

Gibberellin A₁ is required for stem elongation in spinach

(growth retardant/gas chromatography–mass spectrometry/photoperiod/quantification)

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ABSTRACT The effects of the growth retardants 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate (AMO-1618) and calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate (BX-112) on stem elongation were investigated in the rosette plant spinach (*Spinacia oleracea* L.) under long-day (LD) conditions. Stem growth induced by a LD treatment was prevented by both retardants. The inhibition caused by AMO-1618 was reversed by gibberellin A₁ (GA₁) and GA₂₀, whereas the effects of BX-112 were reversed by GA₁ only. Six GAs (GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁, and GA₈) were quantified by gas chromatography–selected ion monitoring using internal standards. Plants treated with BX-112 had reduced levels of GA₁ and GA₈ and accumulated GA₅₃, GA₄₄, GA₁₉, and GA₂₀. The relative levels of four additional GAs (3-epi-GA₁, GA₂₉, GA₆₀, and GA₈₁) were compared by ion intensities only. Relative to GA₈₁, the level of GA₂₉ was decreased by BX-112, whereas the levels of GA₆₀ and 3-epi-GA₁ were increased. Transfer of spinach from short-day conditions to LD conditions caused an increase in all identified GAs of the early 13-hydroxylation pathway with GA₂₀, GA₁, and GA₈ showing the largest increases. These findings support the position that, of the GAs belonging to the early 13-hydroxylation pathway, GA₁ is the primary GA active *per se* for stem elongation in spinach. The increase in endogenous GA₁ in plants in LD conditions is most likely the primary factor for stem elongation.

Long-day (LD) rosette plants, such as spinach, grow vegetatively and do not produce stems under short-day (SD) conditions. Upon transfer to LD conditions, the stems elongate and flower formation takes place. LD rosette plants represent, therefore, a class of plants that are physiological dwarfs during the early stages of their life cycle. There is considerable evidence that photoperiodic control of stem elongation in rosette plants is mediated by gibberellins (GAs). Just as GA can restore the normal phenotype in GA-deficient genetic dwarfs (1), so can application of GA to rosette plants under SD conditions overcome the physiological block and cause stem growth (2). Conversely, treatment with inhibitors of GA biosynthesis suppresses stem elongation under LD (3–5). The promotive effect of LD on stem growth appears to be due to enhanced GA biosynthesis (6), which results in an increase in the levels of endogenous GAs (3, 7).

In many higher plants, including spinach (8), the major endogenous GAs belong to the early 13-hydroxylation pathway (GA₅₃, GA₄₄, GA₁₉, GA₁₇, GA₂₀, GA₁, GA₂₉, and GA₈) (9). Mutations in maize (1), pea (10), and rice (11) block the 3β-hydroxylation step, GA₂₀ to GA₁. These mutants have played a crucial role in establishing that, for the early 13-hydroxylation pathway, GA₁ is the major GA active *per se* for shoot elongation. The other GAs of the pathway are either precursors to GA₁ or inactive GA metabolites. Because no

GA biosynthetic mutants are available in spinach, it has not been possible so far to determine conclusively which GA is intrinsically active. However, the growth retardant calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate (BX-112) and other acylcyclohexanediones are known to inhibit the 3β-hydroxylation step, GA₂₀ to GA₁ (12, 13). Also, the growth retardant 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate (AMO-1618), a quaternary ammonium carbamate, inhibits *ent*-kaurene synthetase A, a step early in the pathway (9). We have used these two chemicals to determine whether one or more GAs are active *per se* in spinach. The effects of BX-112 and of photoperiod on the endogenous levels of the GAs are also presented.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea* L., Savoy Hybrid; Harris Seed Co., Rochester, NY) was grown as described (6). SD conditions consisted of 8 hr of light from fluorescent tubes and incandescent bulbs (450 μmol·m⁻²·s⁻¹) at 23°C, followed by 16 hr of darkness at 20°C. For LD conditions, the 8-hr main light period was followed by 16 hr of weak light from incandescent bulbs (12 μmol·m⁻²·s⁻¹) at 20°C.

