Gibberellins in Immature Seeds of *Pharbitis nil*

Part I. Isolation and Structure of a Novel Gibberellin, Gibberellin A₂₀

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A new gibberellin, gibberellin A₂₀ (GA₂₀), was isolated from immature seeds of morning-glory (*Pharbitis nil*). Its structure was established as 4 α , 7 α -dihydroxy-1 β -methyl-8-methylenegibbane-1 α , 10 β -dicarboxylic acid-1 $\rightarrow$ 4 α lactone (I) on the basis of its physico-chemical analysis as well as chemical evidences. GA₂₀ shows marked growth promoting activities on dwarf maize *d*₂ and *d*₅ but weak activities on *d*₁, rice seedling and dwarf pea.

The presence of gibberellin-like substances in immature seeds of morning-glory (*Pharbitis nil*) has been suggested by Ogawa,^{1,2)} Murakami³⁾ and Zeevaart.⁴⁾ In the preliminary reports,^{5,6)} we have presented the isolation of a novel gibberellin, gibberellin A₂₀ (GA₂₀) (I), from the same seeds together with the assignment of its structure. Here we wish to report the details of the isolation procedure, structural assignment and biological activities of GA₂₀ as well as the co-existence of GA₃ and GA₅.

The immature seeds (60 kg) were extracted with methanol using a blender and the extract was concentrated *in vacuo*. The aqueous residue was extracted with ethyl acetate and *n*-butanol successively. Each fraction was separated into acidic and neutral fractions

by the usual method. As preliminarily reported, the butanol-soluble acidic fraction showed marked growth promoting activity to rice seedlings and was found to contain GA₃ glucoside together with other two water-soluble gibberellins.^{6,7)}

The ethyl acetate-soluble acidic fraction was purified by method illustrated in Fig. 1. At first ten transfers countercurrent distribution was conducted. The active fractions were combined and chromatographed on a charcoal column. Elution with acetone-water mixtures gave active fractions F-I (50:50 and 60:40, acetone-water, v/v) and F-II (70:30, v/v). The presence of different active principles in F-I and F-II was suggested by the histograms obtained from thin layer chromatography and bioassay. Each fraction was successively purified through silicic acid adsorption and preparative thin layer chromatography. Thus F-I showed a fluorescent spot under ultra-violet light at R_{GA3} 1.00 on a thin layer chromatogram treated with 70% sulfuric acid without heating. The extract from this zone exhibited marked activity on dwarf maize *d*₅. The methylated sample obtained from the active zone on treatment with diazomethane revealed a fluorescent spot with

1) Y. Ogawa, *Plant and Cell Physiol.*, **4**, 217 (1963).

2) Y. Ogawa, *Bot. Mag. Tokyo*, **79**, 69 (1966).

3) Y. Murakami, *ibid.*, **74**, 241 (1961).

4) J. A. D. Zeevaart, *Plant Physiol.*, **41**, 856 (1966).

5) N. Takahashi, N. Murofushi, T. Yokota and S. Tamura, *Tetrahedron Letters*, **1967**, 1065.

6) N. Takahashi, N. Murofushi, T. Yokota and S. Tamura, Presented at the VIth International Conference on Plant Growth Substances, Ottawa, Canada, 1967.

7) S. Tamura, N. Takahashi, T. Yokota, N. Murofushi and Y. Ogawa, *Planta*, **78**, 208 (1968).

FIG. 1. Isolation Procedure for Gibberellins in Immature Seeds of Morning-glory.

sulfuric acid on thin layer chromatography and an evident peak on gas chromatography, the R_f value and retention time being identical with those of GA_3 methyl ester. Thus, the presence of GA_3 in the seeds was established.

Purified F-II on a thin layer chromatogram showed a fluorescent spot at R_{GA_3} 1.60 on treatment with sulfuric acid followed by heating. This spot exhibited strong growth promoting activity on dwarf maize d_5 and rather weak activity on d_1 and rice seedling. Since the active principle could not be obtained as crystals by preparative thin layer chromatography, it was converted to the methyl ester, which showed a fluorescent spot at R_{GA_3-Me} 1.82 on a thin layer plate with

sulfuric acid. Preparative thin layer chromatography of the ester followed by recrystallization afforded 7 mg of colorless crystals melting at 181°C . Alkaline hydrolysis of the ester afforded an acid melting at $232\sim 233^\circ\text{C}$, which regenerated the original ester on treatment with diazomethane, indicating that simple hydrolysis occurred. The free acid and its methyl ester thus obtained revealed almost identical R_f values with those of GA_5 and its methyl ester (III) on thin layer chromatograms, and the growth promoting activities of the acid on dwarf maize d_1 , d_5 and rice seedlings resembled those of GA_5 . However, IR spectrum of the ester was different from not only III but also any known gib-

FIG. 2. IR Spectrum of Gibberellin A_{20} (Nujol).

