Gibberellins in Immature Seeds of *Canavalia*

Part I. Isolation and Biological Activity of Gibberellins

A₂₁ and A₂₂

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Two novel gibberellins, GA₂₁ (I) and GA₂₂ (II), were isolated from immature seeds of sword bean, *Canavalia gladiata* DC. The isolation procedure of these substances as well as their growth-promoting effects on dwarf maize mutants *d*₁ and *d*₅, rice, cucumber and dwarf peas (Progress No. 9) are described.

Several crystalline gibberellins have been isolated from higher plants and their structures established. Most of them were obtained from immature leguminous seeds. In 1959 Murakami noticed the presence of gibberellin-like substances in immature seeds of sword bean (*Canavalia gladiata* DC.),¹⁾ an annual leguminous plant which bears large pods of approximately 30 cm in length and 5 cm in width. As preliminarily reported in the previous papers,^{2,3)} we have succeeded in the isolation of two new gibberellins from immature seeds of this plant and determined their structures. In this paper we wish to report the details of their isolation procedure and biological activities.

The acidic fraction of the immature seeds showed a marked growth-promoting effect on dwarf maize mutant *d*₅. As illustrated in Fig. 1, thin layer chromatography (TLC) of this fraction revealed three distinct fluorescent spots, characteristic of gibberellins, under

ultraviolet light after spraying with sulfuric acid and heating. On the other hand, bioassay using *d*₅ dwarf maize mutant indicated the presence of two gibberellin-like substances corresponding to spots II and III, while spot I scarcely showed any activity. Isolation of the gibberellin-like substances was undertaken according to the procedure illustrated in Fig.

FIG. 1. Thin Layer Chromatography and Bioassay of Crude Extract on Dwarf Maize Mutant *d*₅.

- 1) Y. Murakami, *Bot. Mag. Tokyo*, **72**, 36 (1959).
- 2) S. Tamura, N. Takahashi, N. Murofushi, T. Yokota, J. Kato and Y. Shiotani, *Planta*, **75**, 279 (1967).
- 3) N. Takahashi, N. Murofushi, T. Yokota, S. Tamura, J. Kato and Y. Shiotani, *Tetrahedron Letters*, **1967**, 4861.

FIG. 2. Isolation Procedure for GA₂₁ and GA₂₂.

2. At each step of purification, the activity was detected by d_5 bioassay in combination with TLC.

Immature seeds were extracted with acetone using a blender. The ethyl acetate-soluble fraction was obtained by the usual method and applied to countercurrent distribution by the use of ethyl acetate and phosphate buffer (pH 5.5) with eleven transfers. Biologically active solid was recovered from fractions No. 2~10 and extracted with ethyl acetate to give ethyl acetate-soluble and -in-

soluble fractions. The soluble fraction was purified by silicic acid adsorption chromatography. Elution with benzene-ethyl acetate (80:20, v/v) gave an active principle corresponding to spot III, but it could not be obtained as crystals due to its low content. The eluates with benzene-ethyl acetate (45:55~25:75, v/v) contained the substances corresponding to spots I and II, which were separated as crystals respectively with partition chromatography using butanol and Sephadex G-50 treated with phosphate buffer (pH 5.4).

The ethyl acetate-insoluble fraction mentioned above also afforded the compound corresponding to spot I by partition chromatography conducted in the same manner as for the soluble fraction.

The two substances thus obtained were con-

firmed to be new C_{19} gibberellins on the basis of physico-chemical data, behavior on TLC and biological activities, and tentatively named "canavalia gibberellins I and II",²⁾ which were afterwards changed to gibberellins A_{21} and A_{22} (GA_{21} and GA_{22}) according to the

FIG. 3. IR Spectrum of GA_{21} (nujol).

FIG. 4. IR Spectrum of GA_{21} Methyl Ester (nujol).

FIG. 5. IR Spectrum of GA_{22} (nujol).

FIG. 6. IR Spectrum of GA_{22} Methyl Ester (nujol).

proposal of MacMillan and Takahashi.⁴⁾ The IR spectra of GA_{21} , GA_{22} and their methyl esters are shown in Figs. 3~6. As reported in the preliminary report³⁾ the structures I and II have been assigned to GA_{21} and GA_{22}

respectively. The details of the structural elucidation will be described in the succeeding paper.

Biological activities of the new gibberellins were examined by the use of dwarf maize mutants d_1 and d_5 , rice, cucumber and dwarf pea (Progress No. 9). As shown in Table I, GA_{21} exhibited very weak but evident effect on the d_1 and d_5 maize mutants. The application of $100\mu g$ of GA_{21} per plant of both mutants resulted in stem elongation only comparable to that caused by $1\mu g$ of GA_3 . When GA_{22} was applied to d_1 and d_5 mutants at a

dose of $1\mu g$ per plant, stem elongation was about 200% of the control.

GA_{21} showed no effect on rice seedlings at 10 ppm, while GA_{22} caused 130% elongation at 0.3 ppm. The growth of cucumbers was not affected by both compounds. On dwarf peas GA_{21} exhibited very weak activity, elongation being 130% of the control even at a dosage of $100\mu g$ per plant. GA_{22} , on the other hand, caused nearly 200% elongation at $0.01\mu g$.

The action spectrum of GA_{22} , in which activities on rice and cucumber are extremely lower than those on dwarf maize mutants, might be characteristic of gibberellins bearing a hydroxyl at C-7 but not at C-2 on the gibbane ring (V).⁵⁾ It is noteworthy that GA_{21} and GA_{22} with a carboxyl and a hydroxymethyl, respectively, instead of a methyl at C-1 show gibberellin activity. It has been imagined so far that the C-1 methyl is essential for gibberellins to show biological activity, but this hypothesis should be revised hereafter. The structural features of these gibberellins may suggest the catabolic pathways of GA_{20} (pharbitis gibberellin) (III) and GA_5 (IV) which have been confirmed to exist in immature seeds of higher plants such as morning glory.⁵⁾

4) J. McMillan and N. Takahashi, *Nature*, **217**, No. 5124, 170 (1968).

