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# Effect of Photoperiod on the Metabolism of Deuterium-Labeled Gibberellin $A_{53}$ in Spinach[1]

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THOMAS GIANFAGNA[2], JAN A. D. ZEEVAART[3], AND WILLIAM J. LUSK
*Michigan State University, MSU-DOE Plant Research Laboratory, East Lansing, Michigan 48824*

## ABSTRACT

**Application of gibberellin $A_{53}$ ($GA_{53}$) to short-day (SD)-grown spinach (*Spinacia oleracea* L.) plants caused an increase in petiole length and leaf angle similar to that found in plants transferred to long days (LD). [$^2$H]$GA_{53}$ was fed to plants in SD, LD, and in a SD to LD transition experiment, and the metabolites were identified by gas chromatography with selected ion monitoring. After 2, 4, or 6 SD, [$^2$H]$GA_{53}$ was converted to [$^2$H]$GA_{19}$ and [$^2$H]$GA_{44}$. No other metabolites were detected. After 2 LD, only [$^2$H]$GA_{20}$ was identified. In the transition experiment in which plants were given 4 SD followed by 2 LD, all three metabolites were found. The results demonstrate unequivocally that $GA_{19}$, $GA_{20}$, and $GA_{44}$ are metabolic products of $GA_{53}$, and strongly suggest that photoperiod regulates GA metabolism, in part, by controlling the conversion of $GA_{19}$ to $GA_{20}$.**

Stem growth in spinach is photoperiod dependent. In SD, the plants exhibit the typical rosette pattern of growth with short internodes and leaves with short petioles oriented horizontally. In LD, the petioles elongate and the leaves assume a more vertical orientation. In addition, LD induce stem elongation and the formation of flower primordia.

Application of GA[4] to the leaves in SD will substitute for the LD effect on the growth habit, whereas application of the growth retardant AMO-1618, which reduces the levels of the endogenous GA, to LD-grown plants results in reversion to the SD growth habit (12). It is, therefore, possible to reversibly control stem and leaf elongation environmentally by altering the photoperiod, or chemically by using AMO-1618 and GA.

Previous work has indicated that there is little change in the total GA-like activity when plants are transferred from SD to LD (12). When the GA-like activity was fractionated by TLC, however, profound changes were evident as a result of the photoperiod transition. Three zones of biological activity were identified in both SD and LD. Upon transfer to LD, zones labeled I and III declined, and zone II increased in relative activity. The GA responsible for the biological activity were subsequently identified by GC-MS (8). Zone I contained $GA_{19}$, zone II $GA_{20}$, and zone III $GA_{53}$, suggesting that photoperiod controls the conversion of $GA_{19}$ to $GA_{20}$ (9). In all, six GA were identified from spinach shoots, and based upon structural relationships the following probable biosynthetic pathway was proposed: $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{17} \rightarrow GA_{20} \rightarrow GA_{29}$ (Fig. 1). In the meantime, it has been demonstrated with cell-free systems from *Cucurbita* (3) and *Pisum* (6) that conversion of $C_{20}$-GA to $C_{19}$-GA involves elimination of C-20 at the aldehyde state of oxidation. $GA_{17}$, with a carboxyl group at C-20, is therefore now considered a biologically inactive end product of GA metabolism. To test this hypothesis for GA metabolism in spinach and to provide additional information on the relationships between photoperiod, stem growth, and GA metabolism, deuterium-labeled $GA_{53}$ was synthesized and applied to spinach leaves in SD, in LD, and in a SD to LD transition experiment.

## MATERIALS AND METHODS

**Plant Material and Cultural Conditions.** Seeds of spinach (*Spinacia oleracea* L., cv Savoy Hybrid 612; Harris Seed Co., Rochester, NY) were sown on vermiculite and transferred to 340-ml plastic cups 10 d after germination. Plants were watered twice daily with half-strength Hoagland solution, and were maintained in growth chambers under LD or SD as previously described (12). One week prior to [$^2$H]$GA_{53}$ treatment, plants received a daily soil application (10 ml) of 5 mM AMO-1618 to reduce the level of endogenous GA (12), which would interfere with the analysis of $^2$H-labeled compounds by GC-SIM.

