Identification of Six Endogenous Gibberellins in Spinach Shoots

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ABSTRACT

Analysis of highly purified extracts from spinach shoots by combined gas chromatography-mass spectrometry has demonstrated the presence of six 13-C-hydroxylated gibberellins (GAs): GA_{13}, GA_{14}, GA_{15}, GA_{17}, GA_{23}, and GA_{25}. The major GAs were GA_{17}, GA_{19}, and GA_{20}, whereas the other three GAs occurred in trace amounts. Structural considerations suggest that the six GAs identified in spinach are related in the following metabolic sequence: GA_{13} → GA_{14} → GA_{19} → GA_{17} → GA_{20} → GA_{25}.

LD^3 rosette plants respond to a transfer from SD to LD with increased stem elongation and subsequent flower formation. In many instances application of GAs to plants grown under SD will cause stem growth (10). Work in this laboratory with spinach (18, 19) demonstrated that photoperiodic control of stem elongation is mediated by GAs. It was shown that although LD did not act to increase the total GA content of spinach plants, GA turnover was enhanced (18). It was also found that the levels of individual GAs were altered. However, before the mechanism of photoperiodic regulation of GA metabolism can be fully investigated, the chemical identity of the endogenous GAs should be determined. In this paper we report the identification of six GAs in spinach and discuss the possible metabolic relationships between those GAs.

MATERIALS AND METHODS

Plant Material. Seeds of spinach, Spinacia oleracea cv. Savoy Hybrid 612 (Harris Seed Co., Rochester, New York) were sown in the field in early August 1978. Whole shoots were harvested 1.5 months after sowing. The harvested shoots were washed with distilled H_2O and the yellow, senescing leaves discarded. The remaining plant material was frozen in liquid N_2, lyophilized, and stored at -15 C prior to extraction. Approximately 2,500 plants yielded 2.5 kg dried plant material.

Extraction and Purification Procedures. Freeze-dried spinach shoots, in 200-g lots, were homogenized with 4 liters ice-cold 80% aqueous methanol in a Waring Blender. The extract was filtered, and the residue was stirred overnight at room temperature in 100% methanol. After a second filtration, the two extracts were combined and the methanol removed under reduced pressure. An equal volume of 1 M phosphate buffer (pH 8.2) was then added and the resulting mixture was partitioned three times with equal volumes of petroleum ether. The aqueous phase was adjusted to pH 2.5 with 6 N HCl and purified on a charcoal-cellulose column as described before (18), except that 2 g of charcoal were used for every 10 g dry plant material extracted. Elution of GAs was achieved with 80% aqueous acetone. The acetone was removed under reduced pressure. The remaining aqueous residue was adjusted to pH 2.5 with 6 N HCl and partitioned four times with equal volumes of ethyl acetate.

The acidic ethyl acetate fraction obtained after charcoal chromatography was purified further by silicic acid adsorption chromatography as described earlier (18), except that 60% ethyl acetate in chloroform was used as the elution mixture. Two grams of silicic acid (Mallinkrodt, 100 mesh) were used for every 10 g lyophilized plant material extracted.

The eluate from the silicic acid adsorption column was fractionated via preparative reverse phase HPLC using Bondapak C_{18}/Porsil B as described before (11). GAs were eluted from the column with a linear gradient of 95% ethanol (30-100%) in 1% aqueous acetic acid in 25 min at 9.9 ml min^{-1}. Fractions were collected every min from the time of injection and corresponding fractions from 12 HPLC runs were combined and dried. The remaining residues were redissolved in 2 ml ethanol and a small aliquot (1.0%) of each of the combined fractions was tested for the presence of GA-like substances with the d-5 corn bioassay as described before (17). Fractions which contained biological activity, or fractions in which authentic, inactive GAs eluted (11), were further purified by silicic acid partition chromatography. In this procedure the method described by Powell and Tautvydas (13) was followed, except that the stationary phase consisted of water with the pH adjusted to 3.0 by the addition of a few drops of trifluoroacetic acid. Elution of GAs was achieved with a step gradient of increasing concentrations in 5% increments of ethyl acetate in hexane. Both solvents were saturated with water at pH 3.0 before mixing.

Fractions which contained biological activity, or eluted at the same step as authentic GAs, were subjected to final purification using analytical reverse phase HPLC with a Bondapak C_{18} column as described earlier (11). Elution of GAs from this column was achieved with either a 30-100% linear gradient in methanol in 1% aqueous acetic acid (grad A), or a 10-70% linear gradient (grad B). Both gradients were completed in 30 min at 2 ml min^{-1}. Grad B was used only when eluting polyhydroxylated GAs (e.g. GA_{14}, GA_{20}, or GA_{25}).

