Plant Physiol. (1986) 81, 976–979
0032-0889/86/81/0976/04/$01.00/0

Phototropism in Hypocotyls of Radish[1]

I. ISOLATION AND IDENTIFICATION OF GROWTH INHIBITORS, *cis*- AND *trans*-RAPHANUSANINS AND RAPHANUSAMIDE, INVOLVED IN PHOTOTROPISM OF RADISH HYPOCOTYLS

Received for publication December 11, 1985 and in revised form April 9, 1986

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ABSTRACT

Three growth inhibitors which might be involved in phototropism of Sakurajima radish (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) hypocotyls, were isolated as crystalline forms from light-exposed radish seedlings and identified as *cis*- and *trans*-raphanusanins and 6-methoxy-2,3,4,5-tetrahydro-1,3-oxazepin-2-one (designated raphanusamide). The *cis*- and *trans*-raphanusanins inhibited growth of etiolated radish hypocotyls at concentrations higher than 1.5 micromolar, raphanusamide at concentrations higher than 20 micromolar.

Recent evidence on the distribution of growth-regulating substances in phototropically stimulated, dicotyledonous plants is incompatible with the Cholodny-Went theory. This theory (3, 9) ascribes the differential growth at the illuminated and shaded sides of the bending organ to asymmetric distribution of auxin. Franssen *et al.* (5) and Macleod *et al.* (7) found that in different plant species the phototropic response is brought about by cessation of growth at the lighted side and not by acceleration at the shaded one. Furthermore, Bruinsma *et al.* (2), Thompson and Bruinsma (8), and Franssen and Bruinsma (4) demonstrated an equal distribution of endogenous indole-3-acetic acid in phototropically stimulated sunflower seedlings using the indolo-α-pyrone method, and an increase in neutral growth inhibitors, including xanthoxin, at the lighted side. Their findings support the hypothesis of Blaauw (1) that the phototropic response is caused by the local accumulation of growth inhibitor(s) at the lighted side, so that as regulating factor(s) in phototropism growth inhibitor(s) are to be considered rather than auxin.

It was found in our recent preliminary experiments that inhibiting activity for radish hypocotyl growth in the 50 to 60% ethyl acetate in benzene of silica gel column chromatogram was at the lighted sides of phototropically stimulated radish hypocotyls higher than at the shaded ones when etiolated radish seedlings were irradiated with a unilateral white fluorescent light (Fig. 1). This finding suggests that the growth inhibitors(s) in the above fraction may play a role in phototropism of radish hypocotyls. Thus, the growth inhibitor(s) are isolated from a large amount of light-exposed radish seedlings and identified from the data of their ^1H and ^{13}C NMR, mass, IR spectra, and NOE[2] experiments. Their biological activities are also reported.

[1] Partly supported by grants-in-aid for Scientific Research in 1983 to 1985 No. 58540430 to K. H. from the Japanese Ministry of Education, Science and Culture.

[2] Abbreviations: NOE, nuclear Overhauser effect; Rt, retention time.

MATERIALS AND METHODS

Plant Materials. Sakurajima radish seeds (*Raphanus sativus* var. *hortensis* f. *gigantissimus* Makino) were sown on filter paper moistened with water in large trays (37 × 60 × 14 cm^3) and grown in the dark for 2 d at 25°C. Etiolated seedlings were irradiated with white fluorescent light (2 $W \cdot m^{-2}$, Natural Daylight, Toshiba Corp.) for 2 d at 25°C. Ten kg green seedlings were harvested, washed with distilled H_2O, and frozen at −20°C.

Extraction. Frozen seedlings were homogenized in 40 L of cold 70% methanol in a homogenizer. The extract was filtered through Toyo No. 1 filter paper. The filtrate was concentrated *in vacuo* at 40°C to give an aqueous solution. The aqueous solution was added to 1/10 volume of 1 M phosphate buffer (pH 7.8) and partitioned with an equal volume of ethyl acetate three times. The ethyl acetate neutral fraction was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo* at 40°C.