**Synthesis of [$^2$H]$GA_{53}$.** This compound was synthesized microbially by feeding [$^2$H]steviol (13-hydroxy kaurenoic acid) to the fungus *Gibberella fujikuroi*, strain LM-45-399. Steviol was labeled in the 15- and 17-positions by the Wittig reaction of steviol 13-acetate-17-norketone with (methyl-$d_3$) triphenylphosphonium bromide. A mixture of $^2$H-labeled analogs was produced containing from 1 to 4 $^2$H/molecule. This material was fed to the fungus which produced [$^2$H]$GA_{53}$ (0.2–1.2% yield) in the following ratios ($^2H_x$ [%]: $^2H_0$ (2), $^2H_1$ (11), $^2H_2$ (30), $^2H_3$ (37), $^2H_4$ (20). A detailed description of the methods used will be published elsewhere (2).

**Chemical Applications.** [$^2$H]$GA_{53}$ (150 μg) was applied to the leaf blades of four plants (8–10 weeks old) in 15% aqueous ethanol plus 0.05% Tween 20 in a total volume of 0.8 ml. In the experiments comparing GA activity and petiole elongation, $GA_1$, $GA_{20}$, and $GA_{53}$ were applied (3 μg, 50 μl) in 15% aqueous ethanol three times weekly for 3 weeks for a total of 27 μg GA/plant.

**Extraction and Purification of Metabolites.** Whole shoots (50–60 g) were harvested and homogenized with 1 L of ice-cold 80% aqueous methanol in a Waring Blendor and the residue extracted overnight at 4°C. The methanol was removed *in vacuo* at 35°C. To the remaining aqueous phase, an equal volume of 0.5 M phosphate buffer (pH 8.5) was added. The extract was partitioned three times with half-volumes of hexanes. The aqueous phase was next adjusted to pH 3 with 3 N HCl and purified by charcoal (5 g):Celite (10 g) adsorption chromatography. The GA were eluted with 80% acetone. After removing the acetone *in vacuo*, the extract

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[2] Present address: Department of Horticulture, Rutgers University, New Brunswick, NJ 08903.

[3] To whom correspondence should be addressed.

[4] Abbreviations: GA, gibberellin(s); AMO-1618, 2 *iso*propyl-4-dimethyl amino-5-methylphenyl-1-piperidine-carboxylate-methylchloride; GC-SIM, gas chromatography-selected ion monitoring; MeTMSi, methylester trimethylsilyl ether; $M^+$, molecular ion.

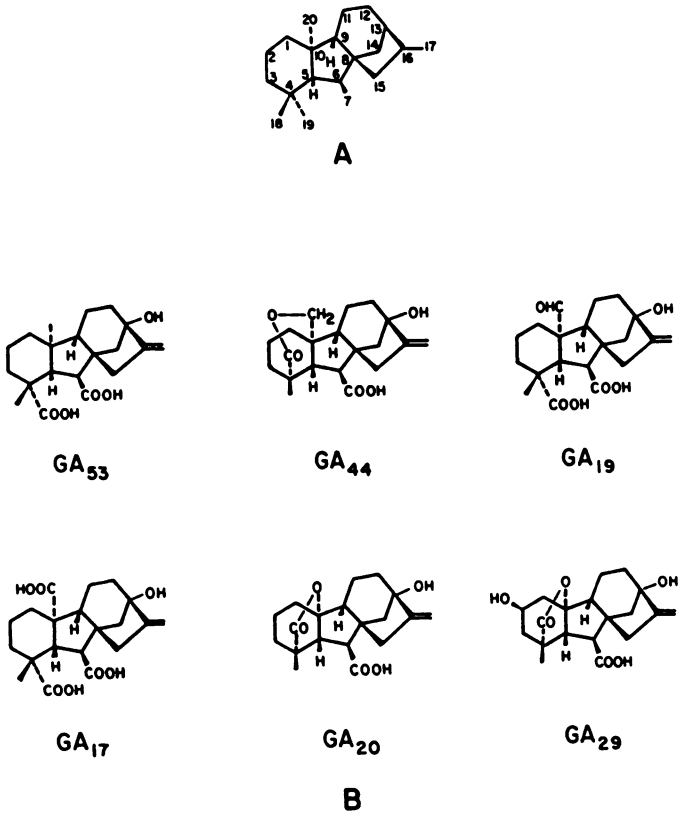


FIG. 1. Numbering system for the *ent*-gibberellane skeleton (A), and the structures of six GA found in spinach (B).

