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LIGHT-INDUCED AUXIN-INHIBITING SUBSTANCE FROM SUNFLOWER SEEDLINGS

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Key Word Index—*Helianthus annuus*; Asteraceae; auxin-inhibiting substance; 8-epixanthatin; light-induced growth inhibition.

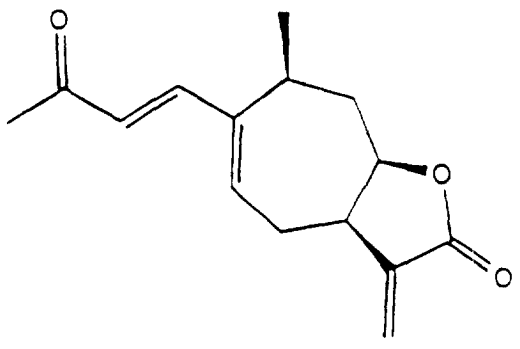
Abstract—A plant growth-inhibiting substance was isolated from de-etiolated sunflower (*Helianthus annuus*) seedlings. Its structure was identified as 8-epixanthatin from IR, ^{1}H NMR and EI mass spectra. 8-Epixanthatin was detected in sunflower and burweed shoots, but not in lettuce, radish, oat and corn. 8-Epixanthatin inhibited auxin-induced growth of sunflower hypocotyl and oat coleoptile sections at concentrations higher than 100 μM and 30 μM, respectively, and the elongation of cress roots at concentrations higher than 30 μM. However, it did not affect gibberellin-induced growth of lettuce hypocotyls and second leaf sheaths of rice at the various concentrations used. The light-grown seedlings were observed to contain a higher level of 8-epixanthatin than the dark control, and the difference in its level was noticeable before the growth inhibition by light appeared. These results suggest that 8-epixanthatin with its auxin-inhibiting activity might contribute to the light-induced growth inhibition of sunflower seedlings. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Phototropism is one of the common growth responses of plants to unilateral irradiation. There have been several cases of evidence that phototropism is caused by the uneven distribution of light-induced growth inhibitors [1], but the chemical nature of such compounds has not yet been elucidated. We have been investigating the relationship between plant growth inhibitors and growth inhibition by light in plants, and have isolated raphanusanin from radish hypocotyls and benzoxazolinones from maize coleoptiles as light-induced growth inhibitors [2–4]. In radish seedlings, raphanusanin causes growth inhibition through interference with the auxin-mediated orientation of microtubules [5]. On the other hand, two sesquiterpene lactones have been isolated from the leaves and stems of sunflower seedlings as auxin-inhibiting substances [6, 7]. Furthermore, xanthoxin and caprolactam have been isolated from sunflower seedlings, as light-induced plant growth inhibitors [8, 9]. We have confirmed the presence of other growth inhibitors in sunflower during the process of the extraction of xanthoxin and caprolactam, but they have not yet been isolated [9]. In the present study, we report on isolation and identification of another plant growth inhibitor in sunflower seedlings and its biological activities.

RESULTS AND DISCUSSION

A plant growth-inhibiting substance was isolated from de-etiolated sunflower seedlings. The EI mass spectrum of the substance exhibited $[M]^+$ at m/z 246, calcd for $C_{15}H_{18}O_3$. The ^{1}H NMR and IR spectra proved that it was 8-epixanthatin (**1**), which had already been isolated from *Xanthium* species [10–12], but its biological activity has not yet been studied. In the



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present study, its biological activity was tested in the cress root elongation test, in auxin bioassays with sunflower hypocotyls and oat coleoptile sections, and in gibberellin bioassays with lettuce hypocotyl and second leaf sheath of rice. Compound **1** inhibited the elongation of cress roots at concentrations higher than 30 μM ($t \geqslant t_{0.05}$), and also inhibited the auxin-induced growth of sunflower hypocotyl sections at concentrations higher than 100 μM ($t \geqslant t_{0.05}$), when evaluated by Students' t-test. Compound **1** also inhibited auxin-induced growth of oat coleoptile sections at concentrations higher than 30 μM ($t \geqslant t_{0.05}$) (Fig. 1). However, gibberellin-induced growth of lettuce hypocotyls and second leaf sheaths of rice was not significantly affected by **1** at the various concentrations