For GA analyses, the plants were cut below the rosette, and older leaves up to the just fully expanded leaf were removed. Plants were harvested at the end of the main light period. Quantitative GA analyses were performed on lots of five plants. Samples were frozen in liquid N₂, lyophilized, and stored at -20°C until analysis. The dry weight per plant was ≈1 g.

Application of Growth Retardants. AMO-1618 was dissolved in distilled water at a concentration of 5 mM. Ten milliliters of this solution was applied per plant via the soil on alternate days. BX-112 was dissolved (20 mg/liter) in distilled water containing 0.05% Tween 20 and 5% ethanol. This solution was sprayed on the experimental plants on alternate days until the leaves were fully wetted.

Application of GAs. Solutions of GAs were prepared in distilled water, containing 0.05% Tween 20 and 5% ethanol. Solutions were applied in 50-μl volumes to the shoot apex and surrounding young leaves on alternate days (when the plants were not treated with BX-112 or AMO-1618), starting on the first LD.

[¹⁴C]GA₂₀ (1.5 GBq/mmol) was prepared as described (14). A solution containing [¹⁴C]GA₂₀ was injected into the petioles and midribs of just fully expanded and younger

Abbreviations: AMO-1618, 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate; BX-112, calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate; GA, gibberellin; LD, long day(s); SD, short day(s); SIM, selected ion monitoring; MS, mass spectrometry.

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leaves. Treatment groups consisted of four control plants and four plants treated with BX-112 for 7 LD. Each group received 13 kBq of [14 C]GA₂₀. The plants were harvested after 2 days.

Extraction and Purification. Extraction, removal of pigments, and purification via a charcoal column were performed as described (8). The GAs were eluted from the charcoal column with 80% acetone into a flask containing 10 ml of 1 M potassium phosphate (pH 8.2). The acetone was removed on a rotary evaporator and more 1 M potassium phosphate was added to give a final concentration of ≈ 0.5 M to enhance partitioning of the more polar GAs into ethyl acetate (15). After adjustment with HCl to pH 2.6, this aqueous phase was partitioned against ethyl acetate [six times, 2:1 (vol/vol)]. The combined ethyl acetate was reduced to a small volume and the extract was applied to a silicic/Celite column, 1:2 (wt/wt). The GAs were eluted with chloroform/ethyl acetate, 3:7 (vol/vol). Five milliliters of water at pH 8.0 was added to the eluate and the organic solvents were removed on a rotary evaporator. The aqueous residue solution was adjusted to pH 8.0 with KOH and the solution was applied to a column of QAE-Sephadex A-25 (3 ml). The column was washed with 3 vol of water at pH 8.0 after which the GAs were eluted with 4 vol of 1 M acetic acid. The eluate was dried on a rotary evaporator

Table 1. Comparison of the effects of the growth retardants AMO-1618 and BX-112 and GA₁ and GA₂₀ on stem and petiole length of spinach

Treatment	Stem length, cm	Petiole length, cm
Untreated	42.1 \pm 4.5	13.1 \pm 0.4
AMO-1618	3.1 \pm 0.9	5.9 \pm 0.2
AMO-1618 + GA ₂₀	44.2 \pm 3.6	10.2 \pm 0.4
AMO-1618 + GA ₁	40.9 \pm 2.5	10.2 \pm 0.3
BX-112	2.4 \pm 0.6	8.5 \pm 0.2
BX-112 + GA ₂₀	3.2 \pm 1.2	9.2 \pm 0.3
BX-112 + GA ₁	41.1 \pm 3.9	15.7 \pm 0.3

Treatments were as in Fig. 1. Two petioles that developed during the experimental treatments were measured on each plant. Measurements were after 22 days. Results are mean \pm SEM for nine plants per group.

and the residue was fractionated with a μ Bondapak C₁₈ column (30 \times 0.4 cm). A 30-min gradient of 20–80% methanol in 1% aqueous acetic acid at a flow rate of 2.0 ml/min was used. Appropriate HPLC fractions were combined (GA₈, GA₂₉, GA₈₁, GA₆₀, and 3-epi-GA₁, 7–14 min; GA₁, 15–16.5 min; GA₂₀, 21.5–22 min; GA₄₄ and GA₁₉, 23–25.5 min; and GA₅₃, 27–30.5 min), dried with a vacuum centrifuge, methylated, and trimethylsilylated.