Table I. *Effect of $GA_1$, $GA_{20}$, and $GA_{53}$ on Leaf Angle and Petiole Elongation in Spinach Grown in SD*

Plants were treated with 3 μg of GA three times weekly for 3 weeks. Measurements taken 30 d after GA treatments initiated.

| Treatment | Petiole Length | Leaf Angle |
|---|---|---|
| | *mm* | *degrees* |
| SD control | 22.7 ± 1.2[a] | 27.6 ± 1.1 |
| $GA_{53}$ | 49.9 ± 1.9 | 51.1 ± 4.0 |
| $GA_{20}$ | 42.6 ± 2.0 | 44.4 ± 3.9 |
| $GA_1$ | 51.8 ± 2.7 | 46.8 ± 3.7 |
| LD | 74.6 ± 3.8 | 56.3 ± 2.8 |

[a] SE. Three leaves were measured on each of three plants.

was acidified and partitioned three times with ethyl acetate. The GA in the ethyl acetate fraction were separated by analytical reverse-phase HPLC using a linear gradient of 30 to 100% aqueous methanol according to the method of Jones *et al.* (5). HPLC fractions were methylated wih ethereal diazomethane and silylated with Tri-Sil (Pierce Chemical Co., Rockford, IL) for analysis by GC-SIM.

**Identification of Metabolites.** $[^2H]GA_{53}$ metabolites were idenified by GC-SIM using a Hewlett-Packard 5985 quadrupole GC-MS equipped with a computer data system. GC conditions were: 3% SP-2100 on 100–200 mesh Gas Chrom Q in a silanized glass column (180 × 0.2 cm), held at 215°C for 6 min, then temperature programmed to 265°C at 4°C/min with a He flow rate of 30 ml/min.

For SIM measurements, each GA was identified by monitoring four prominent ions (dwell time, 500 ms) in the mass spectrum of the trideuterated analog, and calculating the intensity ratio for these fragments. In addition, the intensity ratio of the deuterated $M^+$ cluster ($M^+$+2, $M^+$+3, $M^+$+4) was also monitored for each GA since the $[^2H]GA_{53}$ contained a mixture of deuterated analogs. In the case of $GA_{19}$-MeTMSi, the base peak cluster was used, since the $M^+$ of this compound is in low relative abundance.

## RESULTS

**Effect of GA Applications in SD.** $GA_1$, $GA_{20}$, and $GA_{53}$ applications in SD without AMO-1618 resulted in an increase in petiole elongation and a change in the leaf angle. The effect on leaf angle was evident within 24 h after GA application. The GA applied were equally effective in promoting petiole growth although less effective at the concentration used than LD (Table I).

**$[^2H]GA_{53}$ Metabolism.** $[^2H]GA_{53}$ was applied to the leaves of plants growing in SD and shoots were harvested after 2, 4, or 6 d. Petiole elongation and an increase in leaf angle were evident in each treatment. Four ions in the expected mass spectrum for the trideuterated-MeTMSi derivatives of $GA_{17}$, $GA_{19}$, $GA_{20}$, $GA_{29}$, and $GA_{44}$ were monitored by GC-SIM. During the SD time course, two metabolites were identified after 2, 4, and 6 d, namely, $[^2H_3]GA_{19}$-MeTMSi (Fig. 2), and $[^2H_3]GA_{44}$-MeTMSi (Fig. 3). The ratio of the monitored ions was similar to that previously obtained for the unlabeled analogs of these compounds (8). In addition, the relative intensity ratios of ions in the $M^+$ cluster of $[^2H]GA_{44}$-MeTMSi and in the base peak cluster of $[^2H]GA_{19}$-MeTMSi were nearly identical to that obtained for the $M^+$ cluster of $[^2H]GA_{53}$-MeTMSi (Table II). After 2 LD, one product was identified as $[^2H_3]GA_{20}$-MeTMSi (Fig. 4); none of the other possible metabolites were found. The relative intensity ratio for the ions monitored was in good agreement with previously published results for nonlabeled $GA_{20}$-MeTMSi, and the ratio of the $M^+$

