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ISOLATION AND STRUCTURE ELUCIDATION OF A POTENT GROWTH INHIBITOR, HELIAN, FROM BLUE LIGHT-ILLUMINATED SUNFLOWER (*Helianthus annuus*) HYPOCOTYLS

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Abstract – A potent growth inhibitor was isolated from blue light-illuminated sunflower (*Helianthus annuus*) hypocotyls and identified as 8-(*β*-D-glucopyranosyloxy)-3-hydroxy-1,9,14-pentadecatriene-4,6-diyne (**1**, designated "helian") from analysis of its ^{1}H and ^{13}C NMR spectra, and HRFABMS. Helian **1** showed plant growth-inhibitory activity at concentrations higher than 2.6×10^{-6} mol/L for the cress root growth test. The amount of helian was higher in the illuminated halves than in the shaded ones in phototropically stimulated sunflower hypocotyls. These results suggest that this new potent growth inhibitor, helian, may play an important role on phototropism of sunflower hypocotyls.

INTRODUCTION

Plants, unlike animals, have survival mechanisms for responding sensitively to the environment around them since, once established, they are unable to modify their environment by moving to a different location. The phenomenon of plant seedlings bending toward light to optimize the exposure of their photosynthetic organs is called phototropism, and is a typical example of an environmental response found in plants. Sunflower (*Helianthus annuus*) seedling has been frequently used in studies on phototropic curvature.[1-6] The genus name *Helianthus* is derived from "helio" of sun and "anthos" of flower, and means that a flower

similar to the sun's shape opens toward sunlight. In 1928, using the *Avena* coleoptile curvature test,[7] Went found that more auxin diffuses out of the shaded than out of the illuminated side. This led to the formation of the Cholodny-Went theory:[8] the accumulation of auxin in the shaded side promoted the growth at that side, causing bending toward the illuminated side. Since then, this simple theory has been widely accepted. However, using spectro-fluorometric and immunological methods, two groups independently showed that sunflower seedlings revealed the absence of a lateral gradient in extractable or diffusable endogenous auxin.[4,9] Instead, the presence of a gradient of light-induced growth inhibitor(s) was suggested.[4] Since then, the search for light-induced growth inhibitor(s) has evolved. As light-induced growth-inhibitor(s), caprolactam[10] and 8-epixanthatin[6] were isolated and identified from light-grown sunflower seedlings. We have confirmed the presence of light-induced growth inhibitor(s) showing greater inhibitory activity than caprolactam and 8-epixanthatin during the process of the extraction of caprolactam and 8-epixanthatin. In this paper, we report isolation and identification of growth inhibitor from blue light-illuminated sunflower hypocotyls and its role in phototropism of sunflower hypocotyls.

RESULTS AND DISCUSSION

Phototropic curvature was measured under continuous, unilateral blue illumination. Figure 1 shows that the hypocotyls began to bend toward the light source from 20 min after the onset of phototropic stimulation. Figure 2 shows the HPLC chromatograms of the extracts from the illuminated and shaded

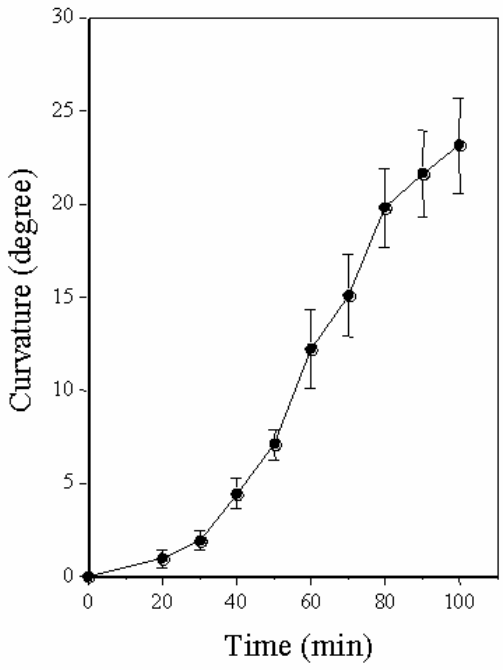

Figure 1. Time course of phototropic response of etiolated sunflower hypocotyls to continuous, unilateral blue illumination. Values are means of 20 seedlings ± SE.