FIG. 3. IR Spectrum of Gibberellin A_{20} Methyl Ester (Nujol).

FIG. 4. NMR Spectrum of Gibberellin A₂₀ Methyl Ester (CDCl₃).

berellin methyl esters. Thus the acid was decided to be a new gibberellin, and named *pharbitis gibberellin*, later, gibberellin A₂₀ according to the proposal by MacMillan and Takahashi.⁸⁾ IR spectra of GA₂₀ and its methyl ester are shown in Fig. 2 and 3.

The occurrence of GA₅ in the fraction containing GA₂₀ was indicated by the presence of weak signals due to III in the NMR spectrum of crude GA₂₀ methyl ester, which revealed a 3H singlet of C-1 methyl at τ 8.78, an AB quartet of C-10 and 10a protons at τ 7.13 and 7.40 and complicated signals of C-2 and C-3 olefinic protons at τ 4.1~4.5. The content of GA₅ is estimated to be less than 10% of GA₂₀ based on comparison of intensities of the signals corresponding to the both esters.

The high resolution mass spectrum of GA₂₀ methyl ester revealed a parent ion peak at 346.177 with the composition of C₂₀H₂₆O₅ (calculated value, 346.178). The NMR spectrum of the methyl ester in deuteriochloroform showed a methoxyl signal at τ 6.33, indicating GA₂₀ to be monobasic. Therefore, the molecular formula C₁₉H₂₄O₅ was assigned to GA₂₀. The IR spectrum of the ester suggested the presence of functional groups illustrated

in Table I. In the high resolution mass spectrum, GA₂₀ methyl ester exhibited prominent peaks due to M-32(CH₄O), M-46(CH₂O₂), M-60(C₂H₄O₂), M-78(C₂H₆O₃), M-104(C₃H₄O₄), M-106(C₃H₆O₄) and M-122(C₃H₆O₅) fragment ions which constitute a characteristic pattern of C₁₉ gibberellin methyl esters.⁹⁾ Appearance of C₁₇ fragment ions as the largest hydrocarbon fragments suggests the presence of a gibbane ring (VI). This is further supported by the presence of the AB quartet due to C-10 and C-10a protons at τ 7.50 and 7.33 ($J=10$ cps), which has been known to be characteristic of a gibbane ring.

TABLE I. FUNCTIONAL GROUPS OF GA₂₀ METHYL ESTER

	IR (cm ⁻¹ , nujol)	NMR (τ , CDCl ₃)
1 OH	3520	
1 Lactone	1778	
1 COOCH ₃	1720	6.33 (3H, s)
1 Double bond (exomethylene)	1670, 882	4.80 (1H, s), 5.10 (1H, s)
1 $\begin{array}{c} \\ -C-CH_3 \\ \end{array}$		8.93 (3H, s)

According to the Hanson's information,¹⁰⁾ the NMR spectrum of GA₂₀ methyl ester measured in deuteriochloroform was compared

8) J. MacMillan and N. Takahashi, *Nature*, **217**, 170 (1968).

9) N. Takahashi, N. Murofushi, S. Tamura, N. Wasada, H. Hoshino, T. Tsuchiya, T. Aoyama and H. Morita, *Tetrahedron Letters*, **1967**, 865.

with that in pyridine in details. The C-1 methyl signal was observed at τ 8.93 in deuteriochloroform and τ 8.85 in pyridine. This slight shift in pyridine suggests the absence of a hydroxyl group at C-2. This is concordant with the observation that an AB quartet due to C-10 and C-10a protons at τ 7.50 and 7.33 ($J=10$ cps) in deuteriochloroform does not shift downfield so much in pyridine. Two 1H singlets due to the C-8 exomethylene exist at τ 5.10 and 4.80 in deuteriochloroform, and at τ 4.94 and 4.42 in pyridine. This downfield shift of one of the exomethylene protons in pyridine can be explained by the presence of a hydroxyl group at C-7. These physicochemical evidences allow to assign the plane structure of II to GA₂₀ methyl ester.