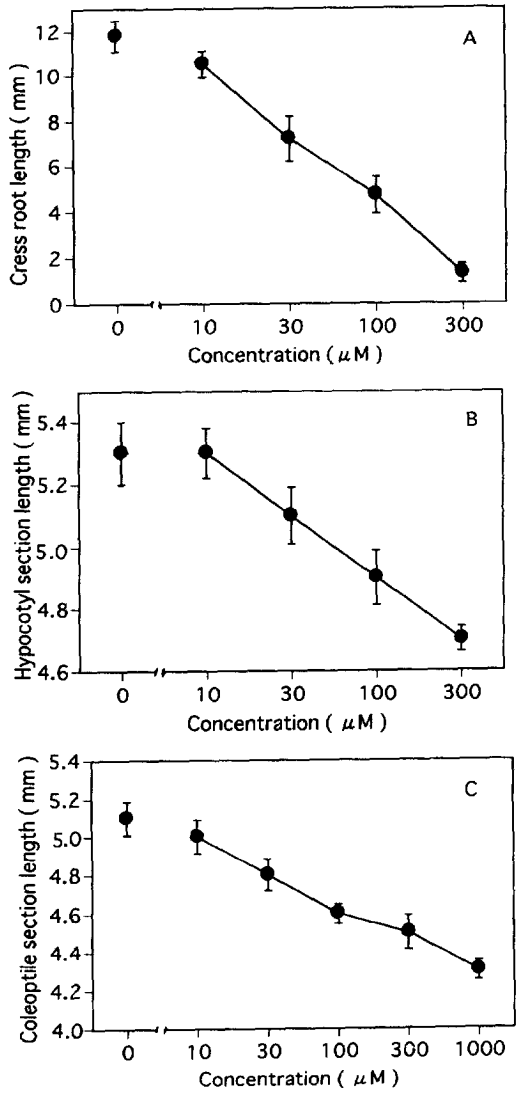

Fig. 1. A; Effect of 8-epixanthatin on the root elongation of etiolated cress seedlings (A), the auxin-induced growth of etiolated sunflower hypocotyl (B) and oat coleoptile sections (C). Each value is the average of 12 (A and B) and 10 measurements (C), respectively; bars indicate s.e.

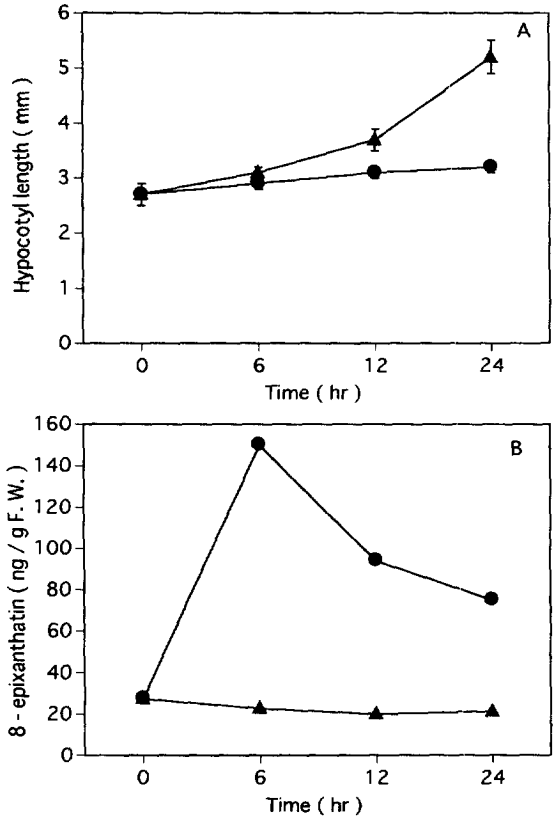

Fig. 2. Time courses in the hypocotyl growth of sunflower seedlings (A) and of the quantitative changes of 8-epixanthatin content in hypocotyls (B) during illumination. ●; light, ▲; dark. Bars indicate s.e.

