Structures of Plant Growth Inhibitors in Seeds of Cucurbita pepo L.†

Hiroshi Fukui, Koichi Koshimizu, Yoshimitsu Yamazaki*1
and Sadakazu Usuda*2

Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Kyoto 606, Japan

Received September 7, 1976

Structures of five plant growth inhibitors isolated from seeds of Cucurbita pepo L. were elucidated, and shown to be 9,10-dihydroxy-12-octadecenoic acid (1), its methyl ester (2), (1S,2S,3S)-3-hydroxy-2-(2′-cis-pentenyl)-cyclopentane-1-acetic acid (3, named cucurbic acid), 3-O-β-D-glucopyranosylcucurbic acid (4) and methyl 3-O-β-D-glucopyranosylcucurbate (5).

The preceding papers reported the isolation2) of three new gibberellins A₃₉, A₄₈ and A₄₉, a new kaurenolide, and six plant growth inhibitors together with three abscisic acid related compounds, and also reported the structures3) of these gibberellins and kaurenolide. This paper is concerned with the structures of the inhibitors (1, 2, 3, 4 and 5).

Compound 1, fine needles (from ethyl acetate-hexane), mp 59−59.5°C, [α]D +10° (c=0.24, MeOH), showed IR absorption bands (CHCl₃) at 3580 (hydroxyl), 2700−2300 and 1715 (a carboxyl), and 1660 cm⁻¹ (a carbon-carbon double bond). Methylation of 1 with ethereal diazomethane gave a monomethyl ester (2), whose PMR spectrum (CDCl₃) exhibited the signals at δ 0.88 (3H, perturbed t., J=7Hz, -(CH₂)n-CH₃), 1.3 (ca. 24H, broad s., methylenes), 2.32 (2H, t., J=7Hz, -CH₂-CH₃COOCH₃), 3.45 (2H, t., J=7Hz, -CH₂CHOHCHOHCH₂-), 3.67 (3H, s., -COOCH₃) and 5.50 (2H, m., -CH₂CH=CHCH₂-). The mass spectrum of 2 had a parent ion at m/e 328, which indicated the molecular formula C₁₉H₃₆O₄. These data revealed that 1 was a singly unsaturated C₁₈ fatty acid having a glycol function.

The methyl ester (2) was oxidized with sodium periodate in aqueous ethanol. The crude products were further oxidized with Jones reagent, esterified with diazomethane and then hydrogenated on platinum catalyst. The two main products were identified as dimethyl azelate and methyl peralgonate by comparing their retention times on GC with those of authentic samples. Ozonolysis of 2 gave two aldehydes, one of which was identified as capronal on GC. Thus, compound 1 was confirmed to be 9,10-dihydroxy-12-octadecenoic acid. The absolute configurations at C-9 and C-10, and the geometry of the double bond remain to be decided.

Compound 2, [α]D +14° (c=1.0, MeOH), was identified as the methyl ester of 1 by comparing their IR, PMR and MS data.

Compound 3, [α]D +25° (c=0.32, MeOH), was found to be a new plant growth inhibitor and named cucurbic acid. Its IR spectrum (CHCl₃) indicated the presence of a hydroxyl (3600 cm⁻¹), a carboxyl (2800−2400 and 1712 cm⁻¹) and a carbon-carbon double bond (1655 cm⁻¹). Methylation of cucurbic acid (3) with ethereal diazomethane gave a monomethyl ester (6), whose PMR spectrum (CDCl₃) showed the signals assignable to a primary methyl (δ 0.94, 3H, t., J=7Hz), methylenes and methines (δ 1.2−2.8, 12H, undissolved m.), a carbomethoxyl (δ 3.65, 3H, s.), a hydroxy-methine proton (δ 3.98, 1H, m.) and two olefinic protons (δ 5.40, 2H, m.). The

† Plant Growth Regulators in Seeds of Cucurbita pepo L. Part III. Some of this work has been presented at “The 8th International Conference on Plant Growth Substances” (see reference 1).
*1 Present address: Fermentation Research Institute, Ministry of International Trade and Industry, Chiba.
*2 Present address: Tokyo Research Laboratories, Kowa Co. Ltd., Tokyo.
mass spectrum of 6 exhibited the parent ion peak at m/e 226. These data indicated the molecular formula C_{13}H_{22}O_{3} for the mono methyl ester (6), and consequently the molecular formula C_{12}H_{20}O_{3} for cucurbic acid (3).

