Identification of Endogenous Gibberellins in Navel Orange Shoots

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ABSTRACT

Eight gibberellins (GAs) were identified from vegetative shoots of navel orange trees (Citrus sinensis L. Osbeck cv Washington) after sequential purification by reverse-phase C18 high performance liquid chromatography, Nucleosil 5CN(H2)2, high performance liquid chromatography, and capillary gas chromatography-mass spectrometry. GA1, GA17, GA18, GA20, GA29, and iso-GA4 were identified based on the full scan mass spectra and Kovats retention indices. GA4 was tentatively identified based on the comparison of the full scan mass spectra with the published spectra. GA4 was tentatively identified from the characteristic masses at the correct Kovats retention index.

The isolation of GA1 from the water sprouts of mandarin orange (Citrus unshiu) was one of the earliest identifications of a GA in higher plants (9). Since then, no further work has been reported on the identification of GAs in the vegetative tissues of citrus. Most of the work on different species of citrus has involved measuring GA-like activity in fruit by means of bioassay. Based on this information, GAs seem to be involved in the regreening of Valencia oranges, which reduces their commercial value (12). Regreening is the increase in the Chl content of the flavedo of the mature fruit caused by certain climatic conditions. Valencia oranges that regreened showed a marked increase in the GA-like activity before the Chl began to increase, while the GA-like activity in non-regreened fruit remained low. The application of exogenous GA3 in April increased the regreening observed in June and July (12). The use of GA3 on citrus is one of the major applications of GAs in agriculture. It is used mainly on navel oranges, 2 weeks before the color change, to improve rind color and firmness of fruit scheduled for late harvest (11). There has also been recent interest in using some of the newer gibberellin inhibitors to control the growth of citrus trees and reduce the pruning costs (1, 2, 13, 16). Given the importance of GAs for citrus growth and fruit quality, we have applied the modern methods of HPLC and GC-MS to the identification of the endogenous GAs of navel orange shoots.

MATERIALS AND METHODS

Plant Material. Navel orange shoots (Citrus sinensis L. Osbeck cv Washington) from the current year’s growth, 10 to 15 cm in length, were collected from a commercial citrus grove at Lindsay, California, in May 1986. The shoots consisted of the stem and several pairs of partially to almost wholly expanded leaves. The shoots were immediately frozen with dry ice. The shoots were crushed, lyophilized, and stored at −14°C prior to extraction.

Pea seeds (Pisum sativum L. cv Progress #9, Vermont Bean Seed Co., Fair Haven, VT) were planted in Supersoil (Rod McAllan Co., San Francisco, CA) at the end of June 1987 and grown in a greenhouse. Immature pea seeds were harvested 5 to 6 weeks after planting, lyophilized, and stored at −14°C.

NH3 Sep-Pak Procedure. To determine the capacity of NH3 Sep-Paks, mixtures of similar amounts of ABA (Lancaster Synthesis, Ltd., Windham, NH) and GA3 (Sigma Chemical Co., St. Louis, MO) were dissolved in 2 mL MeOH. A new Sep-Pak NH3 cartridge (Millipore, Milford, MA) was used for each sample. The NH3 Sep-Pak was washed with 15 mL of MeOH. The 2-mL aliquot was loaded, and the NH3 Sep-Pak was eluted to dryness with 10 mL of MeOH. The NH3 Sep-Pak was then eluted to dryness with 10 mL of 1% (v/v) HOAc/MeOH. The third fraction was eluted with 10 mL of 2% (v/v) HOAc/MeOH. Toluenol (2 mL) was added to each fraction to remove the HOAc as an azetrope. The fractions were evaporated to dryness, under a N2 stream at 60°C, and dissolved in 3 mL of HPLC solvent. The samples were analyzed by means of a Nucleosil 5CN(H2)2 column (20 cm × 4 mm) (Macherey-Nagel, Duren, FRG), which was eluted at 1 mL/min with MeOH containing 0.1% HOAc at room temperature.