Column Chromatography. The ethyl acetate fraction was chromatographed on a column (4 × 60 cm^2) of silica gel (Kieselgel, 70–230 mesh, Merck Corp.) with a benzene-ethyl acetate solvent system in a series of 10% steps (500 ml/step), followed with methanol (1 L). Biological activity was determined using radish hypocotyl growth assay as described in "Bioassay." The active fractions were detected in the 50 to 60% ethyl acetate in benzene and methanol fractions. However, as shown in Figure 1, growth inhibiting factor(s) which might be involved in phototropism of radish hypocotyls, was observed in the 50 to 60% ethyl acetate fraction; therefore, the 50 to 60% ethyl acetate fraction was evaporated to dryness *in vacuo* to give 4.5 g crude oil. It was chromatographed on a column (2 × 60 cm^2) of the same silica gel with chloroform. The active fraction was detected in 600 to 1000 ml of eluates. It was further purified with column chromatography (4 × 60 cm^2 silica gel, *n*-hexane-ethyl acetate) in a series of 10% steps (500 ml/step). The active fraction (40–70% ethyl acetate in *n*-hexane) was evaporated to dryness *in vacuo* to give 3 g crude oil.

TLC. It was purified on preparative TLC (Kieselgel 60 GF_{254} with 0.25 mm thickness from Merck Corp.) with *n*-hexane : ethyl acetate (1:1, v/v). The active zone (R_F 0.5–0.9) was scraped off and eluted with chloroform : methanol (1:1, v/v), giving on evaporation 2.3 g. It was further purified on TLC (Kieselgel 60 GF_{254}) with chloroform : acetic acid (95:5, v/v). The active zone (R_F 0.3–0.8) was scraped off, eluted with chloroform : methanol (1:1, v/v) and evaporated to dryness *in vacuo* to give 1 g yellow oil.

HPLC. It was finally purified by HPLC (Waters Associate System 500, Prep Pak-500/C_{18}, water:methanol, 3:1, v/v, 150 ml/min). Three active fractions were eluted between 10.42 and 16.20, 25.60 and 32.22, and 37.55 and 46.00 min. Each eluate was filtered through a 5 μm pore-size filter (LS-type, Millipore Corp.) and evaporated to dryness *in vacuo* at 40°C. Repeated

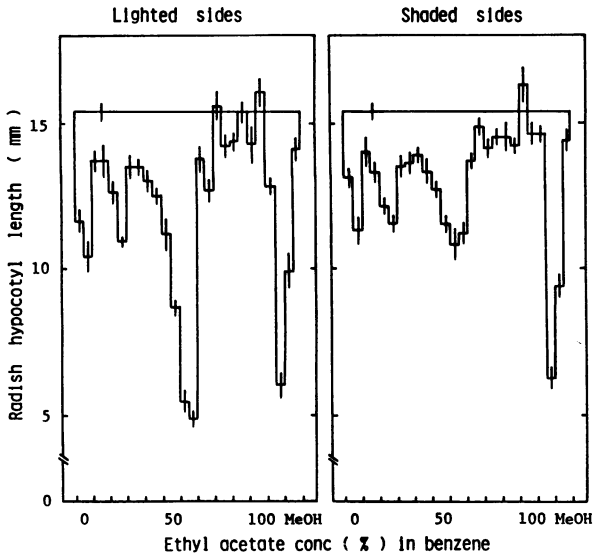

FIG. 1. Radish hypocotyl assays of column chromatograms loaded with extracts from the lighted and shaded halves of radish hypocotyls. Uniform 4-d-old etiolated radish seedlings were illuminated with a unilateral white fluorescent light (0.1 $W \cdot m^{-2}$) for 2 h. The hypocotyls of phototropically stimulated seedlings were bisected into the lighted and shaded sides with a razor under dim green light. The bisected hypocotyls (8 g fresh weight) each were extracted with 400 ml of 70% cold acetone, then fractionated with ethyl acetate to obtain the neutral fraction. This was chromatographed on a column (2 × 60 cm^2) of silica gel (Kieselgel, 70–230 mesh, Merck Corp.) by stepwise elution with benzene containing increasing proportions of ethyl acetate, followed by elution with methanol (200 ml/step). Biological activity was determined by the Sakurajima radish hypocotyl assay as described in "Materials and Methods."