halves of hypocotyls at 20 min after the onset of phototropic stimulation. Differences in some peaks were detected between the illuminated and shaded halves. Among them, at least three peaks (peaks A, B and C) showed growth-inhibiting activity for the cress root growth test. The least polar peak (peak C with retention time (Rt) of 7.7 min) showed the greatest inhibitory activity. Therefore, peak C (1.1 mg) was isolated from blue light-illuminated sunflower hypocotyls (3.2 kg fresh weight). The molecular formula, $C_{21}H_{28}O_7$, of the newly isolated **1** was established by the HRFABMS (pos.) [*m/z* 415.1717 calcd. for

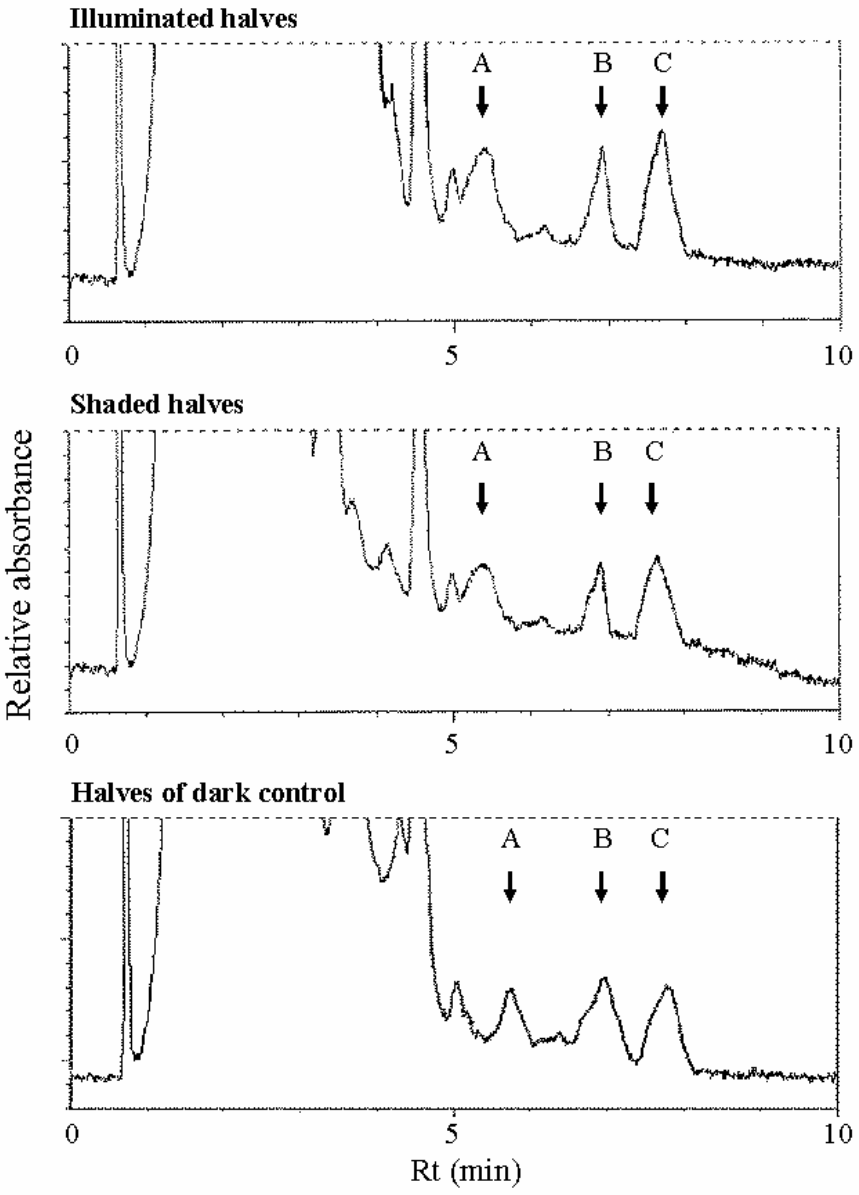

Figure 2. HPLC chromatograms extracts from illuminated and the shaded halves of sunflower hypocotyls at 20 min after onset of continuous, unilateral blue illumination, and in the other halves of dark control. The experiments were repeated three times.

$C_{21}H_{28}O_7Na$, 415.1732]. The ^{13}C NMR spectroscopic data indicated that the molecule possessed four acetylenic carbons at δ_C 80.2 (C-7), 76.5 (C-5), 75.8 (C-4), and 69.9 (C-6), six disubstituted olefinic carbons at δ_C 139.7 (C-14), 138.0 (C-1), 135.1 (C-10), 127.5 (C-9), 116.7 (C-2), and 115.2 (C-15), one

acetal carbon atδ_C 100.9 (C-1'), six oxymethines at δ_C 78.2 (C-4'), 78.1 (C-3'), 74.9 (C-2'), 71.6 (C-5'), 64.3 (C-8), and 63.8 (C-3), one oxymethylene carbon at δ_C 62.8 (C-6') and three methylenes at δ_C 38.8 (C-12), 34.3 (C-13), and 28.2 (C-11) (Table 1).

