Gibberellin in Vetch Seeds

Yosh Kimura

A microgram amount of a substance with the biological, chromatographic, fluorescent, and fluorescence development properties of a gibberellin was isolated from immature seeds of *Vicia villosa* Roth. Its chromatographic properties are identical to those of gibberellin A_5 except for a small R_f difference in one of the solvent systems. The fluorescence development properties do not correspond to gibberellin A_5. Twenty-three gibberellins can be eliminated on the bases of the separation technique and other data, leaving as possible candidates gibberellin A_{20} or an isomer. A parent ion peak of 332, corresponding to the mass of A_{20}, was obtained from the mass spectrum of this compound.

The seeds of the vetch (*Vicia villosa* Roth) were extracted for gibberellins in an attempt to find a readily available local source of various gibberellins. Immature seeds are an especially rich source of these plant growth regulators (Corcoran, 1959) which were isolated originally as metabolic products of a fungus (Yabuta and Sumiki, 1938) prior to its first isolation from higher plants (MacMillan and Suter, 1958).

This paper reports the isolation and properties of a gibberellin A_5-like substance from vetch seeds and its probable identity as gibberellin A_{20}.

EXPERIMENTAL

Extraction and Isolation. Immature vetch seeds (417 grams), were homogenized for 30 minutes in a Lourdes Multi-Mix homogenizer with 830 ml. of methanol and allowed to stand at 2° C. overnight. The liquid was separated by centrifugation and decantation. The extraction was repeated twice more using the same volume of methanol with overnight stirring at room temperature. The combined extracts were evaporated to near dryness under reduced pressure at <35° C. The residue was suspended in 200 ml. of water. Sufficient 10% sodium carbonate was added to bring the pH to 7. After the flask was shaken vigorously to dislodge and disperse all solid particles, the pH was readjusted to 7. The mixture was centrifuged and the supernatant liquid was decanted through a glass fiber filter. The filtrate was acidified to pH 2.5 with 9*N* sulfuric acid and extracted with one 500-ml. and two 200-ml. portions of ethyl acetate. The combined pH 2.5 ethyl acetate extract was evaporated to dryness and redissolved in 5 ml. of ethyl acetate.

The ethyl acetate solution was passed through 1.7 × 21-cm. column of basic aluminum oxide (54 grams of Matheson, Coleman and Bell AX710) and the adsorbed material was eluted with 150 ml. each of ethyl acetate (I), ethyl acetate–methanol 1:1 (II), methanol (III), methanol–water 1:1 (IV), and water (V). The eluates were evaporated under reduced pressure. Each residue was suspended in 5 ml. of water. These suspensions were adjusted to pH 2 with sulfuric acid and extracted three times with 6 ml. of ethyl acetate. The extracts were evaporated and redissolved in 2 ml. portions of methanol. A 10-μl. portion of each solution was used for dwarf pea bioassay.

Biologically active eluate II was streaked on three 0.75-mm. silica gel G (SGG) thin layer chromatographic (TLC) plates and developed in 95:5 ethyl acetate–acetic acid. Gibberellins A_3 (GA_3) and A_7 (GA_7) were placed on narrow strips of each plate as markers. The plate was subsequently scraped in five equal fractions designated *ab*, *cd*, *ef*, *gh*, *ij* in ascending order. The marker strip was left on the plate and sprayed with 70% sulfuric acid to locate standards. Scrapings of each fraction were extracted four times with 35 ml. of methanol. Each extract was evaporated. The residue was suspended in 5 ml. of water, adjusted to pH 2, and extracted four times with 10-ml. of ethyl acetate. After evaporation, each fraction was redissolved in 2 ml. of methanol. Ten microliters were used for pea bioassay.