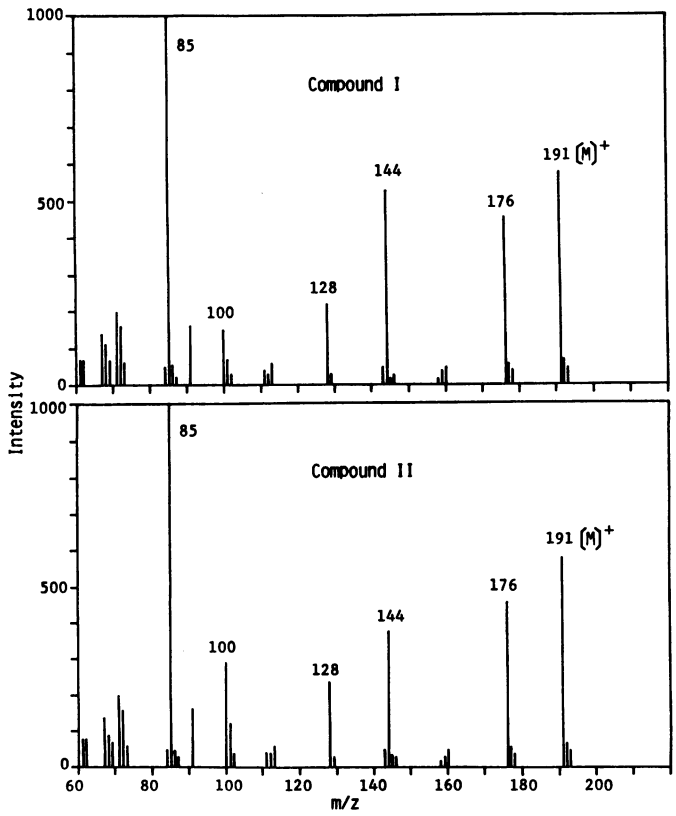

FIG. 2. Mass spectra of compounds I and II.

recrystallization from methanol-water gave 14, 35, and 65 mg of colorless needles. As a matter of convenience, they are named compound III (Rt 10.42–16.20 min, 14 mg), compound II (Rt 25.60–32.22 min, 35 mg), and compound I (Rt 37.55–46.00 min, 65 mg), respectively.

Spectrometric Analyses. IR spectra were obtained in KBr with JASCO IR spectrometer, UV spectra in ethanol with Hitachi 200-20 spectrophotometer. ^1H NMR spectra were recorded at 200 MHz with Hitachi R-900 spectrometer, ^{13}C NMR spectra at 22.6 MHz. Mass spectra were measured at 70 eV with JEOL JMS-D 300.

Bioassay. Ten uniform 2-d-old etiolated Sakurajima radish seedlings were placed on filter paper moistened with 1.3 ml of test solutions in a 4.5-cm Petri dish. Petri dishes were kept in the dark at 25°C for 1 d and then hypocotyl length was measured.

RESULTS AND DISCUSSION

Determination of Stereochemistry of Compounds I and II. Spectrometric data of compound I (Figs. 2–4) absolutely coincide with those of raphanusanin (6) which had previously been isolated as a new light-promoted growth inhibitor from light-exposed radish seedlings and identified as 3-methoxy-4-methylthio-2-piperithione. The mass spectrum of compound II (Fig. 2), was the same as that of compound I (raphanusanin). However, the IR and ^1H NMR spectra of compound II differed somewhat from those of compound I, though many signals were similar (Figs. 3 and 4). From the data of the mass, IR, and ^1H NMR spectra and spin decoupling experiments (data not shown), the planar structure of compound II is identified as 3-methoxy-4-methylthio-2-piperithione (raphanusanin). Therefore, compound II is an isomer of compound I.