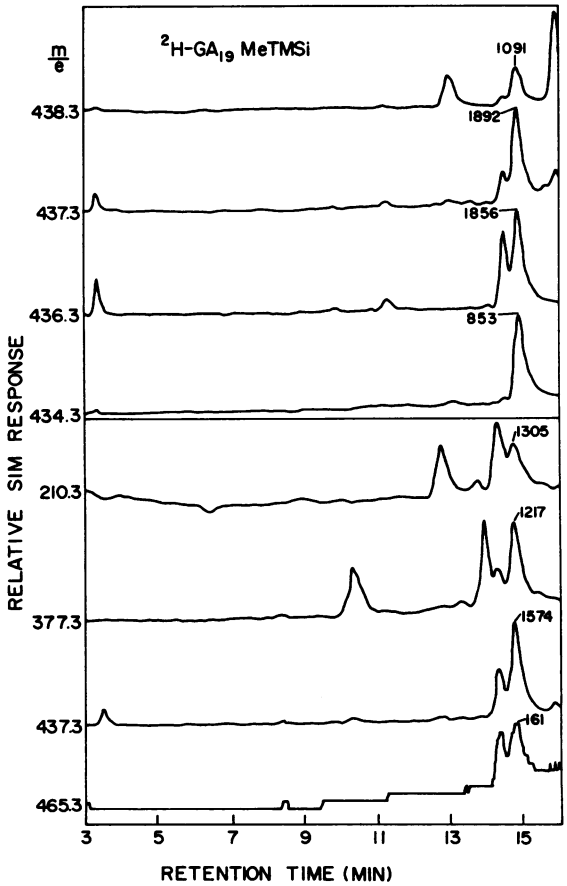


FIG. 2. Ion current chromatogram of $GA_{19}$-MeTMSi. GC-SIM data from two separate runs of the same sample. Run 1 scanned ions at m/e 438.3, 437.3, 436.3, and 434.3. Run 2 scanned ions at m/e 210.3, 377.3, 437.3, and 465.3. Numbers refer to the relative areas of the peaks obtained at 14.7 min.

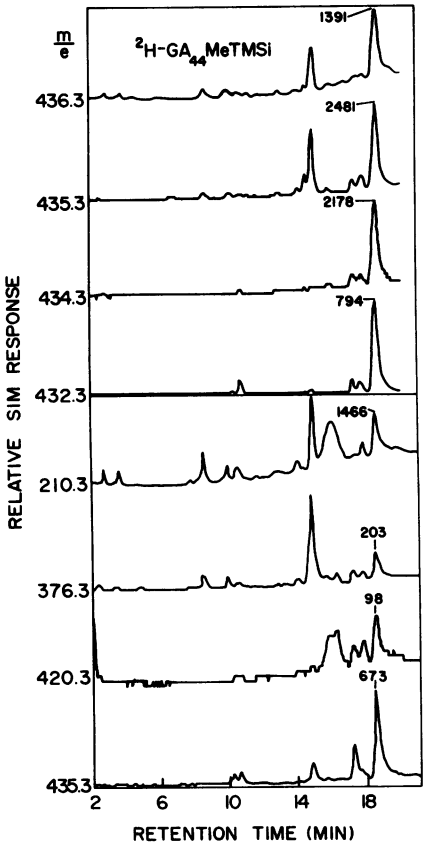


FIG. 3. Ion current chromatogram of $GA_{44}$-MeTMSi. GC-SIM data are from two separate runs of the same sample. Run 1 scanned ions at m/e 436.3, 435.3, 434.3, and 432.3. Run 2 scanned ions at m/e 210.3, 376.3, 420.3, and 435.3. Numbers refer to the relative areas of the peaks obtained at 18.4 min.