5) N. Murofushi, N. Takahashi, T. Yokota and S. Tamura, *Agr. Biol. Chem.*, **32**, 1239 (1968).

TABLE I. GROWTH-PROMOTING ACTIVITIES OF GA_{21} , GA_{22} AND GA_3 ON HIGHER PLANTSA) Activity on dwarf maize mutants, d_1 and d_5

Mutant	Dosage, $\mu\text{g/plant}$	Sum of 1st and 2nd leaf-sheath lengths, mm		
		GA_{21}	GA_{22}	GA_3
d_1	100	88	—	—
	10	70	94	—
	1	52	82	102
	0.1	—	55	77
d_5	100	77	—	—
	10	57	111	—
	1	39	79	110
	0.1	—	42	68

Control: d_1 , 41 mm; d_5 , 31 mm

B) Activity on rice (Norin No. 1)

Dosage, ppm	Length of 2nd leaf-sheath, mm		
	GA_{21}	GA_{22}	GA_3
10	32	—	—
3	31	49	—
1	30	47	67
0.3	—	41	53

Control: 30 mm

C) Activity on cucumber

Dosage, $\mu\text{g/plant}$	Length of hypocotyl, mm		
	GA_{21}	GA_{22}	GA_3
100	37	—	—
10	39	—	—
1	35	34	63
0.1	—	39	39

Control: 37 mm

D) Activity on dwarf pea (Progress No. 9)

Dosage, $\mu\text{g/plant}$	Length of whole stem, mm		
	GA_{21}	GA_{22}	GA_3
100	60	—	—
10	64	—	—
1	51	135	—
0.1	—	90	—
0.01	—	73	119

Control: 41 mm

EXPERIMENTAL

Thin layer chromatography

Thin layer chromatography was conducted by the

use of Silica gel-G (Merck) and a benzene-butanol-acetic acid mixture (70:25:5, v/v). Gibberellins on the chromatograms were detected as fluorescent spots under ultraviolet light after spraying with 70% sulfuric acid and heating at 100°C for 10 min. R_f values of spots I, II and III were 0.2 (cobalt blue), 0.35 (blue) and 0.65 (blue), respectively.

Isolation of GA_{21} and GA_{22}

a) *Separation of acidic fraction.* Nineteen kilograms of immature seeds (each seed, 0.8~3.0 g in weight) were repeatedly extracted with acetone by the use of a blender, and the combined extracts were concentrated under reduced pressure. The resulting aqueous residue was extracted at pH 2.5 with ethyl acetate, which was further extracted with aqueous sodium bicarbonate. The aqueous solution was acidified and re-extracted with ethyl acetate to give the acidic fraction. Evaporation of the solvent afforded a biologically active gum (10.8 g).

b) *Countercurrent distribution.* The acidic fraction was partitioned between 500 ml each of ethyl acetate and phosphate buffer (1 M, pH 5.5), eleven transfers countercurrent distribution being conducted. Organic and aqueous phases of fractions No. 2~10 showing activity were separated, and the combined buffer phases were extracted with ethyl acetate. All the ethyl acetate solutions were combined and concentrated to afford a solid (5.7 g).

c) *Silicic acid adsorption chromatography.* The acidic solid was extracted with two 10 ml portions of hot ethyl acetate to separate soluble and insoluble matters. The combined extracts were chromatographed through a column of silicic acid (Mallinckrodt, 60 g), which was then eluted with benzene-ethyl acetate mixtures. The content of ethyl acetate was increased stepwise by 5% for every 200 ml of eluant. From eluates with benzene-ethyl acetate (45:55~25:75, v/v), an active solid (890 mg) was obtained.

d) *Partition chromatography.* Sephadex G-50 (30 g) was treated with 150 mg of phosphate buffer (1 M, pH 5.4) and packed into a column with butanol saturated with the phosphate buffer. The active solid obtained after silicic acid adsorption chromatography was dissolved in butanol and poured onto the column. Then the column was eluted with butanol saturated with phosphate buffer, and 15 ml of eluate was collected for each fraction. On evaporation of the solvent

fractions No. 4~8 afforded active crystals. Recrystallization from ethanol-hexane gave 28 mg of pure GA₂₂ melting at 213~214°C. It is soluble in methanol and ethanol, and sparingly soluble in acetone, ethyl acetate and water. $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3380, 3240, 1770, 1709, 1670, 1643, 892.

Fractions No. 19~48 afforded another active crystals. Recrystallization from ethanol-chloroform-hexane gave 241 mg of pure GA₂₁ melting at 244~246°C. It is soluble in methanol and ethanol, and sparingly soluble in acetone, ethyl acetate and water. $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3280, 2520 (broad), 1779, 1730, 1682, 877.

When the same procedure was applied to the ethyl acetate-insoluble fraction separated in c), additional 24 mg of pure GA₂₁ was obtained.

same manner as in the case of GA₁₉ (bamboo gibberellin)⁶⁾ using dwarf maize (*Zea mays* L., dwarf mutants *d*₁ and *d*₅), rice (*Oryza sativa* L. cv. Norin No. 1), cucumber (*Cucumis sativus* L.) and dwarf pea (*Pisum sativum*, cv. Progress No. 9).

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Bioassay.

The growth-promoting activities were tested in the

6) S. Tamura, N. Takahashi, N. Murofushi and J. Kato, *Plant and Cell Physiol.*, **7**, 677 (1966).