Extraction and Purification of the GAs from Navel Orange Shoots. Shoots, 500 g freeze-dried plant material, in 100-g lots, were chopped dry in a Waring Blender and transferred to a Waring jar equipped with a Polytron head. Each 100-g lot was homogenized with 1 L of 80% (v/v) aqueous acetone and filtered. The residue was rehomogenized with 1 L of 80% (v/v) aqueous acetone and stirred 30 min before filtering. Each filtrate was stirred with equal amounts of activated charcoal (H2PO4 washed [Sigma] and/or Darco G-60 [Aldrich Chemical Co.,]) and Celite for 30 min. Ninety and 60 g of charcoal/Celite were used for the first and second filtrates, respectively. Each mixture was filtered, and the residue was rinsed with 200 mL of 80% (v/v) aqueous acetone. The filtrates were combined, and the acetone was removed under reduced pressure at 35°C. The aqueous residue, after the addition of 400 mL 0.5 M PO4 (pH 8.0), was partitioned against hexane (3 × 300 mL). The aqueous extract was stirred for 1 h with 30 g PVPP. The mixture was filtered, and the PVPP was rinsed with 200 mL 0.5 M PO4 (pH 8.0). The filtrate and

1 Abbreviations: GA, gibberellin; EtOAc, ethyl acetate; HOAc, acetic acid; KRI, Kovats retention index; MeOH, methanol; PO4, (pH 8.0), potassium phosphate buffer (pH 8.0); PVPP, polyvinylpyrrolidone; Rt, retention time; RRT(GA3), Rt relative to Rt of GA3; MSTFA, N-methyl-N-trimethylsilylfluoroacetamide.

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rinse were combined, adjusted to pH 2.5 with 50% HCl, and extracted with EtOAc (5 × 400 mL). The combined EtOAc extracts were reduced to about 600 mL under reduced pressure at 35°C without letting any portion of the sample go to dryness. The EtOAc was washed with 5% NaCl that had been adjusted to pH 2.5 with 1 N HCl (3 × 300 mL) to remove any remaining PO₄. The EtOAc was then dried over anhydrous sodium sulfate. Combining all five extracts and reducing to dryness resulted in an acidic EtOAc fraction of 1.51 g.

A mixture of 25 g activated charcoal (Darco G-60) and 50 g Celite in 250 mL of water was degassed under vacuum and then packed under low pressure in a glass column (3.1 cm diameter). The combined acidic EtOAc fraction was dissolved in 20 mL of 20% (v/v) aqueous acetone and loaded onto the column. The column was eluted with 400 mL of 20% and then 800 mL of 80% (v/v) aqueous acetone. The acetone was removed under pressure at reduced pressure at 35°C from the 80% eluate, and 20 g PVPP and 250 mL of 0.5 M PO₄ (pH 8.0) were added. The mixture was stirred 15 min and then filtered and rinsed with 100 mL of 0.5 M PO₄. The filtrate was treated the same way with another 15 g PVPP. The pH was adjusted to 2.5 as above, and the filtrate was extracted with EtOAc (5 × 300 mL). The volume of the EtOAc fraction was reduced to 600 mL, and it was washed with 5% NaCl (pH 2.5) and dried. This reduced the weight of the acidic EtOAc fraction to 370 mg.

The acidic EtOAc fraction was dissolved in 40 mL of MeOH and 2-mL aliquots were processed through individual NH₂ Sep-Paks. The 1% (v/v) HOAc/MeOH fractions from all the aliquots were combined and, after 50 mL of toluene had been added to remove HOAc as an azetrop, evaporated to dryness under reduced pressure at 35°C. This reduced the dry weight of the GA fraction to 40 mg.