This conclusion was further confirmed through chemical conversion of III into II by partial hydrogenation. Cross *et al.*¹¹⁾ reported partial hydrogenation of C-3~C-4 double bond in GA₇ methyl ester to afford GA₄ methyl ester by the use of the partially poisoned palladium-barium carbonate catalyst. Application of this procedure to III, however, did not give the anticipated single compound but mixture of equal amounts of II, III, IV and V. The mixture was successfully separated through adsorption chromatography by

the use of silicic acid impregnated with silver nitrate. II thus obtained was completely identical with the methyl ester of natural GA₂₀ in all respects, IR, NMR and melting point. Thus, stereochemical formulas of GA₂₀ and its methyl ester could be established as I and II, respectively.

TABLE II. GROWTH PROMOTING ACTIVITIES OF GA₂₀, GA₃ AND GA₅ ON HIGHER PLANTS

A) Activities on dwarf maize mutants, *d*₁, *d*₂ and *d*₅

Mutant	Dosage (μ g/plant)	Sum of 1st and 2nd leaf-sheath lengths (mm)		
		GA ₃	GA ₅	GA ₂₀
<i>d</i> ₁	1	107	78	51
	0.1	68	52	41
<i>d</i> ₂	1	113	106	101
	0.1	87	89	69
<i>d</i> ₅	1	113	125	107
	0.1	65	75	61

Control: *d*₁, 41; *d*₂, 36; *d*₅, 31 mm

B) Activities on rice seedling (Kotake-tamanishiki)

Dosage (ppm)	Length of 2nd leaf-sheath (mm)		
	GA ₃	GA ₅	GA ₂₀
10	—	66	47
1	63	39	26
0.1	28	23	23

Control: 20 mm

C) Activities on dwarf pea (Progress No. 9)

Dosage (μ g/plant)	Length of whole stem (mm)		
	GA ₃	GA ₅	GA ₂₀
10	—	—	160
1	—	105	111
0.1	210	84	53
0.01	125	53	46

Control: 41 mm

Growth promoting effects of GA₂₀ on higher plants were compared with those of GA₃ and GA₅ using dwarf maize mutants *d*₁, *d*₂, *d*₅, rice seedlings and dwarf peas, according to the methods applied for GA₁₉ (*bamboo gibberellin*).¹²⁾ As shown in Table II, GA₅ and GA₂₀ were highly active on dwarf maize *d*₂ and *d*₅

10) J. R. Hanson, *J. Chem. Soc.*, **1965**, 5036.

11) B. E. Cross, R. H. B. Galt and J. R. Hanson, *Tetrahedron*, **18**, 451 (1962).

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

but far less on d_1 , rice seedlings and dwarf peas. Although both gibberellins showed similar action spectra, differences of activities between these higher plants were more enhanced in the case of GA_{20} than GA_5 . GA_9 and GA_{19} , which contain no hydroxyl group at C-2, show similar activities to that of GA_{20} on these higher plants. This might be due to this structural feature.

EXPERIMENTAL

Thin layer chromatography. Throughout this study, thin layer chromatography was conducted under the following conditions unless otherwise stated: adsorbent, Silica gel G (Merck); solvent, benzene-*n*-butanol-acetic acid (70:25:5, v/v). Gibberellins on thin layer plates were detected by the treatment with 70% sulfuric acid followed by heating at 120°C for 10 min to develop fluorescent spots under ultra-violet light, and bioassays were adopted if necessary.

Gas chromatography. Retention times were determined with a Shimadzu GC-1B gas chromatograph fitted with a hydrogen flame-ionization detector. A silanized glass column, 1.5 m $\times$ 4 mm, was packed with 1.5% SE-30 on Chromosorb W. Retention time of GA_3 methyl ester was 9.1 min under the following conditions: carrier gas, nitrogen flowing at 57.5 ml/min; column temperature, 230°C.

Determination of IR, NMR and mass spectroscopy. Infrared spectra were measured in nujol mulls with a JASCO IR-S KCl spectrometer. NMR spectra were determined with a JNM-4H-100 spectrometer with tetramethylsilane as the internal standard. Mass spectra were determined with CEC-110B and JMS-01S spectrographs equipped with direct inlet systems. The electron accelerating energy was 70 eV and chamber temperature 150~180°C.

Isolation of gibberellin A_{20} . Immature seeds (60 kg) of morning-glory were extracted with methanol using a blender. The ethyl acetate-soluble acidic fraction was obtained from the methanol extract by the usual method. The acidic gum (28.7 g) thus obtained was purified in the following procedures.