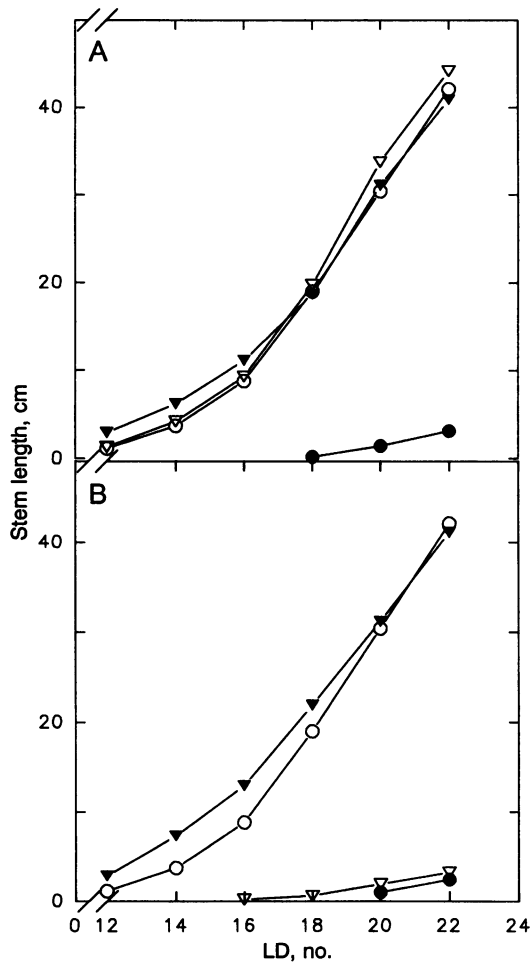


FIG. 1. Time course of stem growth of spinach plants under LD conditions as affected by the growth retardants AMO-1618 (A) and BX-112 (B) and GA₁ and GA₂₀. The growth retardants were applied every other day, starting 3 days before the plants were transferred to LD. GAs were applied 10 times and were alternated with growth-retardant applications, 2 μ g in 50 μ l of solvent, for a total of 20 μ g per plant and nine plants per treatment. (A) ∇ , GA₁; ∇ , GA₂₀; \circ , control; \bullet , AMO-1618. (B) ∇ , GA₁; \circ , control; ∇ , GA₂₀; \bullet , BX-112.

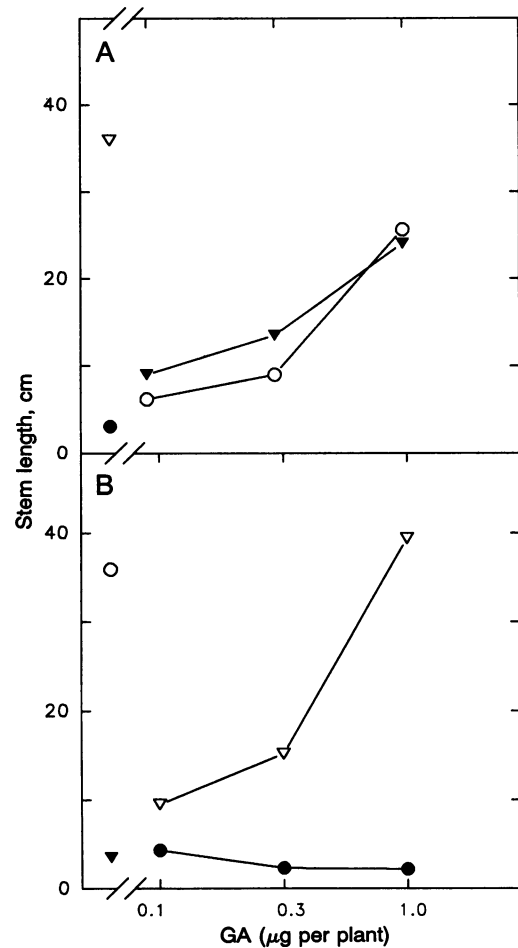


FIG. 2. Reversal of growth inhibition by AMO-1618 (A) and BX-112 (B) with various doses of GA₁ and GA₂₀. The growth retardants were applied every other day, starting 3 days before the plants were transferred to LD. GAs applied 11 times, alternating with growth-retardant treatments. Measurements were after 22 LD on five plants per treatment. (A) ∇ , Control; ∇ , GA₂₀; \circ , GA₁; \bullet , AMO-1618. (B) \circ , Control; ∇ , GA₁; ∇ , BX-112; \bullet , GA₂₀.