The GA fraction was dissolved in 5 mL of the starting solvent and injected onto a Perkin-Elmer C₁₈ preparative column (2.2 × 25 cm) equipped with C₁₈ preparative guard column. The column was eluted at 9.9 mL/min with a 40-min linear gradient from HOAc/MeOH/H₂O (v/v 1:20:79) to 99% MeOH using a Perkin-Elmer Series 10 LC Controller and 2 Series 10 pumps. Fractions were collected every 2 min. The fractions between 6 and 18 min were combined into one fraction and those between 18 and 24 min into another. The fractions between 24 and 38 min were kept separate. MeOH was removed from each fraction under reduced pressure at 35°C and, after adding isopropanol and toluene to remove H₂O and HOAc as azetrope, the fractions were taken to dryness and then dissolved in 3 mL MeOH. A part (0.5 mL) of each fraction was derivatized and analyzed by GC-MS as described below, and the remainder was stored at −15°C. The C₁₈ column was then further separated by means of a Nucleosil 5(CH₃)₂ column (20 cm × 4 mm). Each fraction in 2.4 mL MeOH was loaded onto an NH₂ Sep-Pak and eluted as described above to provide maximum protection of the Nucleosil column. Each 1% (v/v) HOAc/MeOH fraction was passed through a Millere-HV 0.45-μm filter. After adding 2 mL of toluene, the fraction was evaporated to dryness under a N₂ stream at 60°C. The residue was dissolved in 0.5 mL of HPLC solvent. The entire 0.5 mL was injected, and the samples were eluted at room temperature with MeOH containing 0.1% HOAc at 1 mL/min. Five to nine fractions were collected from each injection covering the Rts over which the GAs should elute (18). After all the peaks from the injection had eluted (75–90 min) based on detection of a standard solution of GA₃ injected, and the Rts from the previous injection were divided by the Rt of GA₃ to give the RRT(GA₃). Each fraction was derivatized and analyzed by GC-MS as described below.

**Extraction and Purification of Progress #9 Peas.** Freeze-dried immature pea seeds, 83 g, were extracted the same way as the navel orange shoots up to the charcoal/Celite column. The acidic EtOAc fraction weighed 48 mg. It was dissolved in 6 mL of MeOH, and 2-mL aliquots were loaded onto NH₂ Sep-Paks and sequentially eluted with MeOH and 1 and 2% (v/v) HOAc/MeOH. Each fraction was taken to dryness and dissolved in 20 mL MeOH. One mL of each fraction was derivatized and analyzed by GC-MS. One mL of the 1% (v/v) HOAc/MeOH fraction was evaporated to dryness, dissolved in HPLC solvent, and separated by means of the Nucleosil 5(CH₃)₂ column. Two-mL fractions were collected and derivatized.

**Partial Purification of GA₁, from Gibberella fujikuroi.** The acidic EtOAc extracts from media of Gibberella fujikuroi of work previously done at our laboratory (6) were dried, dissolved in 80% (v/v) aqueous acetone, and treated with charcoal/Celite. The acetone was removed from the filtrate. MeOH equal to the aqueous residue was added, and the solution was passed through a column (1.2 × 3 cm) of 40 μm bonded C₁₈ material (J. T. Baker). The MeOH was removed, the pH was adjusted to 2.5, and the solution was extracted with EtOAc. The EtOAc was evaporated, and the residue was dissolved in EtOAc/CHCl₃ (v/v 60:40) and eluted from a column (3.1 × 23 cm) containing silicic acid/Celite (1:2) with the same solvent. The solvent was evaporated, the residue was dissolved in MeOH, and a small portion was processed on an NH₂ Sep-Pak as above. An aliquot of the 1% (v/v) HOAc/MeOH fraction was separated by means of a Nucleosil 5(N(CH₃)₂) column, and fractions around the Rt of GA₁ were collected and derivatized.

**Synthesis of Iso-GA₃, GA₃ (5 mg) (Sigma) was methylated with diazomethane and shaken 1 h with 0.01 N NaOH to give iso-GA₃ (4).** The reaction mixture was passed through a C₁₈ Sep-Pak, washed with water, eluted with MeOH, evaporated, dissolved in methylene chloride, and filtered through a Milere-HV 0.45-μm filter, and a small aliquot was derivatized.

**Derivatization.** The fractions were evaporated to dryness in 1 mL Reacti-Vials under a N₂ stream at 60°C, dissolved in 0.1 mL MeOH, and methylated with an excess of ethereal diazomethane. After evaporating, 50 μL MSTFA (Pierce Chemical Co.) was added to each vial, and they were heated at 60°C for 45 min. Only 25 μL MSTFA was used for the Nucleosil fractions of the navel orange shoots.