used (data not shown). Figure 2 shows the time courses of the hypocotyl growth of sunflower seedlings and the quantitative changes of the content of **1** in hypocotyls during illumination. The light-grown seedlings contained a higher level of **1** than the dark control, and the difference in the amount of **1** was noticeable before the growth inhibition by light became apparent. Etiolated hypocotyl growth was strikingly inhibited with a lag of 12 hr after the onset of illumination [Fig. 2(A)]. The amounts of **1** were markedly increased at 6 hr after the onset of illumination, then gradually decreased but maintained high levels. In the dark, the contents of **1** remained at the initial level [Fig. 2(B)]. Exogenous concentrations of **1** required to inhibit growth of sunflower sections, are much higher than the endogenous level in light-grown sunflower hypocotyls. However, this does not necessarily mean that **1** does not act in plants as a growth inhibitor. Both the penetration of exogenous **1** and the distribution of endogenous **1** in the plant or the cell are not known. The occurrence of **1** in a variety of plants was also examined. Compound **1** was detected in sunflower and burweed shoots, but not in lettuce, radish, oat and corn.

These results suggest that **1** may play an important

role in light-induced growth of sunflower hypocotyls through suppressing auxin action [5–7].

EXPERIMENTAL

Extraction and isolation. Sunflower (*Helianthus annuus* L. cv. Taiyo, Sapporo Saishuen, Japan) seeds were imbibed for 4 hr in running tap H_2O and spread evenly on wet double-layered Whatman No. 2 filter paper in large trays. The seeds were then covered with two layers of filter paper moistened with H_2O and the trays covered with a pane and incubated at 25° in the dark for 1 day. The germinated seeds were transplanted into large trays containing vermiculite moistened with H_2O and incubated in the dark at 25° for 4 days. Etiolated seedlings were then transferred to white fluorescent light (1.65 μmol m^{-2} s^{-1}) and allowed to grow for 2 days at 25°. De-etiolated seedlings (hypocotyl length *ca* 5 cm) (10 kg fr. wt) were harvested and frozen at −40°. The frozen material was homogenized in 70% Me_2CO with a homogenizer and the homogenate filtered. The extract was reduced at 40° *in vacuo* to give an aq. concentrate. The aq. was adjusted to pH 7.5 with 1 M K_2HPO_4 and extracted ×3 with equal vols of EtOAc. The EtOAc was dried by anhydrous Na_2SO_4 and then evapd to dryness *in vacuo* to give 35.5 g crude material. The crude material was chromatographed on a column (3.5 × 36 cm) of silica gel (Wakogel C-300, Wako) with a *n*-hexane–EtOAc solvent system by increasing the EtOAc concn in a series of 10% steps (200 ml per step) and finally with MeOH (600 ml). Biological activities of the frs were determined in a cress root elongation test. Caprolactam was purified from the 100% MeOH [9]. Inhibitory activities were detected between 40–90% EtOAc in *n*-hexane frs and in the 100% MeOH fr. The former fr. was further chromatographed on a C_{18} Sep-pak cartridge (Waters) with MeOH–H_2O concn in a series of 20% steps (10 ml per step.) The active fr. was detected in 70–90% MeOH in H_2O. This crude extract was purified by HPLC (TSK gel ODS-80 Ts, ϕ21.5 × 300 mm, TOSOH, H_2O–MeOH, 1:1, 5 ml min^{-1}, 214 nm detector). The inhibitory activities were detected in frs with R_ts of 69–77 min and 82–88 min frs. The former fr. contained xanthoxin [8] and unknown growth substances. The latter fr. was purified by HPLC (YMC-Pack R and D ODS, ϕ20 × 250 mm, YMC, Inc., H_2O–MeOH, 11:9, 5 ml min^{-1}, 214 nm detector). An active eluate (R_t 85 min) was evapd to dryness *in vacuo* at 40°, giving 14.8 mg. The active substance was finally purified by TLC (silica gel), *n*-hexane–EtOAc (1:1). The active zone (R_f 0.53) was scraped off and eluted with EtOAc. The eluant was evapd to dryness *in vacuo* at 40°, giving 10.2 mg colourless needles.