A one-tenth aliquot of the biologically active fraction *gh* was streaked on a 0.25-mm. SGG plate. Standards GA_3, GA_5, GA_7, and GA_9 were placed as markers on a strip along one edge. The plate was developed in the upper phase of benzene–acetic acid–water (8:3:5) after overnight equilibration in the vapors of both phases. The plate was scraped off in 10 equal strips from R_f 0 to 1.0 and designated, respectively, as subfractions 1 to 10. Each strip was extracted as before using four 10-ml. portions of methanol and brought to a volume of 1 ml. in ethyl acetate after evaporation of the methanol. Ten microliters were again used for pea bioassay.

Biological Assays, etc. Methods for biological assays, visualization of spots on chromatoplates using 70:30 sulfuric acid–water, and fluorescence emission spectra have been previously cited or described (Kimura, 1967).

Mass Spectrum. A mass spectrum was obtained on the residue in subfraction 4 remaining after TLC and bioassays. After the removal of ethyl acetate, the residue was redissolved in small portions of ethyl ether and transferred with washings to a 1.7-mm. O.D. capillary tubing. The ether in the capillary was evaporated by heating in a stream of warm air to a temperature slightly above the boiling point of ether. A Varian Model M66 mass spectrometer was used at an electron energy of 70 eV and a current of 30 μA. Probe temperature was 150° C; inlet, 100° C.; and analyzer, 110° C.

RESULTS

Dwarf pea bioassays of aluminum oxide column eluates I to V indicated that most of the biological activity was in eluate II (Figure 1*A*). Eluates II, III, and IV were phytotoxic to cucumber seedlings. Fractionation of eluate II by TLC on SGG using ethyl acetate–acetic acid 95:5 followed by pea bioassay (Figure 1*B*) revealed that the main activity was in fraction *gh* adjacent to the standard GA_7 and, consequently, likely to be GA_4, GA_5, GA_6, GA_7, or GA_{14}. Further fractionation of fraction *gh* on SGG with benzene–acetic acid–water followed by pea bioassay (Figure 1*C*) revealed this activity was in subfraction 4 adjacent to GA_5. The R_f value of the suspected GA_5 was identical to that of GA_5 in all but

Western Washington Research and Extension Center, Washington State University, Puyallup, Wash. 98371

Table I. Thin-Layer Chromatographic Comparison of *Vicia* gibberellin (VG) and gibberellin A_5

TLC System	R_f VG	R_f GA_5
Silica Gel PC[a]		
Isopropanol–4.5*N* ammonium hydroxide (3:1)	0.51	0.52, 0.52
Diisopropyl ether–acetic acid (95:5)	0.14	0.14, 0.14
Benzene–acetic acid–water (8:3:5)[b]	0.26	0.21, 0.22
Benzene–propionic acid–water (8:3:5)[b]	0.53	0.51, 0.53
Ethyl acetate–chloroform–acetic acid (15:5:1)	0.37	0.37, 0.36
Ethyl acetate–acetic acid (95:5)	0.47	0.47, 0.46
Methyl acetate–2-propanol–ammonium hydroxide (45:35:20)	0.28	0.29, 0.30
Benzene–1-butanol–acetic acid (80:15:5)	0.40	0.41, 0.41
(70:25:5)	0.55	0.55, 0.56
Kieselguhr G		
Benzene–propionic acid–water (8:3:5)[c]	0.95	0.95, 0.96
Carbon tetrachloride–acetic acid–water (8:3:5)[d]	0.97	0.97, 0.98

[a] Pre-coated, Brinkmann Instruments.
[b] Overnight equilibration, both phases present.
[c] Four-hour equilibration, both phases present.
[d] One-hour equilibration, both phases present.

one of the solvent systems used, namely benzene–acetic acid–water (Table I). Curvature of the solvent front was suspected as a possibility; however, repeated runs indicated the difference to be minor but reproducible and therefore, significant (Figure 2*A*, *C*).

Fluorescence emission spectra of the GA_5 and the suspected GA_5 were similar (Figure 3). However, a close examination of the fluorescence development characteristics showed that a 30-minute heating period at 120° C. was required for fluorescence visualizations (Figure 2*B*, *C*), whereas the fluorescence of GA_5 developed to a visible intensity during the first 10 minutes at 120° C. The period of heating required to induce fluorescence is one of the features by which gibberellins may be distinguished from one another (Jones *et al.*, 1963).