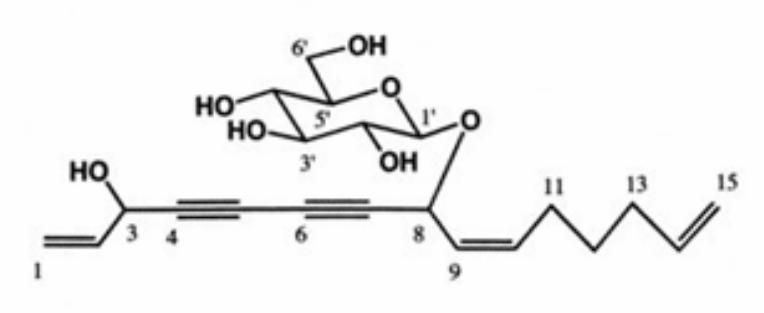

Figure 3. Chemical structure of compound **1**.

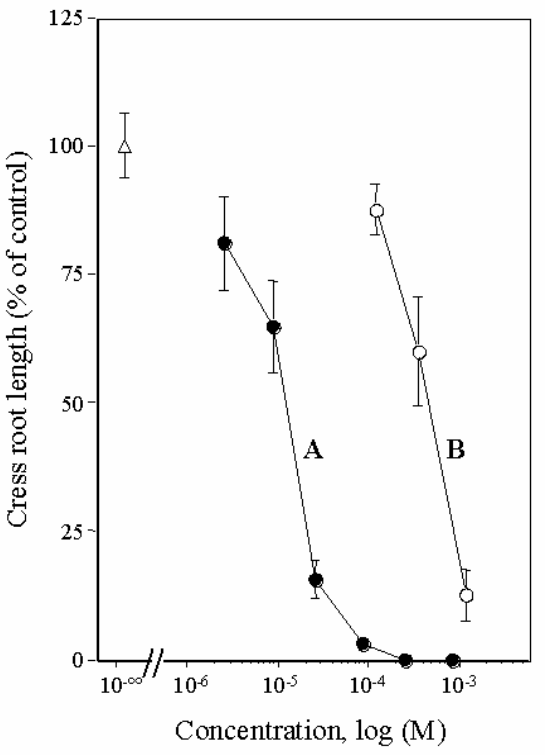

Figure 4. Effects of the isolated **1** and 8-epixanthatin on the cress root growth. A: isolated **1**, B: 8-epixanthatin, Control (open triangle). Each value is the mean of 10 seedlings ± SE. These experiments were repeated three times and the results were similar.

The ^{1}H NMR spectrum showed the presence of eight olefinic protons (Table 1). The Z-geometry of the double bond (C-9,10) was deduced on the basis of the ^{1}H–^{1}H coupling constant ($J_{9,10}$=7.8 Hz) between H-9 and H-10 and the NOESY correlation between H-9 and H-10. The ^{1}H-^{1}H COSY spectrum of **1** indicated the connectivities of C-1 to C-3, C-8 to C-15, and C-1' to C-6'. The sugar component was assigned to be β-glucopyranoside by NOESY correlations of H-1' to H-3' and H-5', and the ^{1}H–^{1}H coupling constants. An HMBC correlation between the anomeric proton (H-1') to C-8 (δ_C 64.3) revealed

that the glucose moiety was connected to C-8. On the basis of this spectroscopic data, the structure of **1** was elucidated as 8-(*β*-D-glucopyranosyloxy)-3-hydroxy-1,9,14-pentadecatriene-4,6-diyne (Figure 3, designated helian).

Biological activities of newly isolated **1** and 8-epixanthatin were tested in the cress root growth assay, which is frequently used to test plant growth inhibitory activity (Figure 4). **1** showed plant growth-inhibiting activity at concentrations higher than 2.6×10^{-6} mol/L, whereas above 1.2×10^{-4} mol/L, 8-epixanthatin inhibited cress root growth.

The concentration (EC_{50}) of samples, which cause 50% inhibition of the root growth of cress seedlings, was estimated from the dose-response curve. The EC_{50} data is shown in Table 2. The activity of **1** was

Table 1. ^{13}C and ^{1}H NMR chemical shifts (values from TMS) and multiplicities of compound **1**.