Table 2. Effect of BX-112 on GA content of spinach plants sprayed with BX-112, starting 2 days before transfer to LD conditions

GA	GA, ng/g (dry weight)	
	Untreated	BX-112
GA ₅₃	11.0	28.1
GA ₄₄	15.9	39.9
GA ₁₉	72.1	100.6
GA ₂₀	23.5	96.5
GA ₁	6.7	1.5
GA ₈	146.9	14.3

Plants were harvested after 9 LD.

Samples containing [¹⁴C]GA₂₀ and its metabolites were purified by the procedures described above, but the effluent from the HPLC column was passed through a radioactive flow detector (Flo-One model, Radioanalytic, Tampa, FL), using a 250- μ l solid scintillator flow cell. Radioactive peaks were collected, dried, methylated, and again purified by reverse HPLC. The radioactive fractions were dried and trimethylsilylated.

GC-MS. Qualitative GC-mass spectrometry (MS) analyses were performed with a JEOL JMS-AX 505H double-focusing mass spectrometer equipped with a Hewlett-Packard 5890A gas chromatograph. The samples were coinjected with dissolved Parafilm to establish retention times relative to *n*-alkane standards in splitless mode into a fused silica HP Ultra 2 (25 m \times 0.32 mm \times 0.52 μ m film thickness) capillary column as described (8).

GC-Selected Ion Monitoring (SIM). For quantification of GAs by GC-SIM (8), the following GAs were added to the extracts as internal standards: [17,17-²H₂]GA₅₃ (99.0% enrichment), [20-²H₁]GA₄₄ (95.7% enrichment), [17,17-²H₂]GA₁₉ (94.2% enrichment), [17,17-²H₂]GA₂₀ (99.3% enrichment), [17,17-²H₂]GA₁ (99.2% enrichment), and [17-¹³C₁]GA₈ (92.3% enrichment). Four or five prominent ions of each GA were monitored with dwell times of 100 ms. The amounts of GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁, and GA₈ were calculated from the peak area ratios 448/450, 432/433, 434/436, 418/420, 506/508, and 594/595, respectively, by the formula in ref. 16, taking into account the abundance of natural isotopes. Labeled standards were not available for 3-epi-GA₁, GA₂₉, GA₆₀, and GA₈₁, so that for these GAs comparison between their relative levels was based on the intensities of the parent ion, M⁺ = 506, in their spectra. All experiments were repeated one to three times, but data from only a single experiment are presented.

Table 3. Identification of GA₆₀ and GA₈₁ as their trimethylsilylated derivatives by GC-MS in extracts from spinach shoots

Material	KRI	Ion <i>m/z</i> (relative abundance)
Authentic GA ₆₀	2631	506 (100), 491 (16), 477 (5), 447 (14), 416 (3), 375 (47), 357 (3), 313 (5), 238 (6), 207 (17), 194 (22)
Extract	2633*	506 (100), 491 (14), 477 (7), 447 (17), 416 (4), 375 (48), 357 (4), 313 (5), 238 (5), 207 (11), 194 (17)
Authentic GA ₈₁	2743	506 (100), 491 (8), 459 (14), 447 (9), 431 (9), 389 (8), 375 (22), 303 (32), 235 (5), 207 (22)
Extract	2743*	506 (100), 491 (8), 459 (8), 447 (5), 431 (5), 389 (5), 375 (36), 303 (21), 235 (4), 207 (14)

KRI, Kovats retention index.

*KRI values were 100 higher than in ref. 8 due to erroneous assignments of *n*-alkane numbers of Parafilm.