One-hour contact of the standard GA_5 in ethyl acetate solution with aluminum oxide and subsequent treatments did not alter either the chromatographic or the fluorescence development characteristics.

The mass spectrum (Figure 4) of the solid in subfraction 4 containing the *Vicia* gibberellin showed a parent ion at *m/e* 332 and prominent peaks at *m/e* 314 ($-H_2O$), 286 ($-HCO_2H$), and 289. These fragment ions correspond to peaks at *m/e* 314 ($-CH_3OH$, base peak), 286 ($-HCO_2CH_3$), and 289 described for GA_{20} methyl ester (MacMillan and Pryce, 1968). However, the peak at *m/e* 289 is much more intense in the spectrum of the *Vicia* gibberellin. Peaks at *m/e* 303 (−43) and 300 (−46) in the spectrum of the GA_{20} methyl ester (mass 346) correspond in the spectrum of the *Vicia* gibberellin to peaks at *m/e* 289 (−43) and 286 (−46). Prominent peaks at the lower end of the spectrum (*m/e* <200) were assumed to be due mostly to the obvious impurities present in the prepared sample as indicated by the yellowish discoloration of the subfraction 4 residue. GA_{20} was unavailable for comparison.

DISCUSSION

Cavell *et al.* (1967) have shown that 17 of at least 24 currently known gibberellins of known structures can be separated by TLC. Furthermore, their data show that by using silica gel and the solvent system, ethyl acetate–chloroform–acetic acid, the 17 gibberellins can be separated into four groups: the GA_8 group, including GA_2 and GA_8;

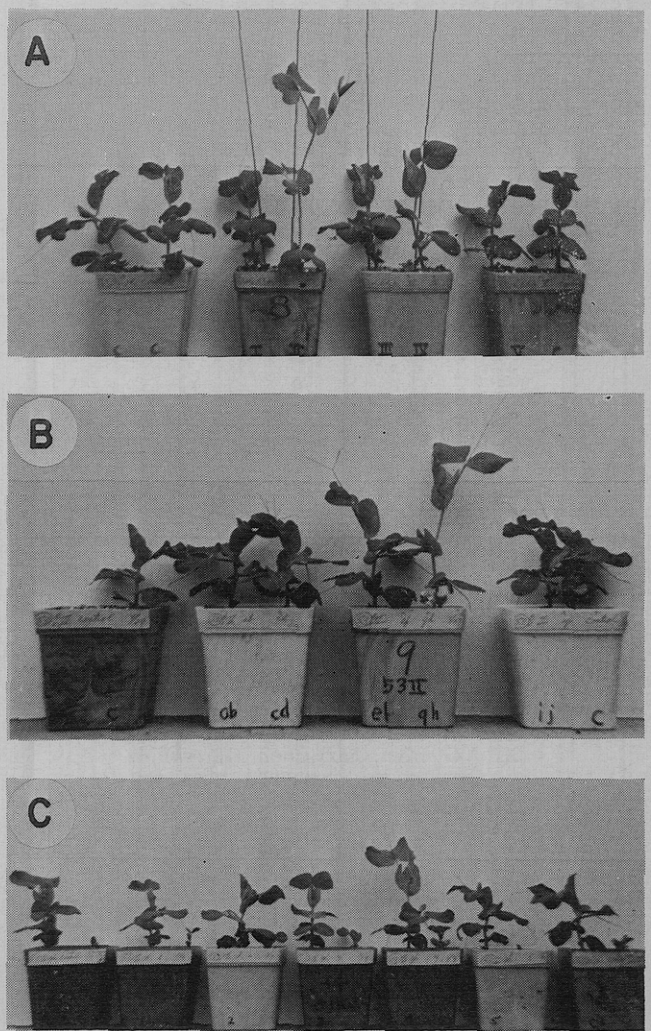

Figure 1. Dwarf pea bioassay of vetch extracts

A. At 8 days after application, plants left to right are: control, control, eluates I, II, III, IV, V, and control
B. At 9 days, left to right: control, fractions (II) *ab, cd, ef, gh, ij*, and control
C. At 4 days, left to right: control, subfractions (*gh*) 1, 2, 3, 4, 5, and control