TLC. Preparative TLC was carried out on glass plates (20 x 20 cm) coated with silica gel H. Partially purified acidic extracts were applied as a narrow band 12 cm long. Authentic GAs were spotted 2 cm to the side of this band. The thin layer plates were developed to 15 cm from the origin in chloroform-ethyl acetate-acetic acid (60:40:5, v/v/v). The resulting chromatogram was divided into 10 equal zones and the silica gel from each zone was scraped off into a test tube. The silica gel was then eluted twice with water-saturated ethyl acetate, and then with acetone. The combined eluates from each of 10 zones were assayed for the presence of GA-like substances using the d-5 corn bioassay. The side of the plate which was spotted with the authentic GAs was left intact and sprayed with a H_2SO_4-ethanol mixture (5:95, v/v). The plate was heated at 100 C for 10 min to visualize the reference GAs.

Derivatization. Appropriate fractions from analytical reverse
phase HPLC were methylated with ethereal diazomethane. The TMS ethers of the methyl esters were prepared by adding 100 μl of a solution containing pyridine-hexamethyldisilazane-trimethylchlorosilane (9:3:1, v/v) to dry methylated samples in Reacti-vials (Pierce).

GLC-MS. Derivatized samples were chromatographed on a Hewlett-Packard 5840-A gas chromatograph with a glass column (183 x 0.2 cm i.d.) packed with 2% SP-2100 on 100/120 Supelcoport. The column temperature was programmed from 170 to 280°C at 10°C min⁻¹ with a 2-min isothermal hold at the beginning of the program and a 5-min isothermal hold at the end. The flow rate of the carrier gas (He) was 25 ml min⁻¹. The GC was connected to a Hewlett-Packard 5983 mass spectrometer by a jet separator and mass spectra were collected every 4.5 s. The ionizing potential was 70 eV.

RESULTS

A sample of field grown spinach, 20 g dry weight, was extracted and subjected to charcoal adsorption chromatography, silicic acid adsorption chromatography, and TLC. Figure 1 shows the presence of two zones of GA-like activity. The zone at Rf 0.2, called fraction I, co-chromatographed with GA1, whereas the less polar zone at Rf 0.4-0.5 (fraction II) co-chromatographed with GA20. These results indicate that the pattern of GA-like activity from field grown spinach is similar to that from plants grown in growth chambers (18, 19).

When fractions I and II were first separated by TLC and then subjected to preparative reverse phase HPLC, fraction I no longer behaved chromatographically like GA1 (Fig. 2A), whereas fraction II still co-chromatographed with GA20 (Fig. 2B). To identify the GAs present in fractions I and II, it was necessary to extract and purify large amounts of the plant material. After charcoal and silicic acid adsorption chromatography, followed by preparative reverse phase HPLC, fractions I and II were purified further by silicic acid partition chromatography. Fractions I and II were eluted with 35 and 40% ethyl acetate in hexane, respectively. Final purification of both fractions I and II was achieved by analytical reverse phase HPLC. Small aliquots (0.5%) of each fraction were assayed for the presence of GA-like substances by the d-5 corn bioassay. Both fractions I and II were contained in single fractions. Figure 3 shows that fraction II co-chromatographed exactly with authentic GA20, whereas fraction I co-chromatographed with none of the available GA standards (11).

The MeTMS derivatives of fractions I and II were prepared. GLC-MS analysis of the fraction containing II showed a well resolved peak with the same retention time as authentic MeTMS-GA20. The mass spectrum of this peak was identical to the spectrum of MeTMS-GA20 (Table I). No other peaks from this fraction had fragmentation patterns recognizable as any of the known GAs. When the derivatized fraction containing I was analyzed by GLC-MS, a single large peak was found with a mass spectrum that closely resembled the published mass spectrum for MeTMS-GA19 (Table I). Unfortunately, a reference sample of GA19 (Fig. 4) was not available for further confirmation. However, on the basis of the similarities between the published spectrum (3) and the mass spectrum obtained from fraction I, we conclude that I is mostly comprised of GA19.

Since GA19 and GA20 are both C-13-hydroxylated GAs, it would seem logical that other C-13-hydroxylated GAs are present in spinach shoots. If these GAs were biologically inactive, or were present in minute quantities, they would escape detection in the bioassay. Fractions resulting from preparative reverse phase HPLC which would contain various C-13-hydroxylated GAs (11), if present in spinach shoot extracts, were purified by silicic acid partition chromatography. Final purification was achieved by

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**Fig. 1.** GA-like activity present in an extract of lyophilized spinach shoots (20 g). The partially purified acidic extract was fractionated by TLC and the resulting chromatogram divided into 10 equal strips. Each strip was eluted and the eluate was assayed for the presence of GA-like substances by the d-5 corn bioassay.

