Isolation and Structures of Gibberellin A₃₅ and Its Glucoside from Immature Seed of *Cytisus scoparius*

Hisakazu YAMANE, Isomaro YAMAGUCHI, Noboru MUROFUSHI
and Nobutaka TAKAHASHI

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

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A new gibberellin, gibberellin A₃₅, and its glucoside were isolated from immature pods of yellow broom (*Cytisus scoparius*) and their structures were elucidated as I and III, respectively.

In the preliminary communication,¹⁾ we reported the isolation of a new gibberellin, gibberellin A₃₅, and its glucoside from immature seeds of yellow broom (*Cytisus scoparius*) and their structural assignment. We report herein the details of this work.

The immature pods (37 kg) harvested in 1970 were extracted with methanol and the extract was concentrated *in vacuo*. The resulting aqueous residue was acidified and extracted with ethyl acetate and *n*-butanol in turn. The both extracts were separated into an acidic and a neutral fractions.

The ethyl acetate-soluble acidic fraction was purified by ten transfers countercurrent distribution between 1 M phosphate buffer of pH 5.4 and ethyl acetate. The biologically active fractions were combined and subjected to charcoal adsorption chromatography (elution with acetone-water), followed by silicic acid adsorption chromatography (elution with ethyl acetate-benzene). Recrystallization of the eluate with ethyl acetate-benzene (50:50) in the silicic acid chromatography afforded 20 mg of a gibberellin-like substance (F-I). The resulting mother liquor of the recrystallization and the eluate with ethyl acetate-benzene (60:40) were combined and purified by the preparative TLC after methylation with diazomethane. Thus the methyl esters of F-I (5 mg) and another gibberellin-like substance (F-II, 6.6 mg) were obtained.

From 40 kg of immature pods of yellow

broom harvested in 1972, we isolated 41 mg of F-II through the careful purification, particularly in the point of pH of the extracts in the concentration process, while the yield of F-I was negligible. Thus F-I was concluded to be an artifact derived from F-II in the purification process. F-II was confirmed to be a new gibberellin from the following evidences, which was named gibberellin A₃₅ (GA₃₅).

GA₃₅ showed mp 252~254°C and formed a monomethyl ester on treatment with diazomethane, whose high resolution mass spectrum exhibited a molecular ion peak at *m/e* 362.1699 with composition C₂₀H₂₆O₆ (calcd. 362.1727). A molecular ion peak at *m/e* 506 in the mass spectrum of TMS-GA₃₅-Me* indicated the presence of two hydroxyl groups in GA₃₅. As shown in Fig. 1, the IR spectrum of GA₃₅ in nujol mull revealed the presence of hydroxyl (3446 cm⁻¹), carbonyl (1730) and exocyclic methylene (1653) groups. The NMR spectrum of GA₃₅-Me* in deuteriochloroform showed a 3H singlet at δ 1.15 due to C-4 CH₃ of the *ent*-gibberellane ring, a pair of 1H doublets at δ 3.26 and 2.74 (*J*=10.5 Hz) characteristic of the C-5, C-6 protons and two 1H singlets at δ 4.91 and 5.04 due to the exocyclic methylene protons. A 1H triplet at δ 3.86 (*J*=2.3 Hz) and a 1H multiplet at δ 4.22 were assigned to two carbinyl protons. The

* Methyl esters of gibberellins and their trimethylsilyl ether are abbreviated as GA_n-Me and TMS-GA_n-Me, respectively.

FIG. 1. IR Spectrum of GA_{35} (Nujol). FIG. 2. Mass Spectrum of GA_{35} -Me. FIG. 3. NMR Spectrum of GA_{35} -Me ($CDCl_3$).

mass and NMR spectra of GA_{35} -Me are shown in Figs. 2 and 3.