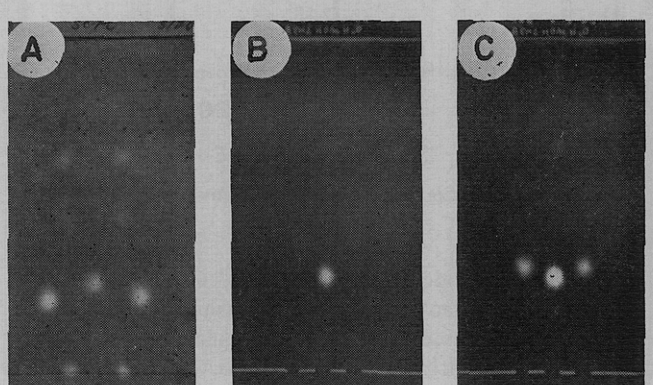

Figure 2. TLC comparison of R_f and fluorescence development characteristics of *Vicia* gibberellin (VG) and GA_5

Silica gel (SG) plates developed in benzene–acetic acid–water (8:3:5) system and transilluminated by long wave ultraviolet light after spraying with 70:30 sulfuric acid–water and heating
A. Precoated plate, 30 minutes at 120° C. Row of intense spots above starting line, left to right: GA_5, VG, GA_5
B. SGG plate, 10 minutes at 120° C, showing GA_5 only
C. Same plate as *B* after 30 minutes at 120° C, left to right: VG, GA_5, VG

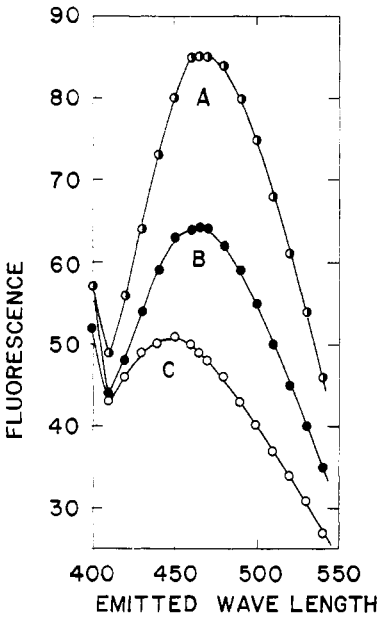

Figure 3. Fluorescence emission spectra of GA_5 and *Vicia* gibberellin (VG). Activation wavelength was 365 mμ