**Fig. 2.** Chromatographic behavior of fractions I (A) and II (B) when separately subjected to preparative reverse phase HPLC as detected by the d-5 corn bioassay. An acidic extract from 20 g dry weight of plant material was prepared, and I and II separated by TLC. Standard GA1 and GA20 were run separately for comparison of the chromatographic behavior.
analytical reverse phase HPLC. Corresponding fractions from the spinach extract where various 13-OH GAs are known to elute in this system (11) were derivatized and analyzed by GLC-MS. Fraction 14 (grad B) and fraction 19 (grad A) contained substances that had retention times and mass spectra identical to those of authentic MeTMS-GA29 and MeTMS-GA17, respectively (Table I). GA17 (Fig. 4) is inactive in the d-5 corn bioassay (16), whereas GA29 (Fig. 4) reportedly has very little biological activity (4). Fractions 17 and 21 (grad A) contained compounds which had mass spectra very similar to those published for MeTMS-GA44 (7) and MeTMS-GA60, formerly called 13-hydroxy GA17 (2), respectively (Table I). Both GA44 and GA60 (Fig. 4) occurred in such low quantities that they would have escaped detection in the bioassay. No GA1, GA5, GA6, or GA8 was detected in these spinach extracts.

![Figure 3](image-url)

**Figure 3.** GA activity as measured by the d-5 corn bioassay associated with 0.5% aliquots of individual fractions resulting from analytical reverse phase HPLC of purified fractions I (A) and II (B) using grad A.

![Figure 4](image-url)

**Figure 4.** Numbering system of ent-gibberellane skeleton (A), and structures of the six GAs identified by GLC-MS in spinach shoot extracts (B).

### Table I. GLC-MS Data Obtained with Samples from Spinach Shoots and with Authentic GAs

In cases where no authentic GAs were available, published data from mass spectral analysis are presented. Fraction numbers refer to fractions eluted from analytical HPLC system.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of Scan</th>
<th>Peaks in Mass Spectrum with Relative Abundances in Parentheses</th>
<th>m/e values</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11.5</td>
<td>462 (M+, 6) 447 (4) 434 (100) 402 (30) 374 (42) 207 (37) 208 (39)</td>
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<tr>
<td>MetTMS-GA19(3)</td>
<td>10.8</td>
<td>462 (M+, 12) 447 (7) 434 (100) 402 (36) 374 (61) 207 (47) 208 (72)</td>
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<tr>
<td>II</td>
<td>10.8</td>
<td>418 (M+, 100) 403 (21) 359 (17) 208 (10) 207 (33)</td>
<td></td>
</tr>
<tr>
<td>MetTMS-GA20</td>
<td>10.8</td>
<td>418 (M+, 100) 403 (17) 359 (16) 208 (10) 207 (20)</td>
<td></td>
</tr>
<tr>
<td>Fraction 19</td>
<td>11.4</td>
<td>492 (M+, 13) 460 (22) 432 (13) 401 (5) 373 (13) 208 (100)</td>
<td></td>
</tr>
<tr>
<td>MetTMS-GA17</td>
<td>11.4</td>
<td>492 (M+, 14) 460 (25) 432 (21) 401 (16) 373 (26) 208 (100)</td>
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</tr>
<tr>
<td>Fraction 14</td>
<td>12.6</td>
<td>506 (M+, 100) 491 (12) 477 (15) 447 (9) 207 (40) 208 (25)</td>
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<tr>
<td>MetTMS-GA29</td>
<td>12.6</td>
<td>506 (M+, 100) 491 (16) 477 (5) 447 (14) 207 (41) 208 (38)</td>
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<td>Fraction 17</td>
<td>13.1</td>
<td>432 (M+, 33) 417 (8) 373 (17) 251 (8) 238 (42) 208 (53) 207 (100)</td>
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<td>MetTMS-GA44(7)</td>
<td>11.2</td>
<td>448 (M+, 31) 419 (10) 416 (23) 389 (44) 208 (80) 207 (100)</td>
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<td>Fraction 21</td>
<td>11.2</td>
<td>448 (M+, 34) 419 (8) 416 (9) 389 (22) 208 (98) 207 (100)</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The above results demonstrate the presence of at least six GAs in spinach shoots: GA58, GA44, GA19, GA17, GA20, and GA29 (Fig. 4). All six GAs have in common a C-13 that is hydroxylated. Only GA20 has an additional hydroxyl group at the 2-β position. Four of the six GAs are C20-GAs: GA58, GA44, GA19, and GA17 with C-20 as a methyl, δ-lactone, aldehyde, or carboxyl group, respectively (Fig. 4). GA20, like GA29, is a C19-GA from which the C-20 has been removed and a 19-C→10-C lactone bridge has been formed. The six same GAs present in spinach shoots have recently also been identified in immature seeds of Vicia faba (14), whereas GA58, GA44, GA19, GA17, and GA29 have been found in young tassels of Zea mays (12). Thus, this combination of 13-C-hydroxylated GAs appears to occur commonly in higher plants and suggests a biogenetic relationship between these various GAs.
If one assumes that the six GAs in spinach are related metabolically in a pathway, one can postulate a sequence based on the sequential oxidation of C-20 from a methyl to a carboxyl group, its subsequent removal, and finally the formation of a C-19 → C-10 lactone bridge, and finally C-2 oxidation. This would indicate the existence of the following pathway: GA_{12} → GA_{13} → GA_{19} → GA_{18} → GA_{17} → GA_{20} → GA_{14} (in the open lactone, or hydroxy-diacid form) → GA_{19} → GA_{17} → GA_{16} → GA_{15} → GA_{14} → GA_{13} → GA_{12}. There is little direct evidence to date from other plant systems for the existence of this kind of a pathway in GA metabolism. Conversions of GA_{20} to GA_{29} have been observed in a variety of higher plant systems including Phaseolus vulgaris seeds (15), immature seeds of Pisum sativum (7) and leaves of Bryophyllum daigremontianum (6). This type of reaction may serve as a deactivation process since GA_{20} and other 2-β-hydroxylated GAs have little or no biological activity (9). In a cell-free system derived from Cucurbita maxima endosperm, C-20 of GA_{12} (a nonhydroxylated analog of GA_{13}) was sequentially oxidized from a methyl (GA_{13}) to an aldehyde (GA_{14}, GA_{15}), and finally to a carboxyl group (GA_{19}, GA_{20}, GA_{21}) (8).