Reduction of 6 with lithium aluminum hydride gave a diol (7), whose PMR spectrum (CDCl₃) showed the replacement of the signals due to –CH₂COOCH₃ in 6 by those due to –CH₂CH₂OH (δ 3.64, t, J = 6 Hz). This result, together with the molecular ion peak at m/e 198 in the mass spectrum of 7, indicated the presence of a partial structure –CH₂COOH in cucurbic acid.

Ozonolysis of 6 gave an aldehyde (8), in whose PMR spectrum (CDCl₃) the signals due to –CH=CHCH₂CH₃ in 6 disappeared, and the signal (δ 9.85, t, J = 1 Hz) attributable to a newly-formed aldehyde –CH₂CHO was observed. The mass spectrum of 8 exhibited a peak at m/e 182 corresponding to the loss of H₂O from the molecular ion, indicating the loss of a fragment =CHCH₂CH₃ on ozonolysis of 6. Thus, cucurbic acid was revealed to have a partial structure –CH₂CH=C=CHCH₂CH₃. The geometry of the double bond was suggested to be cis on the basis of the IR absorption band (1655 cm⁻¹) of 6.

Jones oxidation of 3 gave a keto-acid (9) which showed the IR and UV absorptions [δCHCl₃max 1747 cm⁻¹ and λHexanemax 283 nm (ε 46)] assignable to a cyclopentanone, cucurbic acid being shown to have a cyclopentanol moiety. The three partial structures (–CH₂COOH, –CH₂CH=C=CHCH₂CH₃ and a disubstituted cyclopentanol) described above, accounted for all the numbers of carbons, hydrogens and oxygens in cucurbic acid, and immediately reminded us of jasmonic acid.

Methylation of 9 with ethereal diazomethane yielded a keto-ester (10), whose mass fragmentation pattern was in good agreement with that of methyl (±)-jasmonate (12) kindly provided by Dr. Demole. However, their PMR spectra were slightly different from each other in the region of methylene and methine protons, suggesting that 10 was an epimer of methyl jasmonate. On treatment with dil. HCl, 10 was epimerized at the α-position (C-2) of the carbonyl function to afford a stereoisomer (11), whose IR and PMR spectra were superimposable on those of methyl (±)-jasmonate. The optical rotation of 11, [α]_D² = -54° (c = 0.25, MeOH), was in good agreement with the literature value⁴ for naturally occurring methyl (−)-jasmonate [methyl (1S, 2R)-3-oxo-2-(2’-cis-pentenyl)-cyclopentane-1-acetic acid] and the absolute configurations at C-1 and C-2 were assigned to be S and S, respectively. The absolute configuration at C-3 carrying a hydroxyl was shown to be S on the basis of the data (see EXPERIMENTAL) obtained by the method of Brooks et al.⁵ (a gas chromatographic modification of Horeau’s method⁶). This was also supported by the fact that no lactone formation was occurred by the treatment of cucurbic acid with p-toluenesulfonic acid in benzene.

The PMR spectrum (acetone-d₆) of 4 showed the signals assigned to all protons of cucurbic acid, together with the signals at δ 3.2−4.3 due to ring protons of glucose. A doublet at δ 4.28 (1H, d, J = 8 Hz) was assigned to the anomeric proton of β-D-glucose. Acetylation of 4 gave a tetraacetate, indicating that 4 was a monoglucoside of cucurbic acid. Hydrolysis of 4 with emulsin afforded cucurbic acid and glucose which were identified by comparison (δ[α]D, TLC, PPC and PMR) with authentic samples. Thus, the structure of 4 was characterized as 3-O-β-D-glucopyranosyl-cucurbic acid.

On the basis of the spectral (IR and PMR) data, 5 was suggested to be the methyl ester of 4. Esterification of 4 with diazomethane furnished 5 and the structure of 5 was assigned as methyl 3-O-β-D-glucopyranosyl-cucurbate.