**GC and GC-MS.** The undiluted derivatized samples were injected, using the splitless injection mode, into a fused silica capillary column (30 m × 0.25 mm i.d.) coated with a 0.25-μm layer of SPB-1 bonded phase (Supelco, Inc.). The column, in a Hewlett-Packard 5830A gas chromatograph, was attached to a flame-ionization detector or led directly into the ion source of a VG 7070HS. Helium, at 0.7 mL/min, was used as the carrier gas. The column was held at 60°C for 2 min and then programmed to 160°C at 20°C/min and finally to 290°C at 5°C/min. For GC the injector and detector temperature was 300°C, while for GC-MS, the electron impact ionization energy was 70 eV. The mass spectra were acquired from 20 to 750 amu at 0.7 s/decade. A solution of Parafilm plus triacontane in hexane was co-injected with the sample to determine the KRs (8).

**RESULTS AND DISCUSSION**

Preliminary experiments showed that fractions resulting from reverse-phase HPLC of citrus extracts contained substances that had a deleterious effect on the performance of the Nucleosil 5(CH₃)₂ column. These effects could not be reversed by washing the column. The use of NH₂ Sep-Paks greatly reduced this problem, although a slow reduction in the Rts still occurred. Although the Rts slowly decreased, the relative Rts remained fairly constant. Therefore, the Rts for the Nucleosil 5(CH₃)₂ column were divided by the Rt of GA₃ injected immediately after each sample had finished eluting and given as RRT(GA₃).

Although the NH₂ Sep-Paks were originally introduced to protect the Nucleosil 5(CH₃)₂ column, it was also found that
they caused a great reduction in the dry weight of the samples. To determine how much sample could be loaded onto an NH2-Sep-Pak, mixtures containing ABA and GA3 were tested. ABA was used as one of the tests because its Rt on the Nucleosil 5N(CH3)2 column is less than that of most GAs (18). Based on these results, one NH2-Sep-Pak was used for each 20 mg of acidic EtOAc fraction. When the acidic EtOAc fraction from the navel orange shoots was separated by means of the NH2-Sep-Paks, the dry weight was reduced from 370 mg to 40 mg. After the analysis of the navel orange shoots had been completed, the acidic EtOAc fraction from immature peas was separated into three fractions by means of the NH2-Sep-Paks, and each fraction was analyzed by GC-MS. It was found that 15 to 20% of the GA3 and GA19 eluted with the MeOH. Even if the same loss occurred for the orange shoots, the reduction in dry weight would make this procedure useful. The peas also contained GA29-catabolite, which was more strongly retained than the other GAs. Most of it eluted in the 1% HOAc/MeOH fraction, but 20 to 25% eluted in the 2% HOAc/MeOH fraction. The peas also contained the less polar GAs, GA5, and GA24, which eluted mainly in the 1% HOAc/MeOH fraction but with traces in the MeOH fraction.

A part of each fraction from reverse-phase HPLC was derivatized and analyzed by GC-MS. The results from GC-MS show that there is still too much interference in the chromatograms to identify conclusively all the GAs present. The undervitized portion of each fraction was therefore further separated by means of an analytical Nucleosil 5N(CH3)2 column. Each of the fractions, after formation of the methyl ester trimethylsilyl ether derivatives, was analyzed by GC-MS. GA1, GA8, GA17, GA19, GA20, and GA29 were tentatively identified by comparison of their full scan mass spectra to the published spectra (3, 7). GA3 was tentatively identified by the simultaneous occurrence of eight characteristic masses (5, 17) in the reconstructed mass chromatograms. Iso-GA3 was initially identified as GA3-like because of its mass spectra but it had too short a Rt to be GA3.

After the initial identification of the GAs in navel orange shoots was completed, GAs were isolated from two well-known sources, i.e. immature pea seeds (14) and _G. fujikuroi_, to confirm the identifications. GA1 was partially purified from culture media of _G. fujikuroi_ to separate it from GA3, which interfered with obtaining good mass spectra and KRI. GA17, GA19, GA20, and GA29 were partially purified from immature peas.