Stereochemistry of —OCH_3 and —H at C-3, and/or —SCH_3 and —H at C-4 should be different between compounds I and II. The coupling constants of the H at C-4 coupled to the two H at C-5 were same 4 and 6 Hz in both compounds. The H at C-3 was coupled to the H at C-4, was not diaxial from its coupling constant of 2 Hz (J value for diaxial > 8 Hz). Thus the H at C-4 was not axial. The NOE experiments of compound I and II when SCH_3 was irradiated, gave valuable data to deduce the stereochemistry of them. In compound I, irradiation at δ1.80 (SCH_3) increased 5 to 6% the intensity of OCH_3, but did not change the intensity of H at C-3; —OCH_3 was equatorial. On the contrary the intensity of H at C-3 was increased 2 to 3% by irradiation at δ2.12 (SCH_3) in compound II, but did not change that of OCH_3 indicating that —H at C-3 was equatorial. The data of the NOE experiments showing change in the intensity of either OCH_3 or H at C-3 support also the idea that —SCH_3 was not equatorial. Thus, adopting a chair cyclohexane-like conformation in compounds I and II, they are proposed to be stereoisomeric structures shown in Figure 5; compound I is *cis*-raphanusanin, compound II *trans*-one.

Determination of Chemical Structure of Compound III. The IR spectrum (Fig. 6) revealed the presence of >NH (3170 cm^{-1}), —N—C— (1675, 1540, 1245, and 1135 cm^{-1}), —C=C— (1675 and 950 cm^{-1}), —OCH_3 (1340 cm^{-1}), —O— (1035 and 1025 cm^{-1}) and —C=C— (820 cm^{-1}). UV spectrum has $\lambda_{max}^{ethanol}$ 271 nm (ϵ = 7300). The mass spectrum (Fig. 7) gave m/z (relative intensity) 143 $[M]^+$ (100), 128 $[M-15]^+$ (29), 114 $[M-29]^+$ (20), and 100 $[M-43]^+$ (9). The ^1H NMR spectral assignments are given in Table I. Double triplet at δ2.80 (2H) and triplet at δ3.60 (2H) were assigned to two protons of methylene, respectively. Singlet at δ3.88 (3H) was assigned to three protons of a methoxyl

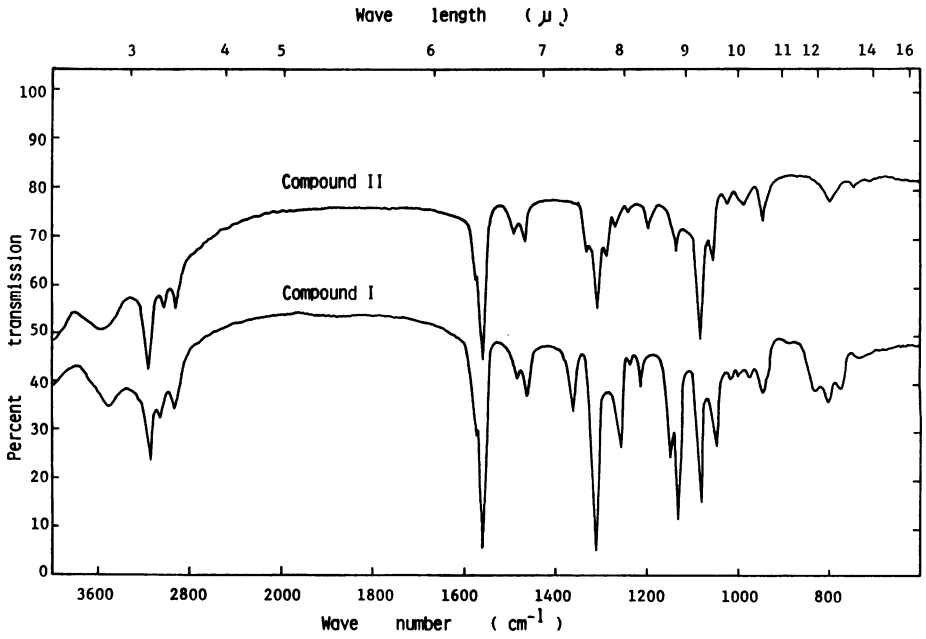

FIG. 3. IR spectra (KBr pressed disc) of compounds I and II.