Biological activities of cucurbic acid (3), methyl cucurbate (6), 3-O-β-D-glucopyranosyl-cucurbic acid (4) and its methyl ester (5) were reported separately.¹¹ Compounds 1 and 2 completely inhibited the growth of rice (Tan-ginbozu) seedlings at a concentration of 10⁻³M.
Plant Growth Inhibitors in \textit{Cucurbita pepo L.} 191

Cucurbic acid (3) and its derivatives, 4 and 5, are the first compounds to have been isolated from natural source. However, there remains the question as to whether the methyl esters, 2 and 5, might be artifacts derived from the original acids, 1 and 4, during the course of extraction and purification, respectively.

Methyl (-)-jasmonate closely allied in structure to cucurbic acid was first isolated from the essential oil of \textit{Jasmarinus grandiflorum} L.\textsuperscript{4} as an odoriferous compound, and has been shown to occur in \textit{Rosmarinus officinalis} L.\textsuperscript{7} The lactone of 5'-hydroxyjasmonic acid has been also found in \textit{J. grandiflorum}.\textsuperscript{8} In addition, the isolation of an inseparable mixture of N-jasmonoyl- and N-dihydrojasmonoyl-isoleucine from \textit{Gibberella fujikuroi} has been reported.\textsuperscript{9} Recently, the free acid (jasmonic acid) has been isolated as a plant growth inhibitor from the culture filtrate of \textit{Lasiodiplodia theobromae},\textsuperscript{10} the fresh leaves and the insect galls of \textit{Castanes crenata} Sieb. et Zucc.,\textsuperscript{11} and the seeds of \textit{Phaseolus vulgaris} L.\textsuperscript{12} It would therefore appear that jasmonic acid derivatives might primarily occur as (1S,2S)-isomers in nature.

**EXPERIMENTAL**

For general experimental details see the preceding paper.\textsuperscript{5}

1. \textit{Gas chromatography (GC)}

A Hitachi 063 gas chromatograph with a hydrogen flame-ionization detector was used. GC was performed on the following columns with a nitrogen flow rate of 30 ml/min: OV–17 (stainless steel column, 3 m × 3 mm i.d., packed with 1\% OV–17 on Gas chrom Q), SE–30 column (stainless steel column, 1 m × 3 mm i.d., packed with 2\% SE–30 on Chromosorb W) and DEGS column (stainless steel column, 1 m × 3 mm i.d., packed with 15\% diethylene glycol succinate on Neopak AS).

2. \textit{Preparation of 9,10-dihydroxy-12-octadecenoic acid derivatives}

\textit{i) Methylolation of 1.} Compound 1 in ether solution was treated with ethereal diazomethane to give the methyl ester (2) as a colorless glassy resin. \textit{IR} \text{cm}^{-1}: 3580, 1746 and 1655.

\textit{ii) Sodium periodate oxidation of 2.} To a solution of 2 (2 mg) in EtOH was added a solution of sodium periodate (10 mg) in water (1 ml). The mixture was allowed to stand at room temperature for 1.2 hr, heated at 40°C for 10 min, and then extracted with ether after addition of water (10 ml). The ether extract was concentrated in vacuo to give a residue which was dissolved in acetone (1 ml). To the acetone solution was added one drop of Jones reagent. The reaction mixture was concentrated to dryness after addition of MeOH. The residue was methylated with excess ethereal diazomethane to give an oily product, which was completely hydrogenated (20 min) over platinum catalyst.

\textit{iii) Ozonolysis of 2.} A solution of 2 (1 mg) in CS\textsubscript{2} was cooled in a salt-ice bath for 10 min and O\textsubscript{3}
was bubbled through for 10 min. Triphenylphosphine (3 mg) was added to the reaction mixture. One of the reaction products was identified as capronal by co-GC with an authentic sample (retention times: 3.2 min on OV-17 at 40°C, 3.1 min on SE-30 at 30°C and 4.3 min on DEGS at 40°C).