Much less is known about the conversion of C_{20}-GAs to C_{19}-GAs. In fact, no C_{19}-GA with a higher oxidation state than a methyl group at C-20 has been found to act as an intermediate in the conversion of C_{20}-GAs to C_{19}-GAs (9). Dockrell and Hanson (5) found that when [3H]kaurene, labeled in the C-20 position, was incubated in cultures of Gibberella fujikuroi, the C-20 carbon was lost as CO_{2} during the formation of C_{19}-GAs. This fact, along with the observation that both oxygen atoms in the C-19 → C-10 lactone bridge of C_{20}-GAs originate from the C-19 carboxyl group of C_{20}-GAs (1), limit the possibilities of a direct precursor to C_{19}-GAs (9). However, the identity of this precursor remains unknown.

In earlier work from this laboratory (18, 19), it was observed that the levels of two GA-like substances in spinach shoots, here called fraction I and II, changed in relation to photoperiod. The biological activity associated with these two GA-like substances can now, for the most part, be attributed to GA_{19} and GA_{20}. The other GAs found in spinach shoots are either present in quantities too low for detection by bioassay, or are biologically inactive in the d-5 corn bioassay. It appears from previous bioassay results (18, 19), that the level of GA_{19} declines with LD treatment, whereas the level of GA_{20} increases during the same period. Moreover, bioassay results indicated that the total GA level remained fairly constant during LD treatment. Since both GA_{19} and GA_{20} are reported to have equal activity in the d-5 corn bioassay (4), the bioassay data should reflect absolute differences in the levels of both GAs. Taken together, these results could be interpreted as a precursor-product relationship between GA_{19} and GA_{20}. Since photoperiodic control of stem growth in spinach is mediated by GAs (18, 19), it is possible that stem growth requires GA_{20} (or some metabolite thereof), and that photoperiod controls stem growth by regulating the conversion of GA_{19} to GA_{20}. However, until the time when a metabolic sequence of GAs can be firmly established, and more precise quantitative data can be obtained for the changes in levels of not only GA_{19} and GA_{20}, but also of the biologically inactive GAs, GA_{18}, and GA_{21}, these ideas will remain mere speculation.

Acknowledgments—We acknowledge with thanks samples of GAs received from: Dr. D. Broadbent, I. C. I. Pharmaceuticals Divisions, Alderley Park, Macclesfield, Cheshire, U. K.; Dr. J. MacMillan, School of Chemistry, University of Bristol, U. K.; Dr. R. P. Pharis, Department of Biology, University of Calgary, Alberta, Canada; Drs. N. Murofushi and N. Takahashi, Department of Agricultural Chemistry, University of Tokyo, Japan; and Drs. H. Fukushima and K. Koshimizu, Department of Food Science and Technology, Kyoto University, Japan.

We would also like to acknowledge the assistance given by Dr. R. K. Chapman in the GLC-MS studies.

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