Gibberellin A₅₉: A New Gibberellin from *Canavalia gladiata*

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Gibberellin A₅₉ has been isolated from immature seeds of *Canavalia gladiata* (sword bean) and its structure was determined to be Δ^2 -gibberellin A₂₁ (1).

Canavalia gladiata is a unique legume in that its immature seed produces C18-oxidized gibberellins, GA₂₁ and GA₂₂.^{1,2)} Such gibberellins have not been found from any other sources including higher plants or fungi. This prompted us to reinvestigate its endogenous gibberellins.

The acidic fraction obtained from immature seeds was purified by silica gel partition chromatography.³⁾ Biological activity of each fraction thus obtained was examined by dwarf rice test (Fig. 1).⁴⁾ The eluate with 45% ethyl acetate which showed the strongest growth-promoting activity was purified by TLC to afford GA₂₂ and a new gibberellin which was

named GA₅₉ based on the MacMillan and Takahashi's proposal.⁵⁾ In the dwarf rice microdrop test⁶⁾ GA₅₉ did not show activity up to 1 μ g per plant (Table I). Therefore, the activity

TABLE I. POTENCY OF GA₅₉ IN THE TAN-GINBOZU MICRODROP TEST

Data expressed as length of 2nd leaf sheath in mm $\pm$ standard error ($n=5$). Controls, 23.7 ± 0.7 .

GA	Dosage, μ g/plant			
	0.001	0.01	0.1	1
GA ₃	32.6 ± 1.9	47.8 ± 2.0	76.4 ± 2.2	70.8 ± 2.0
GA ₅₉		22.0 ± 0.7	22.0 ± 0.4	22.0 ± 2.0
GA ₂₁		23.0 ± 0.3	27.0 ± 0.7	28.6 ± 1.0

FIG. 1. Distribution of Biological Activity after Silica Gel Partition Chromatography.

For details of procedure, see text. 8 g fr. wt. equivalents were used for Tan-ginbozu water-culture test.

FIG. 2. Mass Spectrum of GA_{59} Methyl Ester (40 eV). FIG. 3. 1H NMR Spectrum of GA_{59} Methyl Ester (100 MHz) in $CDCl_3$.

of the 45% ethyl acetate fraction will be due to GA_{22} .

GA_{59} was treated with diazomethane to afford a dimethyl ester. The mass spectrum of the ester, obtained by GC-MS, is shown in Fig. 2. In the high resolution MS the molecular ion peak is observed at m/z 388.1529 ($C_{21}H_{24}O_7$). The base peak at m/z 284.1400 ($C_{18}H_{20}O_3$) is due to elimination of carbon dioxide and methyl formate from the molecular ion. This ion is degraded into an ion at m/z 225.1232 ($C_{16}H_{17}O$) with loss of a methoxycarbonyl, which is confirmed by the presence of a metastable peak at 178.2. Further

loss of water gives rise to a hydrocarbon peak at m/z 207.1086 ($C_{16}H_{15}$), as was indicated by a metastable peak at 190.5. These facts indicate that GA_{59} carries two carboxyls, one lactone, one hydroxyl and two double bonds. Fragmentation pattern down to m/z 250 resembles that of GA_{21} dimethyl ester although peaks due to GA_{59} methyl ester are found at two mass units lower than that of GA_{21} dimethyl ester, suggesting that GA_{59} is a dehydro derivative of GA_{21} .

In the 1H NMR spectrum⁷⁾ of the methyl ester in deuteriochloroform (Fig. 3) the protons at C5 and C6, which are characteristic of

gibberellins, appear as a pair of doublets at δ 3.06 and 3.19 ($J=10$ Hz). Two methoxycarbonyl groups are observed at δ 3.74 and 3.77 but no C-methyl group is detected, indicating that GA₅₉ is a gibberellin with the oxidized C18. The exocyclic methylene protons are observed as a pair of broad singlets at δ 4.95 and 5.26. A large downfield-shift of the latter signal in deuteropyridine (δ 5.63) strongly indicates the presence of a hydroxyl at C13.⁷⁾ The endocyclic olefin protons are observed at δ 6.34 (1H, broad double triplet, $J=10$ Hz)* and 5.91 (1H, double triplet, $J=3, 10$ Hz). It was found in the double resonance experiment (Fig. 3) that these protons are coupled with methylene protons at δ 2.66 (1H, broad doublet, J (geminal)=20 Hz) and 2.33 (1H, broad doublet, J (geminal)=20 Hz). Therefore the double bond must be located only in the A ring. One of the olefinic protons absorbs at the unusually low field (δ 6.34) compared with those of GA₅ methyl ester (5.65),⁸⁾ GA₃₁ (5.67 in deuterioacetone),^{8,9)} and GA₂₂ methyl ester (5.85).^{2,8)} This suggests that this proton should be in the vicinity (*i.e.* C3 position) of the C4 methoxycarbonyl group which is to exhibit a deshielding effect.¹⁰⁾ Thus it is indicated that the double bond is situated between C2 and C3. All evidences above-cited, together with biosynthetic view-points, allow us to assign the structure 1 to GA₅₉.