3. Cucurbic acid and its derivatives

i) Cucurbic acid (3). Cucurbic acid, an intractable gum. PMR (CDCl₃) δ: 0.89 (3H, t., J=7 Hz, -CH₂CH₃) 1.2-2.8 (12H, undissolved m., methines and methyl- enes), 4.00 (1H, m., -CHOH-) and 5.38 (2H, m., -CH=CH-).

ii) Methylation of 3. Cucurbic acid (3) (11 mg) in ether was treated with ethereal diazomethane to give an oil (11 mg), which was chromatographed over silicic acid (0.2 g)-Celite (0.4 g) eluted with benzene containing an increasing concentration of EtOAc by 10% steps. The fractions eluted with 20 and 30% EtOAc were combined and concentrated to give the methyl ester (6) (9 mg) as an oil. IR νmax cm⁻¹: 3600, 1736 and 1653. PMR (CDCl₃) δ: 0.89 (3H, t., J=7 Hz, -CH₂CH₃) 3.62 (3H, s., COOCH₃), 5.31 (2H, m., -CH=CH-). MS m/e: 226 (M+), 208, 153 and 83.

iii) Lithium aluminum hydride reduction of 6. Lithium aluminum hydride (10 mg) was added to a solution of 6 (8 mg) in absolute ether (2 ml). The mixture was stirred for 3 hr at room temperature and concentrated to an amorphous solid, to which water (6 ml). The solution was acidified with dil. HCl and extracted with EtOAc. The EtOAc extract was concentrated and chromatographed over silicic acid (0.2 g)-Celite (0.4 g) eluted with benzene-EtOAc mixtures containing an increasing concentration of EtOAc by 10% steps in 5 ml fractions. The fraction eluted with 20% EtOAc was concentrated to give the starting material (cucurbic acid).

iv) Ozonolysis of 6. A solution of 6 (5 mg) in EtOAc (4 ml) was cooled in a dry ice-acetone bath for 5 min and O₃ was bubbled through for 10 min. After addition of triphenylphosphine (10 mg), the reaction mixture was allowed to stand overnight at room temperature and concentrated to give a gum, which was chromatographed over charcoal (1 g)-Celite (2 g) eluted in 3 ml fractions with CHCl₃ containing increasing concentrations of EtOAc in 20% steps. The fraction eluted with 80% EtOAc was concentrated to give the diol (7) (6 mg) as a colorless oil. IR νmax cm⁻¹: 3450 and 1653. PMR (CDCl₃) δ: 0.90 (3H, t., J=7 Hz, -CH₂CH₃), 3.59 (2H, t., J=7 Hz, -CH₂CH₂OH), 3.95 (1H, m., -CHOH-) and 5.36 (2H, m., -CH=CH-). MS m/e: 198 (M+), 180, 153 and 83.

v) Oxidation of 3 with Jones reagent. To a solution of 3 (12 mg) in acetonitrile (1 ml) was added Jones reagent (0.1 ml). The mixture was kept at room temperature for 30 min. After addition of water (15 ml), the product was extracted with EtOAc. The extract was washed with water, dried over anhyd. Na₂SO₄, concentrated to dryness and chromatographed over silicic acid (0.5 g) eluted with EtOAc-CHCl₃ mixtures to give the keto-acid (9) (10 mg) as an oil. [α]D° +14° (c=0.57, MeOH). ORD (c=0.57, MeOH) [a]D²⁰ (nm): -610° (317), 0° (302) and +750° (279). IR νmax cm⁻¹: 3670, 2700-2300, 1747 and 1718. PMR (CDCl₃) δ: 0.89 (3H, t., J=7 Hz, -CH₂CH₃) and 5.30 (2H, m., -CH=CH-).

vi) Methylation of 9. The keto-acid (9) (6 mg) was dissolved in ether and methylated with excess ethereal diazomethane. The crude product was chromatographed over silicic acid (0.3 g)-Celite (0.6 g) eluted with benzene containing an increasing concentration of EtOAc by 10% steps. The fractions eluted with 10 and 20% EtOAc were concentrated to give the ketoster (10) (5 mg) as an oil. [a]D° +24° (c=0.26, MeOH). ORD (c=0.26, hexane) [α]D²⁰ (nm): -811° (328), -670° (318), 0° (309), +870° (288) and +940° (279). UV νmax nm (ε): 283 (50). IR νmax cm⁻¹: 1740. PMR (CDCl₃) δ: 0.89 (3H, t., J=7 Hz, -CH₂CH₃), 3.62 (3H, s., COOCH₃), 5.31 (2H, m., -CH=CH-). MS m/e: 224 (M+), 193, 156 and 83.