Table I shows the HPLC and GC-MS data of the putative GAs from navel orange shoots and the comparative data for the reference materials. The fractions in which the GAs eluted from the C18 column are in reasonable agreement with the published values (10) if allowance is made for the fact that ABA eluted 1 min later on our system. The only difference is that GA19 might have been expected to elute earlier than the fraction containing GA29. The ranges of the RRT(GA3)s from the Nucleosil

<table>
<thead>
<tr>
<th>GA</th>
<th>Source</th>
<th>HPLC Rt*</th>
<th>RR(GA3)b</th>
<th>KRI</th>
<th>Constituent Ions (Relative Intensity) m/z</th>
<th>RRT(GA3)K</th>
<th>m/z</th>
<th>RRT(GA3)K</th>
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<tr>
<td>GA1</td>
<td>Orange</td>
<td>24–26</td>
<td>0.72–1.08</td>
<td>2684</td>
<td>506 (100) 491 (11) 448 (21) 447 (11)</td>
<td>377 (15) 375 (13) 235 (10) 207 (25)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>506 (100) 491 (12) 448 (19) 447 (9)</td>
<td>377 (13) 375 (11) 235 (9) 207 (32)</td>
<td></td>
<td></td>
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<tr>
<td>GA4</td>
<td>Orange</td>
<td>18–24</td>
<td>0.94–1.08</td>
<td>2825</td>
<td>594 (100) 579 (6) 535 (6) 504 (5)</td>
<td>448 (14) 379 (9) 375 (6) 238 (16)</td>
<td></td>
<td></td>
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<tr>
<td>GA17</td>
<td>Orange</td>
<td>34–36</td>
<td>0.76–0.84</td>
<td>2594</td>
<td>492 (49) 460 (35) 433 (33) 432 (23)</td>
<td>401 (13) 373 (31) 251 (25) 208 (100)</td>
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<td></td>
</tr>
<tr>
<td>Pea</td>
<td>0.67–0.81</td>
<td>2590</td>
<td>492 (37) 460 (24) 433 (24) 432 (21)</td>
<td>401 (13) 373 (26) 251 (21) 208 (100)</td>
<td></td>
<td></td>
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<td>GA19</td>
<td>Orange</td>
<td>32–34</td>
<td>1.07–1.45</td>
<td>2616</td>
<td>462 (7) 447 (7) 434 (100) 402 (54)</td>
<td>375 (59) 374 (67) 259 (31) 207 (43)</td>
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<td>Pea</td>
<td>1.21–1.34</td>
<td>2614</td>
<td>462 (9) 447 (4) 434 (100) 402 (36)</td>
<td>375 (53) 374 (61) 259 (25) 207 (39)</td>
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<td>GA20</td>
<td>Orange</td>
<td>30–32</td>
<td>0.96–1.06 0.67–1.45</td>
<td>2502</td>
<td>418 (100) 403 (15) 387 (2) 375 (50)</td>
<td>359 (13) 301 (11) 208 (11) 207 (30)</td>
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<tr>
<td>Pea</td>
<td>1.07–1.21</td>
<td>2502</td>
<td>418 (100) 403 (15) 387 (1) 375 (55)</td>
<td>359 (16) 301 (18) 208 (16) 207 (42)</td>
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<td>418 (100) 403 (17) 387 (2) 375 (58)</td>
<td>359 (16) 301 (15) 208 (14) 207 (36)</td>
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<tr>
<td>GA29</td>
<td>Orange</td>
<td>18–24</td>
<td>1.08–1.21 1.21–1.35</td>
<td>2694</td>
<td>506 (100) 491 (13) 477 (4) 447 (8)</td>
<td>389 (10) 375 (11) 303 (24) 207 (32)</td>
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<tr>
<td>Pea</td>
<td>1.21–1.34 1.34–1.45</td>
<td>2694</td>
<td>506 (100) 491 (13) 477 (4) 447 (10)</td>
<td>389 (12) 375 (14) 303 (33) 207 (47)</td>
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<td>GA44</td>
<td>Orange</td>
<td>32–34</td>
<td>0.69–1.07</td>
<td>2812</td>
<td>432 (98) 417 (28) 403 (24) 389 (31)</td>
<td>373 (34) 238 (60) 208 (61) 207 (100)</td>
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<td></td>
</tr>
<tr>
<td>Pea</td>
<td>0.81–0.94</td>
<td>2811</td>
<td>432 (57) 417 (8) 403 (3) 389 (3)</td>
<td>373 (15) 238 (37) 208 (45) 207 (100)</td>
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<tr>
<td>Iso-GA3</td>
<td>Orange</td>
<td>18–24</td>
<td>0.67–0.81 0.81–0.94</td>
<td>2645</td>
<td>504 (100) 489 (9) 475 (19) 472 (4)</td>
<td>457 (6) 445 (12) 414 (5) 370 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>504 (100) 489 (11) 475 (19) 472 (4)</td>
<td>457 (7) 445 (15) 414 (5) 370 (24)</td>
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</tbody>
</table>