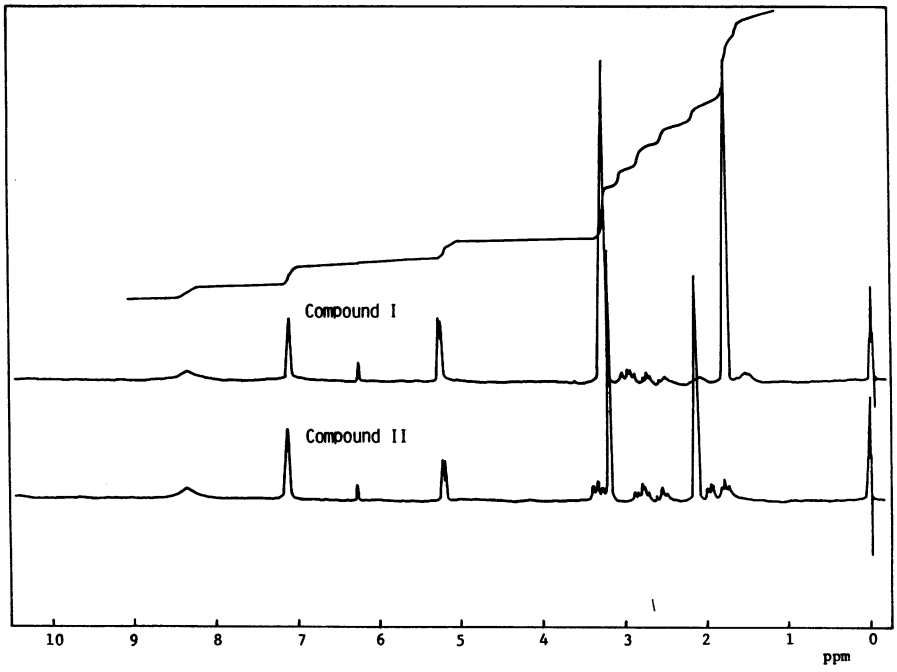

FIG. 4. ^1H NMR spectra (in C_6D_6) of compounds I and II.

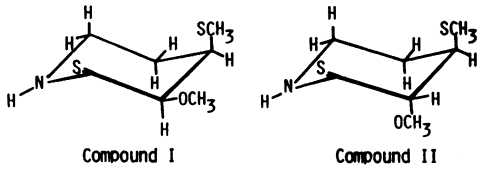

FIG. 5. Stereochemistry of compounds I and II.

group, triplet at δ7.40 (1H) to one proton of methine, and broad signal at δ 8.37 (1H) to one proton of —N—C—. Methylene (C-4) could be deduced to be very adjacent to —N—C— from its chemical shift (δ = 3.60 ppm), the attachment of —OCH_3 to C-6 from chemical shift (δ = 3.88 ppm) of —OCH_3 and J value (3 Hz) of proton at C-7. Irradiation of the triplet at δ3.60 simplified the double triplet at δ2.80 (2H) into a doublet. Irradiation of the double triplet at δ2.80 caused the triplets at δ3.60 (2H) and 7.40 (1H) to change to singlets. These data suggested the structure of compound III as a 6-methoxy-2,3,4,5-tetrahydro-1,3-oxazepin-2-one (tentatively designated raphanusamide) (Fig. 8). The structure was further supported by the ^{13}C NMR spectrum (Table I).

Biological Activities of *cis*- and *trans*-Raphanusanins and Raphanusamide. The effects of *cis*- and *trans*-raphanusanins and raphanusamide on the growth of etiolated radish hypocotyls are shown in Figure 9. The *cis*- and *trans*-raphanusanins are much more effective than raphanusamide in inhibiting the hypocotyl growth. The *cis*- and *trans*-raphanusanins inhibited the hypocotyl growth at concentrations higher than 1.5 μM, raphanusamide at concentrations higher than 20 μM.