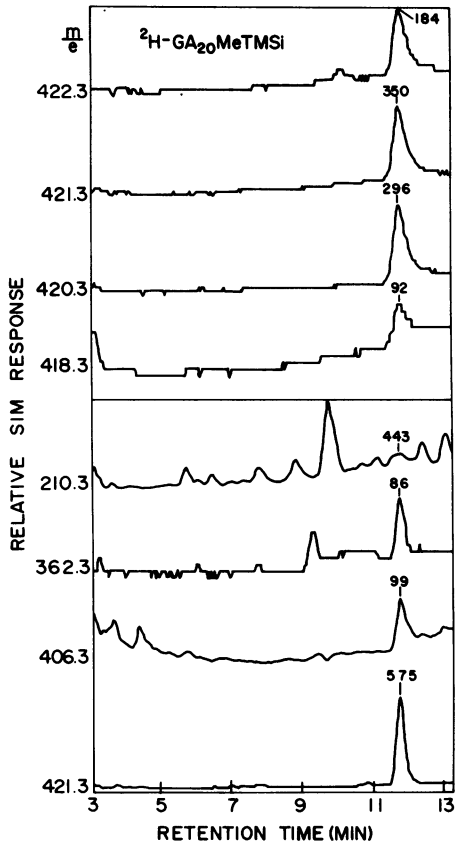


FIG. 4. Ion current chromatogram of $GA_{20}$-MeTMSi. GC-SIM data are from two separate runs of the same sample. Run 1 scanned ions at m/e 422.3, 421.3, 420.3, and 418.3. Run 2 scanned ions at m/e 210.3, 362.3, 406.3, and 421.3. Numbers refer to the relative areas of the peaks obtained at 11.7 min.

Table II. *Ratio of Peak Areas in the $M^+$ Cluster Region for $GA_{19}$, $GA_{20}$, $GA_{44}$, and $GA_{53}$*

| Compound (MeTMSi) | Ratio of Peak Areas | | |
|---|---|---|---|
| | $(M^+{+}2)$:$(M^+{+}3)$:$(M^+{+}4)$ | | |
| $GA_{19}$[a] | 38 | 39 | 23 |
| $GA_{20}$ | 36 | 42 | 23 |
| $GA_{44}$ | 36 | 41 | 23 |
| $GA_{53}$ | 35 | 42 | 23 |

[a] The base peak cluster region of $GA_{19}$ was used for this calculation.

cluster similar to the relative intensity ratio of the $M^+$ cluster of [$^2$H]$GA_{53}$-MeTMSi (Table II). In the third treatment, plants were given 4 SD followed by 2 LD. In this case, all three previously identified metabolites ([$^2$H]$GA_{19}$-MeTMSi, [$^2$H]$GA_{20}$-MeTMSi, [$^2$H]$GA_{44}$-MeTMSi) were found, with the levels of [$^2$H]$GA_{19}$-MeTMSi and [$^2$]$GA_{44}$-MeTMSi somewhat lower than those found after 6 SD.

## DISCUSSION

$GA_{53}$ at the dose applied was as biologically active as $GA_{20}$ and $GA_1$ in the spinach system in SD. Higher doses would most likely have resulted in petiole elongation equal to the LD treatment. In contrast, Bearder *et al.* (1) reported that $GA_{53}$ was relatively inactive in the dwarf rice assay. We have tested $GA_{53}$ in several bioassay systems and have found this compound to be highly active in the dwarf rice and *d-5* corn tests. In *Agrostemma*, however, $GA_{53}$ was much less active than $GA_{20}$, which in turn was less active than $GA_1$ in promoting stem growth in SD (T. Gianfagna and J. A. D. Zeevaart, unpublished results). Given the fact that [$^2$H]$GA_{53}$ is not converted to the presumably bioactive [$^2$H]$GA_{20}$ in spinach under SD, it was surprising that $GA_{53}$ did cause petiole growth. This implies that, at least in spinach, $GA_{53}$ is in itself biologically active, rather than deriving its biological activity as a result of metabolic conversion. In SD, endogenous levels of $GA_{53}$ would be too low to induce stem growth (8). The other $C_{20}$-GA produced in SD from $GA_{53}$, namely $GA_{44}$ and $GA_{19}$, may also be responsible for petiole elongation. On the other hand, Kurogochi *et al.* (7) suggested that in the rice plant $GA_{19}$ derives its biological activity from conversion to $GA_{20}$ and $GA_1$.