* Preparative C18 HPLC. b Analytical Nucleosil 5N(CH3)2 HPLC. c Data from Crozier and Durley (5) and Takahashi et al. (17).
the fractions. shoot growth twice as contamination from earlier found at GA20, estimated from collected after subtraction GA44, GA12, are determined not this of the GAs masses confirmed based on the comparison of the full mass spectra (data not shown) and the KRIs. Table I shows the comparison of selected characteristic masses of the GAs from navel orange shoots to those of the reference sources. The relative intensities of the masses for GA44 from the shoots do not agree well with those from peas because it was just barely detectable. But the simultaneous peaking of the eight characteristic masses in the mass chromatograms at the correct KRI allows the tentative identification of GA44 in orange shoots. No reference material was available for GA8, so the identification, which rests on the comparison of the full spectra to the published spectra (3) and of the characteristic masses (5, 17), must also remain tentative at this time.

GA29 was the most abundant GA in navel orange shoots, while GA44 was at the limit of detectability. The amounts of the GAs were estimated from the total ion current after background subtraction and the flame ionization detector response for GA29, GA20, and iso-GA3. The relative amounts of GAs in orange shoots are GA29 > GA20 > iso-GA3 > GA1 = GA17 > GA19 = GA44 > GA12. There is about twice as much GA29 as iso-GA3 and twice as much GA29 as GA20. This estimate does not take into account the differences in recovery of different GAs, which were not determined in this study. Also, the less polar GAs, i.e. GA8 or GA12, would not be found by our procedure because the C18 fractions collected after 36 min were insufficiently pure for GC-MS even after chromatography on the Nucleosil column. The shoots used in this experiment were from the current year’s growth but were almost fully expanded at the time of harvest. This may account for the relatively high amounts of biologically inactive GA29. A similar pattern occurs in peas as they mature (14).

Commercially, in California, GA3 is applied to navel orange trees either before the fruit changes color (October/November) or after full fruit color develops (December/January). Although the shoots were harvested in May, it is possible that the iso-GA3 found in the shoots resulted from the degradation of GA3 applied earlier in the season. There was no evidence of GA3 in any of the fractions. Until samples can be obtained that are free of the possible contamination from the commercial application of GA3, it is useless to speculate about its natural occurrence in citrus.

In addition to the identified GAs shown in Table I, several other compounds which had GA-like mass spectra were seen in the shoot extracts. They did not appear to be any of the known GAs but two of them had spectra similar to other dihydroxylated GAs. There was insufficient material to identify any of these compounds at this time.

GA1, GA8, GA17, GA19, GA20, GA29, and GA44 are all members of the early 13-hydroxylation pathway. The pathway proceeds from GA44 to GA19, to GA20, and finally to GA1, which is the only GA active per se in the control of shoot elongation (15). The earlier members of the pathway have biological activity because they are metabolized to GA1, GA8 and GA29 are biologically inactive GAs arising from the 28-hydroxylation of GA1, and GA29, respectively. GA20 is also a biologically inactive GA arising from GA11 by the addition of the C20-aldehyde to an acid group. Therefore, it appears that the early 13-hydroxylation pathway is the major biosynthetic pathway of GAs in navel orange shoots.

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