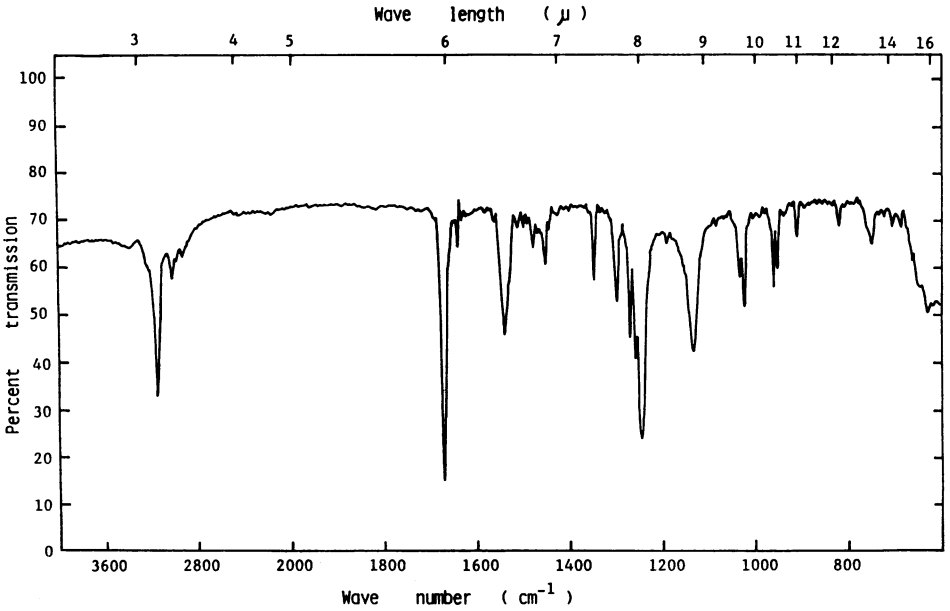

FIG. 6. IR spectrum (KBr pressed disc) of compound III.

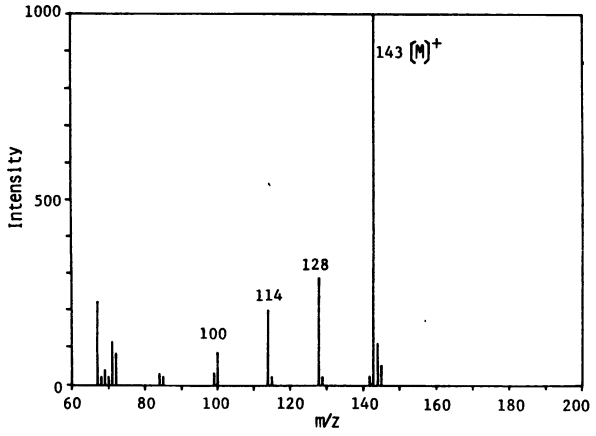

FIG. 7. Mass spectrum of compound III.

Table I. *^{1}H and ^{13}C NMR Chemical Shifts (δ Values from TMS) and Multiplicities (J Values in Hz) of Compound III (in $CDCl_3$) Signals*

Position	^{1}H	^{13}C
1		
2		137.9 (*s*)
3	8.37 (*br*)	
4	3.60 (*t*, 9)	62.0 (*t*)
5	2.80 (*d*, *t*, 3, 9)	24.5 (*t*)
6		120.5 (*s*)
7	7.40 (*t*, 3)	156.7 (*d*)
8	3.88 (*s*)	49.0 (*q*)

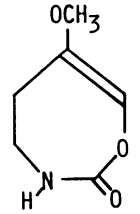

FIG. 8. Chemical structure of compound III.

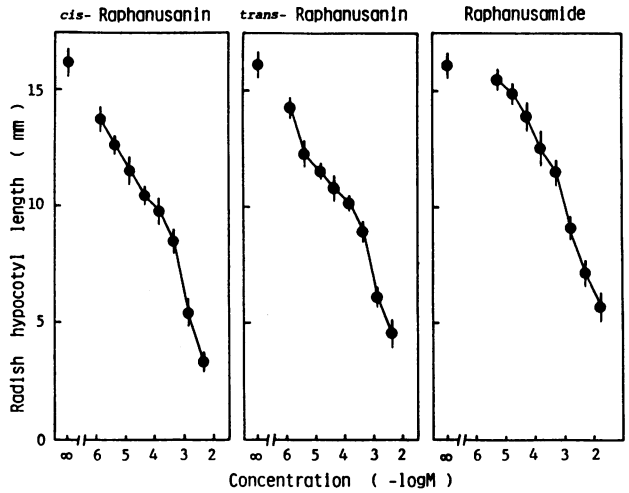

FIG. 9. Effects of *cis*- and *trans*-raphanusanins and raphanusamide on the hypocotyl growth of etiolated Sakurajima radish seedlings. Each value is the mean of 10 triplicates ± SE.

Acknowledgment—We wish to thank Miss C. Tanoue for excellent technical assistance.

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