vii) Epimerization of 10. One drop of 1 N HCl was added to a solution of 10 (6 mg) in benzene. The solution was stirred for 1 hr. The epimerization product was purified by column chromatography over silicic acid (0.2 g)-Celite (0.4 g) eluted with benzene-EtOAc mixtures containing an increasing concentration of EtOAc by 10% steps in 10 ml fractions. The fraction eluted with 20% EtOAc was concentrated to give the stereoisomer (11) (5 mg) as an oil. [α]D° -54° (c=0.25, MeOH). ORD (c=0.25, MeOH) [α]D²⁰ (nm): -1680° (312), 0° (298) and +1760° (275). IR νmax cm⁻¹: 1743, 1653, 1460, 1440, 1410, 1378, 1340, 1265, 135, 133, 121, 109, 95 and 83 (base peak). The optical rotation was in good agreement with that of methyl (-)-jasmonate. The other spectral data were identical with those of methyl (±)-jasmonate.

viii) Attempted lactonization of cucurbic acid (3). The mixture of 3 (6 mg) and p-toluenesulfonic acid (0.5 mg) in benzene (3 ml) was heated on a boiling water bath until the volume of the solution diminished to about 0.5 ml. The product was purified by column chromatography on silicic acid (0.2 g)-Celite (0.4 g) eluted with benzene-EtOAc mixtures containing an increasing concentration of EtOAc by 10% steps in 5 ml fractions. The fraction eluted with 20% EtOAc was concentrated to give the starting material (cucurbic acid).

4. The absolute configuration at C-3 of cucurbic acid (3)

i) Preparation of (±)-α-phenylbutyric anhydride.
The mixture of (±)-α-phenylbutyric acid (3 g) and acetic anhydride (15 ml) was heated in a Claisen flask (50 ml) under atmospheric pressure at 160°C on an oil bath until no more distillate came out. This procedure was repeated after addition of fresh portion (15 ml) of acetic anhydride to the flask. The remaining oil in the flask was distilled at 230°C under reduced pressure (35 mmHg) to give (±)-α-phenylbutyric anhydride (2 g), which was stored in a brown-colored ampule. IR ν<sub>max</sub> cm<sup>-1</sup>: 3080, 3060, 3020, 1815, 1747, 1605, 1455 and 700.

**ii) Application of Horeau-Brooks method.**<sup>5,6</sup>
Methyl cucurbate (6) (10 µmole) in dry pyridine (7 µl) was treated with (±)-α-phenylbutyric anhydride (1.0 molar excess) and kept in a sealed vial at 40°C for 1.5 hr. A parallel reaction was carried out with cyclohexanol. (+)-(R)-α-Phenyethylamine (6 µl) was added and mixed thoroughly. After 15 min, the mixture was diluted with dry EtOAc (400 µl) and a sample was analysed by GC at 190°C on a 2 m column packed with 1 % OV-17 on Chromosorb W. The relative proportions of the amides of (−)-(R)- and (+)-(S)-α-phenylbutyric acid were indicated by the areas of their respective peaks (retention times: 9.4 min and 10.5 min, respectively). The percentage area representing the (−)-(R)-acid was assessed. Subtraction of the corresponding value from the reaction with cyclohexanol gave the increment (+4.5). This value means that the absolute configuration at C-3 was S.

5. 3-O-β-α-11-D-Glucopyranosyleucaric acid and its derivatives

**i) 3-O-β-α-11-D-Glucopyranosyleucaric acid (4).** 3-O-β-α-D-Glucopyranosylcucurbic acid (4), an intractable gum. [α]<sub>D</sub> = −23.4° (c = 4.5, MeOH). IR ν<sub>max</sub> cm<sup>-1</sup>: 3400, 1718, 1410, 1075 and 1020. PMR (acetone-de) δ: 0.93 (3H, t., J = 7 Hz), 1.2–2.6 (12H, undissolved m.), 3.2–4.3 (6H, undissolved m.), 4.29 (1H, d., J = 7 Hz) and 5.40 (2H, m.).