F-I and F-I-Me showed mp 269~271 and 208~210°C, respectively. The high resolution mass spectrum of F-I-Me indicated a molecular ion peak at m/e 362.1725 with composition $C_{20}H_{26}O_6$ (calcd. 362.1727), suggesting that it is an isomer of GA_{35} -Me. As shown in Fig. 4, F-I-Me exhibited an intense molecular ion peak at m/e 362 and prominent peaks at M-18 (H_2O), M-32 (CH_4O), M-43 (C_3H_7), M-46 (CH_2O_2), M-50 (CH_6O_2) and M-60 ($C_2H_4O_2$) in the mass spectrum, which constitute a pattern characteristic of the mass

spectra of gibberellin methyl esters²⁾ as in the case of GA_{35} -Me. The mass spectrum of TMS-F-I-Me revealed a molecular ion peak at m/e 434, indicating the presence of a hydroxyl group in F-I. The IR spectrum of F-I-Me in nujol mull revealed the presence of hydroxyl (3520 cm^{-1}), γ -lactone (1773) and methoxycarbonyl (1710) groups in F-I, but no double bond. The IR and NMR spectra of F-I-Me are illustrated in Figs. 5 and 6.

The NMR spectrum of F-I-Me in deuteriochloroform exhibited a 3H singlet at δ 1.22 due to C-4 CH_3 , a 3H singlet at δ 1.43 characteristic of C-16 CH_3 in GA_2 and GA_{10} type gib-

FIG. 4. Mass Spectrum of F-I-Me.

FIG. 5. IR Spectrum of F-I-Me (Nujol).

FIG. 6. NMR Spectrum of F-I-Me (CDCl₃).

berellins,³⁾ a pair of 1H doublets at δ 3.22 and 2.53 ($J=6.5$ Hz) due to the C-5, C-6 protons and a 1H triplet at δ 3.90 ($J=2.3$ Hz) due to a carbinyl proton. A 1H triplet at δ 4.53, which is distinctive in its low chemical shift,

was deduced to be due to >CH-OR .

Based on these evidences, it is concluded that GA₃₅ and F-I contain the functional groups summarized in Table I. The comparison of the functionalities between GA₃₅ and

TABLE I. FUNCTIONAL GROUPS IN GA₃₅ AND F-I

	GA ₃₅	F-I
Sec. -OH	2	1
-CH-OR	0	1
γ-Lactone	1	1
-COOH	1	1
>C=CH ₂	1	0
-C-CH ₃	1	2

F-I suggests the possibility that F-I was derived from GA₃₅ by the ether formation between one of hydroxyl groups and C-16 in GA₃₅, resulting in the formation of cyclic ether and disappearance of a double bond in F-I. This was confirmed by the quantitative conversion of GA₃₅ to F-I by boiling GA₃₅ in 0.3 N methanolic hydrochloric acid for 1 hr. Such facile ether formation can be expected only between C-11β or C-12β hydroxyl group and C-16. The possibility for C-12β hydroxyl group in GA₃₅ was excluded from the following observation. It has been known that the C-12β hydroxyl group in the known compound (IV) causes down-field shifts of exocyclic methylene protons to show 2H broad singlet at δ 5.16 in the NMR spectrum in deuterochloroform,⁴⁾ while the signals due to the exocyclic methylene protons of GA₃₅-Me did not show such down-field shifts.

The formation of ether linkage between C-11β and C-16 in the conversion of GA₃₅ to F-I causes the conformational change of the ring C from boat to chair, resulting in the change of the dihedral angle between C-5 H and C-6 H and the conversion of C-11α H from axial to equatorial. The small coupling constant between C-5 H and C-6 H ($J=6.5$ Hz) in F-I and the down-field shift of C-11α H can be well explained by this conformational change.

It should be noted that GA₃₅ showed an intense M-18 peak in the mass spectrum, which has been considered to be characteristic feature of gibberellin methyl esters containing C-12 hydroxyl group.⁵⁾

The presence of a hydroxyl group at C-3β in GA₃₅ and F-I was shown by a down-

field shift of C-4 CH₃ (0.3 ppm) and C-5 H (0.5 ppm) in the NMR spectra of F-I-Me in the change of the solvent from deuterochloroform to deuteropyridine.⁴⁾

Thus the structures of GA₃₅ and F-I were elucidated as I and II, respectively.