Atom	^{13}C (in CD_3OD, 150 MHz)	^{1}H (in CD_3OD, 600 MHz)
1(a)	116.7	5.44 (1H, *dd*, *J* = 17.4 and 1.2 Hz)
(b)		5.24(1H, *dd*, *J* = 10.2 and 1.2 Hz)
2	138	5.95 (1H, *ddd*, *J* = 15.6, 10.2 and 5.4 Hz)
3	63.8	4.88 (1H, *m*)
4	75.8	
5	76.5	
6	69.9	
7	80.2	
8	64.3	5.58 (1H, *d*, *J* = 8.4 Hz)
9	127.5	5.59 (1H, *dd*, *J* = 8.4 and 7.8)
10	135.1	5.67 (1H, *dt*, *J* = 7.8 and 7.2 Hz)
11	28.2	2.22 (2H, *m*)
12	38.8	2.19 (2H, *m*)
13	34.3	2.13 (2H, *m*)
14	139.7	5.87 (1H, *ddt*, *J* = 17.4, 10.2 and 7.2 Hz)
15(a)	115.2	5.06 (1H, *dd*, *J* = 17.4 and 1.8 Hz)
(b)		4.99 (1H, *dd*, *J* = 10.2 and 1.8 Hz)
1'	100.9	4.64 (1H, *d*, *J* = 7.8 Hz)
2'	74.9	3.24 (1H, *dd*, *J* = 8.4 and 7.8 Hz)
3'	78.1	3.43 (1H, *dd*, *J* = 10.8 and 8.4 Hz)
4'	78.2	3.32 (1H, *m*)
5'	71.6	3.30 (1H, *m*)
6'(a)	62.8	3.93 (1H, *dd*, *J* = 11.7 and 1.8 Hz)
(b)		3.71 (1H, *dd*, *J* = 11.7 and 6.0 Hz)

about 38-fold higher than that of 8-epixanthatin. The EC_{50} of caprolactam is estimated from the literature,[10] although its activity was determined using the cress hypocotyl growth test. Comparing with **1** and 8-epixanthatin, caprolactam exhibited very lower activity and its EC_{50} is 3.3×10^{-3} M. The distribution of endogenous **1** in the illuminated and shaded halves of sunflower hypocotyls 20 min after the onset of continuous, unilateral blue illumination, was determined using physicochemical assay (Table 3). The amount of **1** in the illuminated half was 1.6 times larger than in the shaded half and dark control. Finally, together with the results obtained here, it is concluded that this newly isolated potent, growth inhibitor helian may play an important role in phototropism of sunflower hypocotyls, supporting the Bruinsma-Hasegawa theory.[11,12] That is, phototropism is caused by an unequal distribution of light-induced growth inhibitor(s) antagonizing auxin on the illuminated and the shaded sides, not by auxin. The isolation and identification of peaks A and B are in progress.

Table 2. Inhibitory activity of the isolated **1**, 8-epixanthatin and caprolactam.

Compounds	EC_{50}/M
Isolated **1**	1.2×10^{-5} a)
8-Epixanthatin	4.6×10^{-4} a)
Caprolactam	3.3×10^{-3} b)

a) EC_{50} represents the concentration of samples, which cause 50% inhibition of the root growth of cress seedlings.

b) The EC_{50} of caprolactam is estimated from the dose-response curve against cress hypocotyl growth test, obtained by Hasegawa et al. (1983).

Table 3. The content of endogenous **1** in the illuminated and the shaded halves of sunflower hypocotyls unilaterally exposed to blue light for 20 min, and the content in the halves of dark control. Average data from three independent experiments in duplicate.

	Illuminated half	Shaded half	Control half
1 (ng/half-hypocotyl)	10.4 ± 0.8	6.5 ± 0.1	6.4 ± 0.1