**ii) Methylation of 4.** Compound 4 (20 mg) was dissolved in MeOH (20 ml) and methylated with excess ethereal diazomethane. The crude product was chromatographed over silicic acid (2 g)-Celite (4 g) eluted with 6 % MeOH. The fraction eluted with 6 % MeOH was concentrated with EtOAc-MeOH mixtures containing an increasing concentration of MeOH by 3 % steps in 20 ml fractions. The fraction eluted with 30 % EtOAc was concentrated to give the tetraacetate (10 mg) as an intractable gum. IR ν<sub>max</sub> cm<sup>-1</sup>: 1760–1740, 1655, 1440, 1370 and 1040. PMR (CDCl<sub>3</sub>) δ: 0.92 (3H, t., J = 7 Hz), 1.97 (3H, s.), 1.98 (3H, s.), 2.02 (3H, s.), 2.04 (3H, s.), 3.65 (3H, s.), 4.18 (2H, m.), 4.46 (1H, d., J = 8 Hz, anomeric proton) and 4.85–5.55 (5H, m.).

**iv) Enzymatic hydrolysis of 4.** Compound 4 (170 mg) was dissolved in 15 ml of pH 4 phosphate buffer (prepared by mixing 16.4 ml of 0.2 M AcOH and 3.6 ml of 0.2 M NaOAc). To the solution was added 20 mg of emulsin and the reaction mixture was allowed to stand at 37°C for 4 hr. A precipitate was filtered off and the filtrate was extracted with EtOAc. The EtOAc layer was concentrated to give an intractable gum (70 mg), whose optical rotation and PMR spectrum (D<sub>2</sub>O) were identical with those of cucurbitic acid (3). The aqueous layer was concentrated to give an intractable gum (70 mg), whose optical rotation and PMR spectrum (D<sub>2</sub>O) were identical with those of D-glucose. The yields of the two hydrolysis products mean that 4 is a monoglucoside of cucurbitic acid.

The Rf values of the product obtained from the aqueous layer were compared with those of D-glucose, D-mannose, D-galactose and D-fructose on the following chromatographic systems.

**Thin-layer chromatography**<sup>85</sup>; Kieselguhr G (20 g) was mixed with 40 ml of pH 5 phosphate buffer (prepared by mixing equal volumes of 0.1 M H<sub>3</sub>PO<sub>4</sub> and 0.1 M Na<sub>2</sub>HPO<sub>4</sub>). The plates were spread to a thickness of 0.25 mm and dried overnight in the air. Samples spotted on the plate were developed with the following solvent systems; (A) n-BuOH: acetonitrile: phosphate buffer (pH 5) = 4: 5: 1, (B) n-BuOH: EtOAc: phosphate buffer (pH 5) = 6: 4: 1.

The sugars and the hydrolysis product were detected after spraying with an aniline-phtalate reagent (prepared by mixing 0.93 g of aniline and 1.66 g of phthalic acid with 100 ml of water presaturated with n-BuOH) followed by heating at 130°C for 3 min. Rf values of D-glucose, D-mannose, D-galactose and D-fructose were 0.55, 0.61, 0.61 and 0.46 in solvent system A, and 0.63, 0.73, 0.53 and 0.68 in solvent system B, respectively. The hydrolysis product gave the same Rf values as D-glucose in both solvent systems.

**Paper chromatography**<sup>86</sup>; The samples spotted on Whatman No. 1 paper (50 x 50 cm) were developed with the solvent system; EtOAc: pyridine: water = 12: 5: 4. The samples were detected by heating at 100°C for 3 min after dipping a solution of 85 % H<sub>2</sub>PO<sub>4</sub> (4 ml), aniline (0.4 ml) and diphenylamine (0.3 g) in acetic (40 ml). The Rf values and colors of D-glucose, D-mannose, D-galactose and D-fructose were 0.40 (blue), 0.47 (gray), 0.38 (blue) and 0.47 (brown), respectively. The hydrolysis product gave the same Rf value and color as D-glucose.
Acknowledgements. We wish to thank Dr. T. Ueno, Pesticide Research Institute, Faculty of Agriculture, Kyoto University, and Dr. A. Kato, Faculty of Pharmaceutical Sciences, Kyoto University, for measuring mass spectra, and Miss S. Yamashita of our department for obtaining PMR spectra. We are also grateful to Dr. E. Demole of Firmenich and Cie, for kindly furnishing us with a sample of methyl (+)-jasmonate and for useful discussions.

REFERENCES