The *n*-butanol-soluble acidic fraction was purified by the following procedure. In the first process charcoal chromatography was carried out. The fractions eluted with acetone-water (30:70~40:60) were combined and subjected to partition chromatography using 1 M phosphate buffer of pH 5.7 on Sephadex G-50 (elution with *n*-butanol-ethyl acetate), successively to charcoal chromatography by gradient method (elution with acetone-water).

The active fractions were further purified by silicic acid adsorption chromatography (elution with methanol-chloroform), followed by preparative paper chromatography. Thus 130 mg of a new gibberellin glucoside was isolated. The compound yielded GA₃₅ on cellulase hydrolysis, and F-I and glucose on acid hydrolysis. The NMR spectrum of the compound in deuteroacetone containing deuterium oxide exhibited a 1H doublet at δ 4.43 ($J=7$ Hz) due to an anomeric proton characteristic of β-glucopyranoside. The trimethylsilyl ether of its methyl ester showed a molecular ion peak at m/e 884 in the mass spectrum, indicating it to be GA₃₅ glucoside.

GA₃₅ glucoside pentaacetate yielded F-I acetate (V) on 0.1 N methanolic hydrochloric acid hydrolysis. This indicates that the new

FIG. 7. NMR Spectrum of GA₃₅ Glucoside Pentaacetate (CDCl₃).

glucoside is 11-O- β -D-glucosyl GA₃₅. This assignment was supported by a decoupling experiment, shown in Fig. 7, which showed a ¹H multiplet due to C-11 α proton remained in the neighborhood at δ 4.22 in the NMR spectrum of GA₃₅ glucoside pentaacetate in deuteriochloroform.

It is worthy to note that the main endogenous gibberellins in immature seed of yellow broom are GA₃₅ and its glucoside, providing the first example of C-11 hydroxygibberellin, and the seeds contain a fairly large amount of glucoside even in undeveloped stage.

The growth promoting effects of GA₃₅, F-I and GA₃₅ glucoside in seven bioassay systems were reported separately.⁶⁾

EXPERIMENTAL

IR spectra were measured in nujol mull with a JASCO IR-S KCl spectrometer. NMR spectra were determined with a JEOL JNM-4H-100 spectrometer using tetramethylsilane as the internal standard. Mass spectra were obtained with a Hitachi RMU-6L spectrometer equipped with a direct inlet system. The electron accelerating voltage was 70 eV and chamber temperature, 180°C. High resolution mass spectra were measured with a Hitachi RMH-2 spectrometer with a direct inlet system under the following conditions: electron accelerating voltage, 70 eV; chamber tempera-

ture, 180°C.

Gas chromatography

A Hitachi K-53 gas chromatograph with a hydrogen flame-ionization detector was used. A silanized glass column, 1 m $\times$ 3 mm i.d., was packed with 2% OV-1 on Chromosorb W (mesh 80~100). Retention times were determined under the following conditions: carrier gas, nitrogen flowing at 29.0 ml/min, column temperature, 197°C; GA₃₅-Me, 10.6 min; F-I-Me, 6.1 min; GA₁-Me, 10.1 min.

Isolation of F-I and methyl esters of F-I and GA₃₅ from the seeds harvested in 1970

Immature pods (37 kg) of yellow broom harvested in 1970 were extracted with methanol using a blender. The methanol extract was separated into ethyl acetate and *n*-butanol-soluble acidic fractions by the usual method. The ethyl acetate-soluble acidic fraction (67.7 g) was purified through the following procedure.

a) *Countercurrent distribution.* The ethyl acetate-soluble acidic gum was purified by ten transfers countercurrent distribution between each 300 ml of ethyl acetate and 1 M phosphate buffer (pH 5.4). Among eleven fractions thus obtained, No. 7~10 (24.7 g) were active on the dwarf rice bioassay (Tan-ginbozu) and showed gibberellin-like spots on TLC.