$GA_{19}$, $GA_{20}$, and $GA_{44}$ have been conclusively identified as metabolic products of $GA_{53}$ by $^2$H-labeling and GC-SIM. Each labeled compound had: (a) a GLC retention time similar to the previously identified unlabeled GA-MeTMSi; (b) similar relative intensity ratios for the four selected ions (3 atomic mass units higher) identified for each unlabeled GA-MeTMSi; and (c) similar ratios for the $M^+{+}2$, $M^+{+}3$, and $M^+{+}4$ ions in the $M^+$ cluster region as [$^2$H]$GA_{53}$-MeTMSi. The last finding demonstrates that the labeled products must have been derived from [$^2$H]$GA_{53}$ and that the $^2$H-metabolites were not appreciably diluted by endogenous GA.

The data strongly support the suggestion by Hedden *et al.* (4) that $GA_{53}$ is the precursor for other, more polar 13-hydroxylated GA. In addition, the finding that $GA_{53}$ was metabolized only to $GA_{44}$ and $GA_{19}$, even after 6 SD, whereas $GA_{20}$ is synthesized after only 2 LD, extends the results of Metzger and Zeevaart (9) who found higher levels of $GA_{19}$ in SD than in LD, and higher levels of $GA_{20}$ in LD than in SD.

Time course (9) studies strongly suggested that $GA_{19}$ is a precursor for $GA_{20}$, and that photoperiod controls this conversion. Results from the SD to LD transition experiment also support this contention. It cannot be ruled out, however, that $GA_{20}$ is produced by an alternate pathway from $GA_{53}$ in LD, since both $GA_{44}$ and $GA_{19}$ were still present after the LD transition. Quantitative analyses of metabolic products after feeding [$^2$H]$GA_{53}$ and [$^2$H]$GA_{19}$ (presently unavailable) will be necessary to prove that $GA_{19}$ is the precursor for $GA_{20}$, and to define the role of photoperiod in this metabolic step.

[$^2$H]$GA_{29}$ was not detected in either SD or LD, although endogenous levels increase in LD (9). Metzger and Zeevaart (10) have shown, however, that [$^3$H]$GA_{20}$ is converted to a radiolabeled metabolite which co-chromatographs with $GA_{29}$. In addition, Sponsel and MacMillan (11) have demonstrated the conversion of [$^2$H]$GA_{20}$ to [$^2$H]$GA_{29}$ in peas.

We have no information on the biosynthesis of $GA_{53}$. In the fungus, the 3-hydroxy isomer of $GA_{53}$ ($GA_{14}$) is synthesized from $GA_{14}$-aldehyde (4); however, neither $GA_{14}$-aldehyde nor the putative $GA_{53}$-aldehyde have been found in higher plants.

Finally, the results presented demonstrate for the first time, the conversion of an endogenous $C_{20}$-GA ($GA_{53}$) to an endogenous $C_{19}$-GA ($GA_{20}$) by intact green plants. Graebe *et al.* (3) have previously shown that $GA_{36}$ is converted to $GA_4$ by an *in vitro* system from *Cucurbita* endosperm, and $GA_{19}$ to $GA_{20}$ by a cell-free system from immature *Pisum* seeds (6). In addition, $GA_{53}$ was found to be biologically active in spinach, and its metabolism to $GA_{44}$, $GA_{19}$, and $GA_{20}$ regulated by photoperiod.

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