EXPERIMENTAL

Phototropic experiment

Sunflower (*Helianthus annuus*) seeds were spread evenly on moist vermiculite in tray and incubated at 25˚C in the dark for 3 days. The germinated seeds were transplanted under dim green light (0.01 μmol m^{-2} s^{-1}) in a row in small trays (6.4 × 14.5 × 3.5 cm) containing moist vermiculite and kept in the dark at 25˚C for 2 day. For phototropic stimulation, the rows of uniform etiolated seedlings were exposed to unilateral blue light (λ_{max}: 448 nm) for 100 min. Incident energy was 0.05 μmol m^{-2} s^{-1} at the plant level. The illumination was given over the whole length of the seedlings. The hypocotyl curvature was measured at 10-min intervals using an infrared-imaging system. Images of the seedlings were recorded throughout an experiment while being monitored in real time. Manipulations were carried out under dim green light. After the onset of phototropic stimulation, 65 phototropically stimulated hypocotyls were harvested at 0, 20, 30 and 60 min. Hypocotyl sections from 0 to 1.5 cm below the hook were excised and bisected into illuminated and shaded halves with a razor blade under dim green light. Bisected hypocotyls were immediately frozen in liquid N_2 and stored at -40˚C until use. Frozen materials were homogenized in acetone-H_2O (4:1, 50 mL) using a homogenizer. The filtered samples were evaporated to dryness *in vacuo* at 40˚C. The samples, dissolved in MeOH, were subjected to HPLC (TSK gel, ODS-100V, Tosoh, Japan, 4.6 × 150 mm, 35% CH_3CN in H_2O, 1 mL/min, detector at 205 nm). The distribution of endogenous **1** in the illuminated and shaded halves of sunflower hypocotyls 20 min after the onset of continuous, unilateral blue light illumination, and in the dark-kept control was performed by measuring the area of peak and calibrating. All experiments were repeated three times.

Isolation of growth inhibitors from blue light-illuminated sunflower hypocotyls

Five-day-old etiolated seedlings (hypocotyl length, ca. 3.5-4.0 cm) were cultured under blue light (λ_{max}: 448 nm, 0.05 μmol m^{-2} s^{-1}) for 30 min. The blue light-illuminated hypocotyls (3.2 kg fresh weight) were harvested, frozen in liquid N_2 and stored at -40˚C. The frozen samples were homogenized in 4 L of cold aqueous acetone (15:85) with a homogenizer. The filtered extract was evaporated to dryness *in vacuo* at 40˚C. The crude material was applied to C_{18} Sep-pak cartridge columns (Waters), and eluted with 0%, 20%, 40%, 60%, 80% and 100% MeOH in H_2O (40 mL per step). The 60% and 80% fractions (5.84 g), which contained the peaks A, B and C (Figure 2), were combined and repeatedly purified using C_{18} Sep-pak cartridge column. The 60% eluate (147.4 mg) which showed the highest inhibitory activity for the cress root growth test, was purified by HPLC (ODS-120A, Tosoh, Japan, 7.8 × 300 mm, 37% CH_3CN in H_2O, 2 mL/min, detector at 205 nm). The active eluate (Rt. 20-22 min, 11 mg) was further purified by HPLC (ODS-120A, Tosoh, Japan, 7.8 × 300 mm, 0-10 min; 37% CH_3CN in H_2O, 10-20 min; linear gradient from 37% to 60% CH_3CN in H_2O, 2 mL/min, detector at 205 nm). The retention time of peak C,

which showed the highest growth inhibitory activity, was 19-22 min. The eluate (2.5 mg) was finally purified by HPLC (ODS-120A, Tosoh, Japan, 7.8 × 300 mm, 35% CH_3CN in H_2O, 2 mL/min, detector at 205 nm). The retention time of 27.6-28.2 min was evaporated to dryness in vacuo at 40˚C to give 1.1 mg (**1**).

Spectrometric analyses

Optical rotation was measured on a DIP-370 (Jasco). IR spectrum was measured on a FT/IR-300 (Jasco) and UV spectrum on a UV-3100PC (Shimadzu). ^1H and ^{13}C NMR spectra were measured and recorded on a Varian Unity INOVA 600 spectrometer in CD_3OD at δ_H 3.35 ppm and δ_C 49.8 ppm were used as internal references for NMR spectra. FABMS were recorded on a JMS-SX102/GCG spectrometer. The molecular formula, $C_{21}H_{28}O_7$, of the newly isolated **1** was established by the HRFABMS (pos.) [m/z 415.1717 calcd. for C21H28O7Na, 415.1732]. $[\alpha]_D^{19}$ –15.4° (c 0.65, MeOH); UV (MeOH) λ_{max}: 232.0 (ε 4030), 267.0 (ε 2300), 284.0 (ε 2100); IR (KBr) ν_{max}: 3429, 2919, 2368, 2330, 1074 cm^{-1}. For the ^1H and ^{13}C NMR spectroscopic data of **1**, see Table 1. 8-Epixanthatin was isolated according to the method reported previously.[6] ^1H and ^{13}C NMR spectral data of isolated sample coincided with those reported in the literature (data not shown).

Bioassay

Ten cress (*Lepidium sativum* L.) seeds were placed on filter paper moistened with 0.5 mL of test solution in a 2.7-cm Petri dish. Petri dishes were kept in the dark at 25˚C for 26 hours, the root length was measured.

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