b) *Charcoal column chromatography.* The active gum obtained in a) was dissolved in acetone-water (20: 80) and charged on a column packed with 200 g of charcoal (Wako, for chromatography). The column

was eluted with 4 liters of acetone-water (20: 80) and then acetone content was increased by 5% steps for every 4 liters. Active substances were eluted with acetone-water (35: 65~45: 55, 3.2 g).

c) *Silicic acid adsorption chromatography.* The active fraction in b) were dissolved in ethyl acetate-benzene (20: 80) and charged on a column packed with 33 g of silicic acid (Mallinckrodt). The column was eluted with 200 ml of ethyl acetate-benzene (20: 80) and with the same solvent system with increasing ethyl acetate content by 10% steps for every 200 ml. From the eluate with ethyl acetate-benzene (50: 50), 20 mg of F-I (prisms, mp 269~271°C) was isolated by the fractional crystallization with ethyl acetate and hexane. F-I, $\text{IR}_{\text{max}}^{\text{NaCl}} \text{ cm}^{-1}$: 3480, 1760, 1715.

d) *Preparative thin-layer chromatography.* The mother liquor of the recrystallization in c) and the eluate with ethyl acetate-benzene (60: 40) were methylated with diazomethane and subjected to preparative TLC under the following conditions: adsorbent, Kieselgel GF₂₅₄ (Merck); solvent system,* ethyl acetate-chloroform-acetic acid (20: 8: 1). The zones with *R_f* 0.53 and 0.58 were scraped and eluted with ethyl acetate to give 6.6 mg of GA₃₅-Me (non-crystalline solid) and 5 mg of F-I-Me (prisms, 208~210°C), respectively. F-I-Me, $\text{NMR } \delta_{\text{Me}_4\text{Si}}^{\text{CD}_3\text{D}_5\text{N}}$: 1.43 (3H, s, C-4 CH₃), 1.52 (3H, s, C-16 CH₃), 2.87 (1H, d, *J*=6.5 Hz, C-6 H), 3.72 (1H, d, *J*=6.5 Hz, C-5 H), 3.65 (3H, s, -COOCH₃), 4.08 (1H, t, *J*=2.3 Hz, C-3 H), 4.57 (1H, t, *J*=3 Hz, C-11 H).

Isolation of GA₃₅ from the seeds harvested in 1972

The ethyl acetate-soluble acidic fraction obtained from the seeds harvested in 1972 was purified through the above mentioned procedures. The active fractions in silicic acid adsorption chromatography were subjected to preparative TLC. The band at *R_f* 0.37 was scraped and eluted with ethyl acetate, followed by crystallization to yield 41 mg of GA₃₅ (needles, mp 252~254°C). $\text{NMR } \delta_{\text{Me}_4\text{Si}}^{\text{CD}_3\text{COCD}_3}$: 1.12 (3H, s, C-4CH₃), 2.59 (1H, d, *J*=11 Hz, C-6 H), 3.23 (1H, d, *J*=11 Hz, C-5 H), 3.72 (1H, ill-defined multiplet, C-3 H), 4.10 (1H, m, C-11 H), 4.86 and 5.00 (a pair of 1H broad singlets, >C=CH₂).

Isolation of GA₃₅ glucoside

a) *Charcoal column chromatography.* The *n*-butanol-soluble acidic fraction (98.4 g) dissolved in water was charged on a column packed with 200 g of charcoal. The column was eluted with 4 liters portions of acetone-water mixture increasing acetone content by 5% steps for every 4 liters. The active substance was eluted with acetone-water (30: 70~40: 60).

b) *Partition chromatography.* The active gum (6.3 g) obtained in a) was dissolved in 20 ml of water, and to this solution 4 g of Sephadex G-50 was added. The gel thus obtained was charged onto a top of column packed with 26 g of Sephadex G-50 impregnated with 130 ml of 1 M phosphate buffer of pH 5.7, and the column was eluted with 1 liter of *n*-butanol-ethyl acetate (5: 95) increasing *n*-butanol content by 5% steps for every 1 liter. The eluates (1.27 g) with *n*-butanol-ethyl acetate (15: 85~25: 75) contained the biologically active substance.

