non-enzymatically.

Key words: FMN; near-ultraviolet light (NUV); *Catharanthus roseus*; dimeric indole alkaloids (DIAs)

Dimeric indole alkaloids (DIAs) produced by *Catharanthus roseus*, including vinblastine, anhydrovinblastine, and leurosine (Fig. 1), have antineoplastic activities,[1)] but these alkaloids are very expensive because of their low concentrations in plants. Hence, the biosynthetic pathway of DIAs has been investigated actively. DIAs were synthesized by a coupling reaction between catharanthine and vindoline with oxidization of catharanthine as the first step. Kutney *et al.* have proposed that this initial step is catalyzed by enzyme including peroxidase.[2)] In contrast, we have found that the coupling reaction occurred *in vitro* in the presence of FMN and Mn^{2+} and in the absence of any enzymes after irradiation by near-ultraviolet light (NUV), with a peak at 370 nm.[3,4)] Moreover, we also found that the amount of DIAs in the leaves of multiple shoot cultures and intact plants specifically increased with NUV irradiation.[4,5)] This study was the first step to determine whether the coupling reaction occurs non-enzymatically *in vivo*. We analyzed the concentrations of alkaloids in plant leaves after NUV irradiation at 4 °C, a temperature at which the rate of enzymatic reactions was slow.

Catharanthus roseus was obtained following ref. 4 and grown outdoors in soil to 2 months of age. The leaves were sliced along the center vein to obtain two sections. Paired leaf sections were floated on water and irradiated with NUV (FL15BA-37K, Matsushita Electric, Tokyo) as treatment and white light (FL10ENW, Matsushita Electric) as the control for 1, 3, 5, and 7 d. Freshly harvested leaf sections were used as the 0 d control without any light treatment. After irradiation, the leaf sections were frozen in liquid N_2 and crushed. The crushed material was added to 500 µl of methanol and sonicated for 5 min. The mixture was centrifuged at 18,000 *g* at 4 °C for 5 min, and the supernatant was collected. These extraction steps were repeated at 3 times. Ten µl of the extract was subjected to HPLC as outlined below. HPLC analyses were performed following ref. 6 except for the following point: the mobile phase was acetonitrile/triethylamine/water (60/0.006/40). Detection of DIAs (anhydrovinblastine, leurosine, and vinblastine) was by fluorescence emission excited at 305 nm. One section was irradiated with white light (20 W/m^2) as a control. The other section was irradiated with NUV (5 W/m^2). The temperatures for light irradiation were set at 25 °C, at which both the enzymatic and non-enzymatic coupling reactions occurred, or at 4 °C. Changes in the alkaloid concentrations of *C. roseus* leaf sections irradiated with NUV at 4 °C and at 25 °C are shown in Fig. 2. At both temperatures, even at 4 °C, the concentrations of catharanthine and vindoline decreased and the total DIAs content increased after irradiation with NUV. Significant changes in the alkaloid concentrations at 4 °C were observed at 5 d after irradiation, but those at 25 °C were observed immediately. The catharanthine and vindoline concentrations as irradiated with NUV decreased as compared with white light after 7 d of irradiation at 4 °C and 25 °C respectively, whereas the concentrations of DIAs reached maximum at 5 and 7 d of irradiation at 4 °C and 25 °C respectively. NUV, with one-fourth the light intensity of white light, induced DIA synthesis.

The important point is that the coupling reaction occurred *in vivo* depending on the NUV at 4 °C. Additionally, two *in vitro* experiments based on the coupling reaction were performed at 4 °C. One was an enzymatic reaction due to horseradish peroxidase which is known as the coupling reaction *in vitro*, and the other was a non-enzymatic reaction that we have reported which occurred in the presence of FMN and Mn^{2+} under NUV light irradiation. These reactions *in vitro* were performed at the highest rate and yield. *In vitro*

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Abbreviations: NUV, near-ultraviolet light; DIAs, dimeric indole alkaloids