Table 4. Comparison of the ion intensities at *m/z* = 506, the M⁺ of the trimethylsilylated derivatives of GA₆₀, GA₂₉, GA₈₁, and epi-GA₁, by GC-SIM analysis in extracts from spinach plants after exposure to 9 LD or from extracts of plants after 9 LD and treated with BX-112

Treatment	Intensity, arbitrary units			
	GA ₆₀	GA ₂₉	GA ₈₁	3-epi-GA ₁
9 LD	92 (9)	360 (34)	1071 (100)	59 (5)
9 LD + BX-112	1667 (61)	356 (13)	2718 (100)	989 (36)

Losses were not taken into account and only horizontal comparisons can be made. The same material as in Table 2 was used. Numbers in parentheses are percentages relative to the intensities of GA₈₁.

RESULTS

When AMO-1618 and BX-112 were applied to spinach plants, LD-induced stem elongation was suppressed (Fig. 1). Leaves of plants treated with AMO-1618 had a darker green color than those treated with BX-112. Petiole growth was less inhibited by BX-112 than by AMO-1618 (Table 1). Although the plants treated with growth retardants showed little stem elongation, inflorescences were produced.

In plants treated with AMO-1618, applied GA₁ and GA₂₀ were equally effective in overcoming the growth inhibition, but with BX-112, only GA₁ not GA₂₀ restored normal stem and petiole growth (Fig. 1 and Table 1). GA₁ (1 μ g) applied 11 times to BX-112-treated plants induced stem growth equal to that of the control; similar treatments with GA₂₀ gave no growth response. In contrast, GA₁ and GA₂₀ were equally effective in reversing the growth inhibition of AMO-1618-treated plants (Fig. 2).

In extracts from spinach plants treated with BX-112, the levels of GA₁ and GA₈ were reduced, whereas the levels of GA₅₃, GA₄₄, GA₁₉, and GA₂₀ were increased (Table 2). In addition to the previously identified GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁, 3-epi-GA₁, GA₂₉, and GA₈ (8), GA₆₀, and GA₈₁ were also identified by full-scan GC-MS (Table 3). The relative levels of 3-epi-GA₁, GA₂₉, GA₆₀, and GA₈₁ (internal standards not available) were estimated from the ion intensities at *m/z* 506 (the M⁺ for each of these GAs) by GC-SIM for control plants and plants treated with BX-112. In both extracts, GA₈₁ was most abundant; GA₆₀ and 3-epi-GA₁ were minor components in extracts of untreated plants. After BX-112 treatment, GA₆₀ and 3-epi-GA₁ increased relative to GA₈₁, whereas GA₂₉ decreased relative to GA₈₁ (Table 4).

All five dihydroxy-GAs (GA₁, 3-epi-GA₁, GA₂₉, GA₆₀, and GA₈₁) present in spinach are metabolites of GA₂₀ (Table 5 and Fig. 3). In plants treated with BX-112, no [¹⁴C]GA₁ or [¹⁴C]GA₈ was detected. The specific activity of each metab-

Table 5. Effects of BX-112 on metabolism of [¹⁴C]GA₂₀ in spinach plants

GA	KRI	[M+8] ⁺	
		Control	BX-112
[¹⁴ C]GA ₂₀ (substrate)	2578	426 (17)	
[¹⁴ C]GA ₂₀	2576	426 (13)	426 (10)
[¹⁴ C]GA ₁	2724	514 (15)	ND
[¹⁴ C]GA ₈	2845	602 (9)	ND
[¹⁴ C]GA ₆₀	2635	514 (10)	514 (6)
[¹⁴ C]GA ₂₉	2739	514 (10)	514 (8)
[¹⁴ C]GA ₈₁	2750	514 (12)	514 (6)
3-epi-[¹⁴ C]GA ₁	2859	514 (16)	514 (11)

GAs were identified by retention times and full-scan GC-MS of their trimethylsilylated derivatives. Ions shown are molecular ions, [M+8]⁺, of species containing four ¹⁴C atoms. The relative abundance is with respect to M⁺ (% M⁺) of nonlabeled species and is in parentheses. ND, not detected; KRI, Kovats retention index.