The other fractions obtained after the partition chromatography were analyzed as follows. The 50% ethyl acetate fraction was purified by TLC, methylated and analysed by GC-MS, resulting in the identification of GA₂₁ (major) and GA₅₉. The 24% ethyl acetate fraction was methylated and purified by TLC

to afford a compound, which was identified as GA₅₉ dimethyl ester based on GC-MS. Since the *R_f* value of the original acid was similar to that of GA₅, but much larger than that of GA₅₉, it is tentatively assigned as a mono-methyl ester of GA₅₉. This fact may suggest occurrence of a glucosyl ester of GA₅₉ in the seed since the GA₅₉ monomethyl ester is likely to be formed by methanolysis of the conjugate during the methanol extraction. The inhibitory activity found in the 12% and 15% ethyl acetate fractions were ascribed to abscisic acid based on TLC analysis.

EXPERIMENTAL

NMR spectra were taken with a JEOL FX-100 spectrometer. Unless otherwise stated the data shown in the text was obtained in CDCl₃ using tetramethylsilane as an internal standard. GC-MS was carried out with a Hitachi RMU-6L spectrometer (40 eV) equipped with a Hitachi 063 gas chromatograph. A glass column packed with 2% OV-1 (1 m × 3 mm) was used. High resolution mass spectra were taken with JEOL JMS-D300 spectrometer (90 eV).

TLC was performed using Merck silica gel TLC plates with solvent A (EtOAc-CHCl₃-AcOH=15:5:1) and solvent B (CHCl₃-MeOH-AcOH-H₂O=80:20:5:2). Elution was effected with MeOH. After the MeOH was removed, the residue was mixed with std. NaCl soln, acidified (pH 2) with dil. HCl and extracted 5 times with EtOAc to give gibberellin fractions. Gibberellins and ABA were detected as fluorescent spots under an UV light after heating the TLC plate treated with 70% H₂SO₄.

Bioassay was carried out using the Tan-ginbozu water culture method⁴⁾ or microdrop method.⁶⁾

Methylation was performed with ethereal diazo-methane.

Purification procedure.

Extraction. Immature seeds (400 g) were extracted with MeOH. The extract, after evaporating the MeOH, was partitioned 3 times against EtOAc at pH 3. The EtOAc phase was fractionated into an acidic and neutral fraction.

Partition chromatography.³⁾ The acidic fraction was purified on a column (2.7 × 22 cm) made of 50 g silica gel impregnated with 31.3 ml of 0.5 M aq. formic acid which was beforehand saturated with 1% EtOAc in hexane. Hexane-EtOAc mixtures saturated with 0.5 M formic acid were used for elution with increasing EtOAc contents (Fig. 1). Eluates were collected in 400 ml fractions.

TLC. The eluate with 12% and 15% EtOAc showed a yellow-green fluorescent spot at *R_f* 0.42 on TLC (solvent

* This proton was observed as a sharp double triplet ($J=2, 10$ Hz) when measured in deuteropyridine (see EXPERIMENTAL).

A), which indicated the presence of *cis,trans*-ABA. Thus the inhibitory activity of these fractions may be due to *cis,trans*-ABA.

The eluate with 24% EtOAc showed a GA-like spot at *Rf* 0.37 (cf. *Rf* of GA₅=0.40, *Rf* of GA₂₁=0.10) on TLC (solvent A). This fraction was esterified and purified by TLC (ether; *Rf* ca. 0.5) to afford GA₅₉ dimethyl ester.

The eluate with 45% EtOAc was subjected to TLC (solvent B). The zone at *Rf* 0.54~0.61 afforded GA₂₂, which was identified by GC-MS of its methyl ester, whilst the zone at *Rf* 0.22~0.28 yielded GA₅₉ (less than 1 mg), mp 248°C (decomposition). GA₅₉ was converted into a dimethyl ester. NMR (in C₅D₅N): 3.34 (1H, d, *J*=10), 3.50 (1H, d, *J*=10), 3.65 (3H, s), 3.68 (3H, s), 5.06 (1H, broad s), 5.63 (1H, broad s), 5.85 (1H, dt, *J*=3, 10), 6.50 (1H, dt, *J*=2, 10). MS (high resolution): 388.1529 (388.1523 calcd for C₂₁H₂₄O₇), 284.1400 (284.1411 calcd for C₁₈H₂₀O₃), 225.1232 (225.1279 calcd for C₁₆H₁₇O), 207.1086 (207.1173 calcd for C₁₆H₁₅), 200.0844 (200.0838 calcd for C₁₃H₁₂O₂). Trimethylsilyl derivative of the ester showed the following MS in GC-MS: 460 (M⁺, 38%), 445 (4), 428 (7), 401 (13), 356 (8), 343 (12), 238 (6), 235 (5), 208 (15), 207 (22), 193 (15), 180 (7), 167 (11), 141 (7), 139 (4), 75 (18), 73 (100).

The eluate with 50% EtOAc was purified by TLC (solvent A). The zone at *Rf* 0.1 afforded a mixture of GA₂₁ (major) and GA₅₉. The zone with a higher *Rf* value yielded needles which crystallised from H₂O and melted (decomposed) at 259°C. From the mass spectrum (M⁺ 170) this compound was identified as gallic acid.

Chromatographic behaviors of GA₂₁, GA₂₂ and GA₅₉. *Rf*s in TLC (solvent B): 0.50 (GA₂₂), 0.28 (GA₂₁), 0.22 (GA₅₉). *Rt*s in GC (2% QF-1, 230, 37 ml/min N₂): 14.1 (GA₂₂ Me), 18.8 (GA₂₁ Me), 19.6 min (GA₅₉ Me).

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