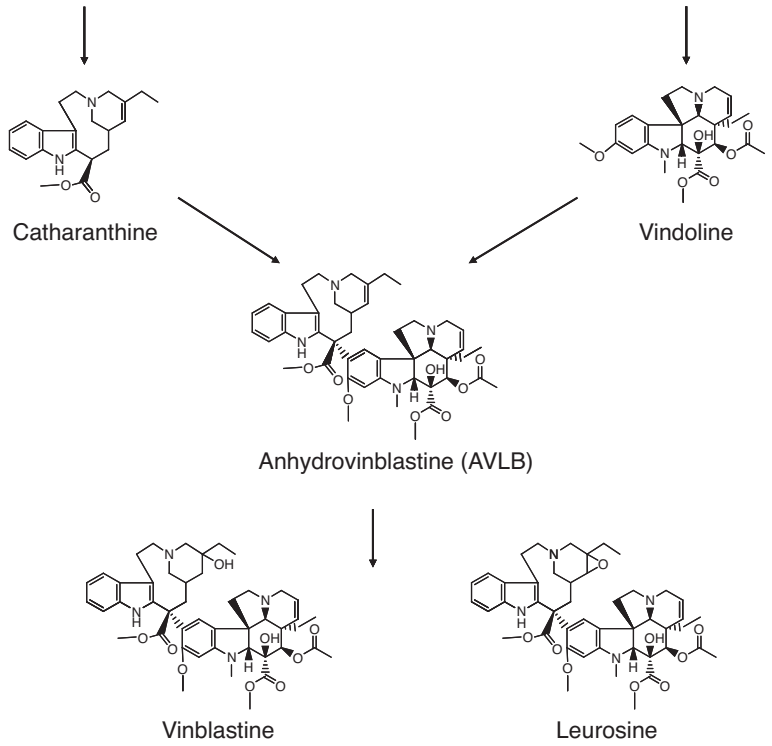

Fig. 1. Biosynthesis of DIAs (vinblastine, anhydrovinblastine, and leurosine) from Monomeric Precursors Catharanthine and Vindoline.