c) *Charcoal column chromatography by gradient method.* The active fraction obtained by b) was charged on a column packed with 10 g of charcoal and the column was eluted by gradient method using 500 ml of water and 500 ml of acetone (Fraction No. 1~20). Based on TLC, Fr. No. 6~8 were found to contain gibberellin-like substance.

d) *Silicic acid adsorption chromatography.* The active fractions in c) were dissolved in 5 ml of methanol-chloroform (10: 90) and charged on a column packed with 10 g of silicic acid. The column was eluted with 50 ml of methanol-chloroform (10: 90) increasing methanol content by 5% steps for every 50 ml. The eluates with methanol-chloroform (15: 85~20: 80, 362.4 mg) contained the active component.

e) *Preparative paper chromatography.* The active solid obtained by d) was subjected to preparative paper chromatography using Toyo filter paper No. 51 developed with isopropanol-7 N NH₄OH-water (8: 1: 1, v/v). The band at *R_f* 0.28 afforded 130 mg of GA₃₅ glucoside. $\text{IR } \nu_{\text{max}}^{\text{NaCl}} \text{ cm}^{-1}$: 3360 (broad), 1750, 1707, 1100~1000. $\text{NMR } \delta_{\text{Me}_4\text{Si}}^{\text{CD}_3\text{COCD}_3}$: 1.13 (3H, s, C-4 CH₃), 4.43 (1H, d, *J*=7 Hz, anomeric proton), 4.88 and 5.02 (a pair of 1H br. s, >C=CH₂). TMS-GA₃₅ glucoside-Me, MS *m/e* 884 (*M*⁺).

Enzymatic hydrolysis of GA₃₅ glucoside

GA₃₅ glucoside (10 mg) in 1 M acetate buffer (1 ml, pH 4.5) was mixed with 20 mg of cellulase (Sigma) and kept at 37°C for 16 hr. The resulting solution was acidified with sulfuric acid at pH 3 and extracted with ethyl acetate. The solid obtained after evaporating the solvent *in vacuo* was purified by preparative TLC, followed by recrystallization from ethyl acetate-hexane to yield needles of GA₃₅ (3 mg, mp 252~254°C).

Acid hydrolysis of GA₃₅ glucoside

GA₃₅ glucoside was hydrolyzed with 1 N HCl in a sealed tube at 100°C for 1 hr. F-I was recovered from ethyl acetate-soluble acidic fraction. The aqueous residue was evaporated to dryness, dissolved in dry pyridine and treated with silylating reagent.⁷⁾ Thus TMS-glucose was detected by GC.

* This solvent system for TLC was used throughout this study.

Acetylation of GA₃₅ glucoside

GA₃₅ glucoside (16 mg) was treated with pyridine-acetic anhydride (2: 1) overnight at room temperature. The crude acetate was purified by recrystallization from ethyl acetate-hexane to yield GA₃₅ glucoside pentaacetate as needles (14.8 mg, mp 175~177°C). IR $\nu_{\text{max}}^{\text{nujol}}$ cm⁻¹: 1777, 1748, 1708, 1653. NMR $^{\text{CDCl}_3}_{\text{Me}_4\text{Si}}$: 1.10 (3H, s, C-4 CH₃), 2.70 (1H, d, *J*=10 Hz, C-6 H), 3.15 (1H, d, *J*=10 Hz, C-5 H), 4.62 (1H, d, *J*=7 Hz, anomeric proton). GA₃₅ glucoside-Me pentaacetate, MS *m/e*: 734 (M⁺)

Acid hydrolysis of GA₃₅ glucoside pentaacetate

GA₃₅ glucoside pentaacetate was hydrolysed with 0.1 N methanolic HCl in a sealed tube at 100°C for 1 hr. The acidic products obtained from hydrolysate were methylated with diazomethane and purified by preparative TLC. F-I-Me acetate zone at *Rf* 0.77 was scraped and eluted with ethyl acetate. The product was confirmed to be F-I-Me acetate by comparing the mass spectrum with that of the authentic sample prepared by treatment of F-I-Me with acetic anhydride in pyridine at room temperature. MS *m/e*: 404 (M⁺).

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