Determination of endogenous 8-epixanthatin in light- and dark-grown sunflower hypocotyls. Uniform de-etiolated sunflower seedlings (hypocotyl length *ca* 3 cm) grown in vermiculite in the dark for 4 days at 25° were irradiated (16.0 μM m^{-2} s^{-1}) or kept in the dark at 25° during 1 day. About 30 mm hypocotyl sections below the hook of light- and dark-grown seedlings were harvested and frozen at −20°. The frozen materials were extracted and fractionated as described above to obtain the neutral. The crude material was purified by silica gel Sep-pak cartridge (Waters) with a *n*-hexane–EtOAc solvent system by increasing the EtOAc concn in a series of 10% steps (10 ml per step). The 40–90% fr. was further purified by C_{18} Sep-pak cartridge with MeOH–H_2O solvent system by increasing the MeOH concn in a series of 20% steps (10 ml per step). The 50–100% fr. was subjected to HPLC (TSK gel ODS-80 Ts, ϕ4.6 × 250 mm, TOSOH, H_2O–MeOH, 1:1, 1 ml min^{-1}, 214 nm detector). In this purification process, the recovery of **1** was 69.1–73.2%. The endogenous **1** was determined by measurement of the peak area (R_t, 19.0 min) and calculation of the recovery.

Occurrence of 8-epixanthatin in plants. Four-day-old etiolated lettuce, radish, oat and corn seedlings and 6-day-old etiolated burweed seedlings were grown under white fluorescent light (16 μM m^{-2} s^{-1}) for 1 day and their shoots were used as materials. The determination of the amounts of **1** was the same described above.

Bioassays of compound. Twelve cress (*Lepidium sativum* L.) seeds were placed in a 3 cm Petri dish containing test soln and incubated in the dark at 25° for 36 hr, and the lengths of the cress roots were measured. In the case of the sunflower hyptocotyl and the oat coleoptile section tests, 4.0 mm-sections of 4-day-old etiolated sunflower hypocotyls or oat coleoptiles were incubated in 1% sucrose soln (pH 5.6) containing 1 μM IAA and various concs of **1** at 25° for 6 hr. After the incubation, the length of each section was measured. In the gibberellin assay, lettuce seeds were incubated with H_2O containing 0.1 or 1 μM gibberellin (GA_3) and various concs of **1** in a 3 cm Petri dish at 25° for 2 days in the dark and further for 2 days under the light at 25°. In the rice second leaf sheath assay, rice seeds were spread evenly on wet filter paper in a tray for 2 days under the light at 25°. The germinated seeds were transplanted in a 3 cm Petri dish and incubated with H_2O containing 0.1 or 1 μM GA_3 and **1** for 2 days under the same condition. The lengths of lettuce hypocotyls or second leaf sheaths of rice were measured.

8-Epixanthatin (**1**). IR ν_{max} cm^{-1}: 1760 (γ-lactone), 1710, 1665, 1620, 1590 (C=C, C=O). EI MS *m/z*: 246 $[M]^+$ ($C_{15}H_{18}O_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 6.98 (1H, *d*, *J* = 16.0 Hz, H-2), 6.32 (1H, *d*, *J* = 3.0 Hz, H-13), 6.20 (1H, *dd*, *J* = 9.0 and 6.5 Hz, H-5), 6.14 (1H, *d*, *J* = 16.0 Hz, H-3), 5.57 (1H, *d*, *J* = 3.0 Hz, H-13), 4.65 (1H, *ddd*, *J* = 12, 8.5 and 2.5 Hz, H-8), 3.40 (1H, *m*, H-7), 2.83 (1H, *ddq*, *J* = 12, 7 and 6 Hz, H-10), 2.60 (1H, *ddd*, *J* = 14, 11.5 and 9 Hz, H-6β), 2.50 (1H, *ddd*, *J* = 14, 6.5 and 5 Hz, H-6α), 2.26 (3H, *s*, H-15), 2.19 (1H, *ddd*, *J* = 14, 6 and 2.5 Hz, H-9α), 1.91 (1H, *ddd*, *J* = 14, 12 and 12 Hz, H-9β), 1.18 (3H, *d*, *J* = 7 Hz, H-14).

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