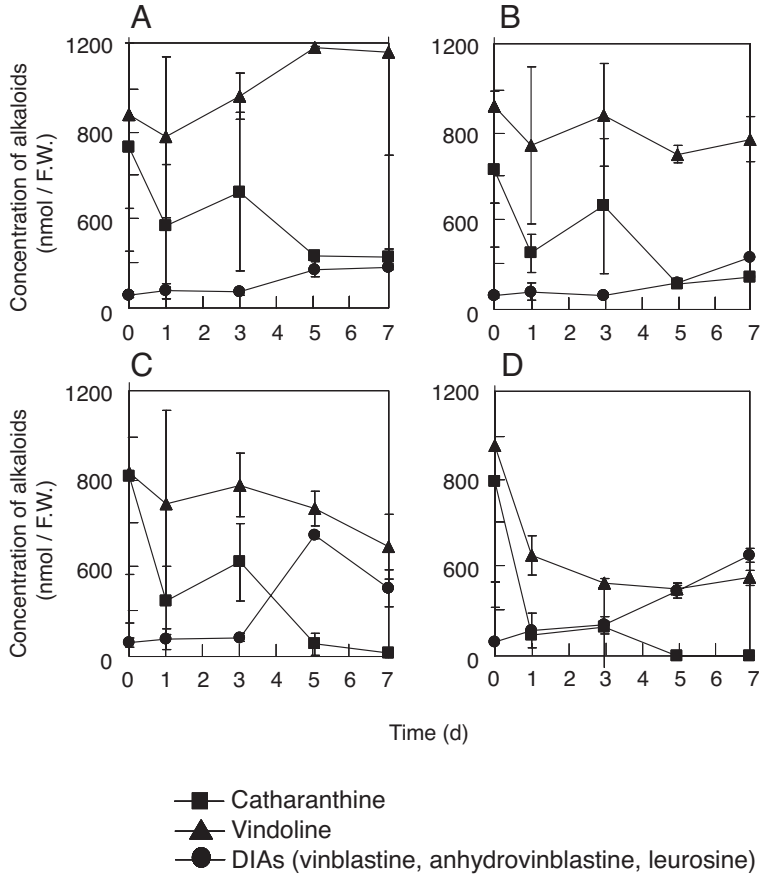

Fig. 2. Changes in Alkaloid Concentrations in Plant Leaves at 4 °C (A, C) and 25 °C (B, D) under Irradiation by NUV.
Leaf sections were irradiated with NUV as the treatment (C, D) and with white light as the control (A, B) for 1, 3, 5, and 7 d. Freshly harvested leaf sections were used as the 0 d control without light treatment.

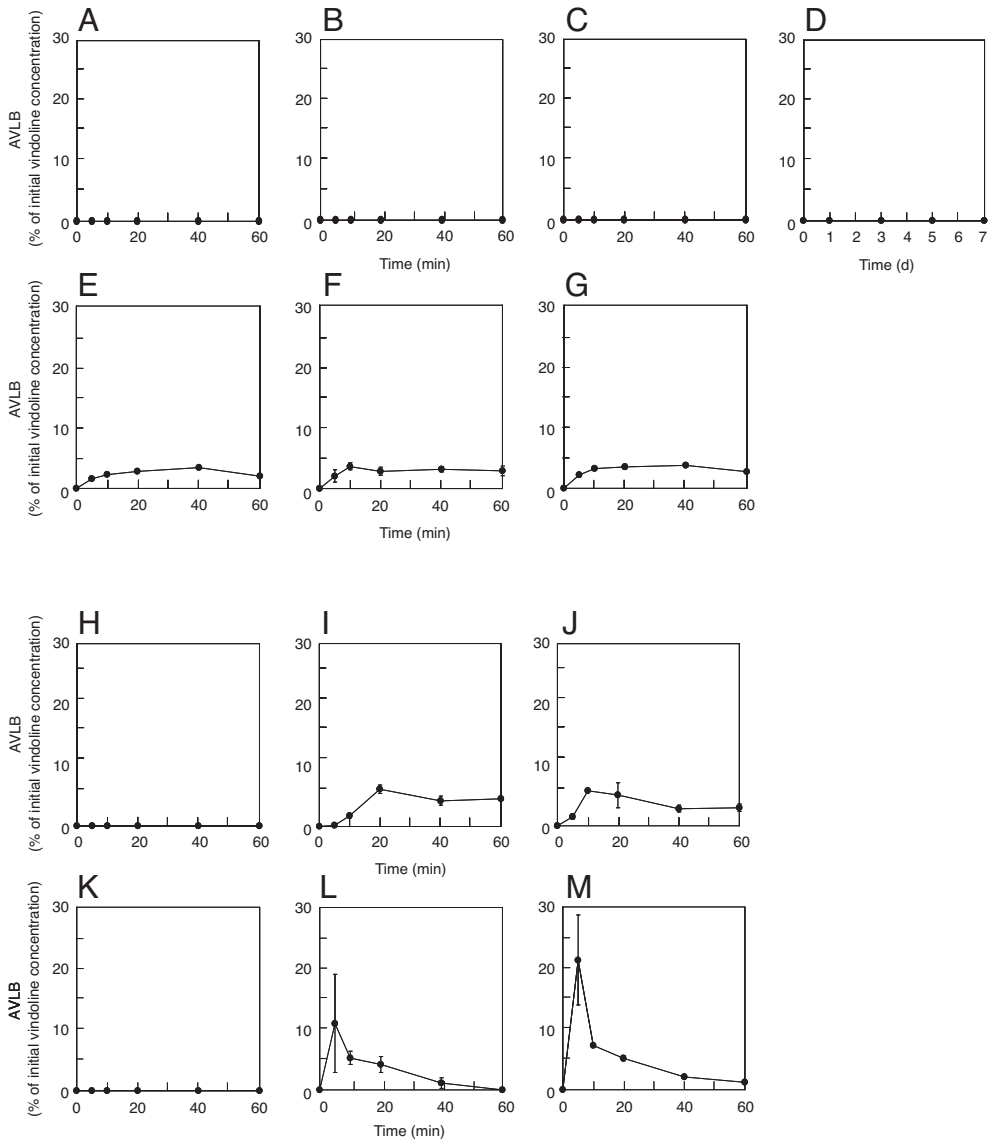

Fig. 3. Effects of Temperature and Light on the Enzymatic and Non-Enzymatic Coupling Reaction of Anhydrovinblastine.