A) Countercurrent distribution. The crude material was distributed between each 500 ml of ethyl acetate and 1 M phosphate buffer (pH 5.5). After ten transfers countercurrent, first and eleventh fractions were excluded. Aqueous phases of fractions No. 2~10 were combined, acidified to pH 3.0 and ex-

tracted with three 1500 ml portions of ethyl acetate. The all ethyl acetate layers were combined and evaporated to afford 14.1 g of biologically active gum.

B) Charcoal column chromatography. The gum thus obtained was dissolved in water-acetone mixture (80:20, v/v), and charged on a column packed with 100 g of charcoal (Wako, activated charcoal for chromatography). The column was eluted with 1 liter of water-acetone (80:20, v/v) and then acetone content was increased by 10% step for every 1 liter. Elution with acetone-water (50:50 and 60:40, v/v) gave F-I (1.8 g) and acetone-water (70:30, v/v) F-II (1.5 g).

C) Silicic acid adsorption chromatography. F-I and F-II were purified by different columns of silicic acid (30 g). Each column was eluted with 100 ml portions of benzene-ethyl acetate mixture increasing ethyl acetate content. The most active fraction (62 mg) was obtained from F-I by the elution with benzene-ethyl acetate (45:55 and 40:60, v/v) and that (110 mg) from F-II with benzene-ethyl acetate (65:35~55:45, v/v).

D) Preparative thin layer chromatography. Each active fraction obtained from F-I and F-II was spotted on thin layer of silica gel (0.5 mm in thickness). After development with benzene-*n*-butanol-acetic acid (70:25:5, v/v), the active zones (R_f 0.42 in F-I and R_f 0.67 in F-II) were cut off and extracted with ethyl acetate to afford active gum, which was treated with diazomethane. The methyl ester obtained from F-I was subjected to thin layer chromatography as well as gas chromatography and identified as GA_3 methyl ester. The ester from F-II was further purified with preparative thin layer chromatography by the use of ethyl ether-benzene (4:1, v/v). The zone corresponding to R_f 0.4 was extracted with ethyl acetate to afford 11 mg of rods, which were then recrystallized from acetone-*n*-hexane to give 7 mg of pure GA_{20} methyl ester, mp 181°C. Found: mol. wt. 346.177 (MS). Calcd. for $C_{20}H_{26}O_5$: mol. wt. 346.178. IR_{max}^{nujol} cm^{-1} : 3520, 1778, 1720, 1670, 882. NMR (in $CDCl_3$) τ : 8.90 (3H, s), 6.33 (3H, s), 5.10 (1H, s), 4.80 (1H, s).

Hydrolysis of GA_{20} methyl ester. To a solution of 5 mg of GA_{20} methyl ester in 0.3 ml of ethanol, was added 3 ml of 0.1 N aqueous sodium hydroxide solution. On standing at 30°C for 20 hr, the solution was neutralized to pH 7 with dil. H_2SO_4 and concentrated. The aqueous residue was extracted with ethyl acetate to give acidic and neutral frac-

tions according to the usual method. On evaporation of the solvent, the acidic fraction gave 3mg of crude crystals, which were then recrystallized from acetone-*n*-hexane to afford 2mg of GA₂₀ as rods melting at 232~233°C. IR $\begin{smallmatrix} \text{Nujol} \\ \text{max} \end{smallmatrix}$ cm⁻¹: 3420, 1767, 1738, 1664, 878. GA₂₀ thus obtained regenerated GA₂₀ methyl ester on treatment with diazomethane.

Preparation of GA₂₀ methyl ester from GA₅ methyl ester. GA₅ methyl ester (148 mg) was hydrogenated over 2% palladium on barium carbonate (300 mg) in ethyl acetate (18 ml) and pyridine (1.2 ml) under atmospheric pressure until one molar equivalent hydrogen (10.5 ml, at 20°C) was absorbed. After removal of the catalyst the solvent was evaporated and the residue was charged into a column of silicic acid (40 g, impregnated with 10 g of silver nitrate). The column was successively eluted with 500 ml of benzene-ethyl acetate (95:5, v/v, fractions No. 1~30) and 500 ml of the same solvent system with a varied composition (92:8, v/v, fractions No. 31~60). The components in each fraction were examined by thin layer chromatography using silicic acid impregnated with silver nitrate and NMR spectra. Fractions No. 6~20 were found to contain IV and V, fractions No. 21~24 II, fractions No. 25~35 II and III, and fractions No. 36~47 III.

Then fractions No. 21~24 were combined and evaporated to afford 22 mg of crystals. Recrystallization from acetone-*n*-hexane yielded 20 mg of rods melting at 183°C. Its IR and NMR spectra were completely identical with those of GA₂₀ methyl ester obtained from immature seeds of morning-glory.

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