A. GA_5
B. VG from subfraction (II, *gh*) 4
C. Background, precoated silica gel

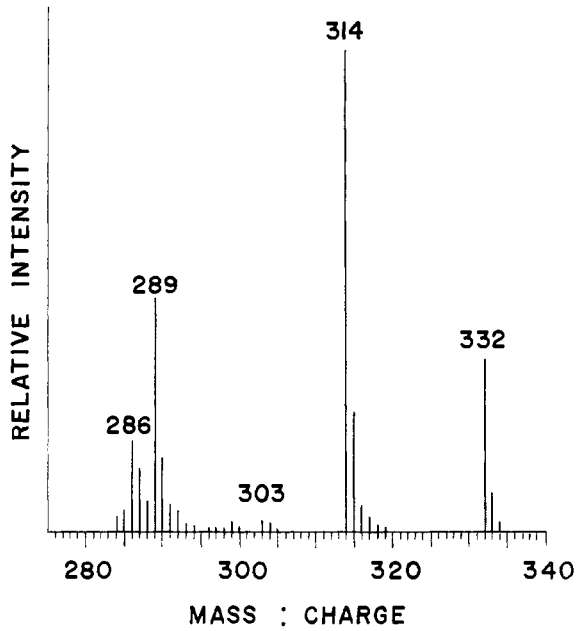

Figure 4. Upper portion of mass spectrum of *Vicia* Gibberellin

the GA_3 group, including GA_1, GA_3, GA_{10}, GA_{13}, GA_{18} (Lupinus I, see MacMillan and Takahashi, 1968, for assignment of trivial names GA_{18} to GA_{23}), and GA_{19} (Bamboo); the GA_7 group, including GA_4, GA_5, GA_6, GA_7, and GA_{14}; and the GA_9 group, including GA_9, GA_{11}, GA_{12}, and GA_{15}. These facts were considered in the isolation of the gibberellin in *V. villosa* using a similar solvent system. Fraction II *gh* which was biologically active on peas, thus was assumed to contain one or more of the GA_7 group. The GA_7 group can best be separated into its components on silica gel using benzene–acetic acid–water. Fractionation using this system placed the source of biological activity in subfraction 4 adjacent to GA_5 on the marker strip.

Of the 17 gibberellins considered by Cavell, the only gibberellin occurring ahead of and in the immediate vicinity of GA_5 on silica gel using benzene–acetic acid–water is GA_{10}, a GA_3 group gibberellin by the above classification. GA_{61} (Galt, 1968) is a dihydroxy gibberellin, an isomer of GA_1, and therefore a presumed GA_3 group gibberellin. GA_{17} (Pryce and MacMillan, 1967) is a tricarboxylic, monohydroxy gibberellin, an isomer of the GA_3 group gibberellin, GA_{13}. GA_{18} and GA_{19} have already been considered. However, GA_{20}, the Pharbitis (Takahashi *et al.*, 1967a), has a structure identical to that of GA_5, with the exception of the missing 2, 3 double bond and, therefore is a possible candidate for the *Vicia* gibberellin. GA_{21} and GA_{22}, the Canavalia I and II (Takahashi *et al.*, 1967b), are similar in structure to GA_5; the GA_{21} differs from the GA_5 by a missing 2,3 unsaturation, and the presence of a carboxylic group instead of a methyl group on the 1-carbon. The GA_{22} differs by a hydroxy group on the 1-methyl carbon. The GA_{21} can be dismissed as a possibility because of its extremely weak activity on dwarf peas and the GA_{22} because of its additional hydroxy group which would place it in the GA_3 group. GA_{23} (Koshimizu *et al.*, 1968) is a dihydroxy analog of the monohydroxy GA_{19}. The greater polarity due to the presence of the one additional hydroxy group places it in a group with GA_8. GA_{24} (Harrison *et al.*, 1968) is the hydroxyl-free analog of GA_{19}. The GA_{24}, therefore, is less polar than GA_{19} (GA_3 group) and presumably belongs in the GA_7 group, with the *Vicia* gibberellin. However, on silica gel in diisopropyl ether–acetic acid system, GA_{19} has an R_f value of 0.8 while *Vicia* gibberellin has a value identical to that of GA_5 or 0.16. Since GA_{24} is less polar than GA_{19}, by one less hydroxy group, the R_f value of GA_{24} in this system can be predicted to be greater than 0.8. In a similar set of analogs, including the dihydroxy GA_{18}, monohydroxy GA_{14} and the hydroxyl-free GA_{12}, the respective R_f values in the same solvent system are 0.04, 0.26, and 0.67.

Thus, of 24 gibberellins with known structures, 23 can be eliminated. The remaining choice, GA_{20}, which bears a similar relationship to GA_5 that GA_1 bears to GA_3, can be expected to have chromatographic properties very similar to GA_5. The difficulty of resolving the derivatives of the GA_5, GA_{20} pair by gas chromatography has been noted recently by MacMillan and Pryce (1968). A molecular weight of 332 obtained from the mass spectral data and the similarity of the fragmentation pattern to that of GA_{20} methyl ester are consistent with the choice of this remaining gibberellin. The *Vicia* gibberellin, therefore, must be either GA_{20} or an unreported A_{20} isomer.

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