Enzymatic reaction, vindoline and catharanthine (150 μM) were reacted in 0.1 M Tris–HCl buffer (pH 7.0) in the presence of 18 U/ml horseradish peroxidase, and 1 mM H_2O_2 under dark (A, D, E) and irradiation with white (B, F) or NUV light (C, G) at 4 °C (A, B, C, D) and 25 °C (E, F, G). Non-enzymatic reaction, vindoline and catharanthine (150 mM) were reacted in 0.1 M Tris–HCl buffer (pH 7.0) in the presence of 100 μM FMN and 1 mM $MnCl_2$ under dark (H, K) and irradiation with white (I, L) or NUV light (J, M) at 4 °C (H, I, J) and 25 °C (K, L, M).

enzymatic reactions were performed following ref. 6, except for the following: the reaction was initiated by adding 300 μl of 180 U/ml peroxidase (Amresco, Solon, OH). The non-enzymatic reaction was carried out using the reaction mixture containing 1 mM of $MnCl_2$ and excluding H_2O_2. The reaction was started by adding 30 μl of 10 mM FMN. The DIAs produced by *in vitro* coupling reactions were converted to AVLB by reduction of $NaBH_4$. Figure 3 shows the time-courses of AVLB production by the *in vitro* enzymatic and non-enzymatic coupling reactions. Catharanthine and vindoline of the enzymatic reaction at 25 °C and the non-enzymatic reaction at 4 °C and 25 °C did not occur at all at 60 min. The catharanthine and vindoline of the enzymatic reaction at 4 °C were still present at 60 min and reduced for 24 h, but AVLB was not synthesized. Although the enzymatic reactions occurred at 25 °C under both lighting conditions, the enzymatic reactions at 4 °C were suppressed completely. In contrast, the non-enzymatic reactions occurred at both temperatures under NUV and the rate of the coupling reaction increased at 25 °C, as did the yield of AVLB at 4 °C under irradiation of NUV. Our *in vivo* experiments indicated that NUV was required for the increase in DIAs levels at low temperature, whereas our *in vitro* experiments indicated that the non-enzymatic coupling reaction at low temperature proceeded by NUV irradiation while the enzymatic coupling reaction at low temperature did not proceed.

It is apparent that the enzymatic reaction was suppressed at 4 °C and that the synthesis of DIAs might have occurred non-enzymatically *in vivo*. To confirm this possibility, we should perform experiments that to relate *in vitro* and *in vivo* to determine whether this non-enzymatic reaction occurs *in vivo*. For example, we should verify that the peroxidase in *Catharanthus roseus* suppress activity at 4 °C as well as horseradish peroxidase does. There is no report that peroxidases *in vivo* increase the activity due to NUV at 4 °C, but we need to analyze the peroxidase activity in *Catharanthus roseus*. Also, we could not compare the light intensity of NUV

in vitro and *in vivo* because there are various pigments *in vivo*, and we suggest that NUV does not infiltrate *in vivo* as compared with *in vitro*. Moreover, it is necessary to verify whether the flavins *in vivo* promoted the coupling reaction. Flavins are the main absorbents of NUV, and the coupling reaction *in vivo* might relate to NUV-excited flavins or the flavin receptor, whereas there is a possibility that irradiation of NUV promotes the redox state and the coupling reaction might not depend on flavins. If the coupling reaction *in vivo* relates to flavins, the flavins might eliminate the oxidation energy generated by NUV in the cell by biosynthesis of DIAs.

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