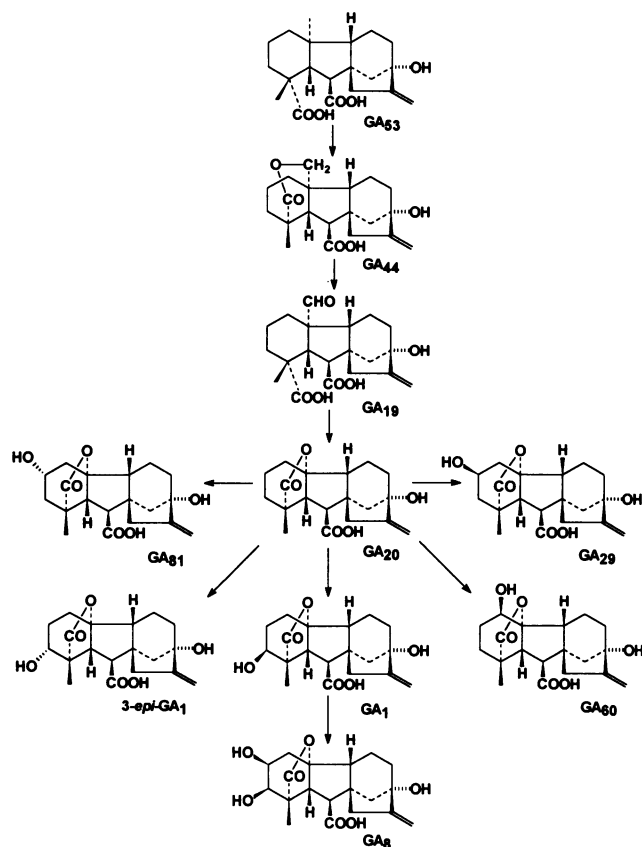


FIG. 3. Last stage of GA biosynthetic pathway in spinach. The growth retardant BX-112 specifically blocks 3β -hydroxylation of GA_{20} to the biologically active GA_1 .

olite of [^{14}C] GA_{20} was lower in extracts from BX-112-treated plants than from controls (Table 5).

When spinach plants were exposed to increasing numbers of LD, there was an increase in GA content that was highest for GA_{20} (16.5 times), GA_1 (5.1 times), and GA_8 (14.5 times) (Table 6). With increasing numbers of LD, the level of GA_{29} relative to GA_{81} decreased, whereas the levels of GA_{60} and 3-epi- GA_1 to GA_{81} remained constant (Table 7).

DISCUSSION

LD-induced stem elongation in rosette plants is preceded by extensive cell divisions in the subapical meristem (7). Native GA is apparently a limiting factor for subapical meristematic activity. The evidence presented here shows that 3β -hydroxylation of GA_{20} to GA_1 is necessary for stem elongation in spinach: (i) Inhibition of stem growth by BX-112 was reversed by GA_1 only, not by its immediate precursor, GA_{20} . In contrast, growth inhibition by AMO-1618 was reversed by both GA_{20} and GA_1 (Figs. 1 and 2). (ii) Metabolism of

Table 6. Changes in GA levels in spinach after exposure to various numbers of LD

GA	GA, ng/g (dry weight)			
	0 LD	4 LD	8 LD	12 LD
GA_{53}	5.9	6.6	7.9	7.1
GA_{44}	2.2	8.8	15.2	17.4
GA_{19}	15.0	27.5	54.2	45.0
GA_{20}	1.4	6.9	18.1	23.1
GA_1	1.0	3.5	3.7	5.1
GA_8	18.0	82.8	153.6	263.9

Stem elongation started after 10 LD.

Table 7. Comparison of the ion intensities at $m/z = 506$, the M^+ for the trimethylsilylated derivatives of GA_{60} , GA_{29} , GA_{81} , and epi- GA_1 , as measured by GC-SIM in extracts from spinach plants exposed to various numbers of LD

LD, no.	Intensity, arbitrary units			
	GA_{60}	GA_{29}	GA_{81}	3-epi- GA_1
0	21 (14)	216 (143)	151 (100)	6 (4)
4	30 (18)	217 (133)	163 (100)	9 (5)
8	95 (16)	501 (87)	578 (100)	20 (3)
12	107 (15)	530 (74)	719 (100)	26 (4)

Losses were not taken into account and only horizontal comparisons can be made. The same material as in Table 6 was used. Numbers in parentheses are percentages relative to the intensities of GA_{81} .

[^{14}C] GA_{20} to [^{14}C] GA_1 and to [^{14}C] GA_8 was inhibited by BX-112 to levels below the limit of detection by GC-MS (Table 5). (iii) The levels of GA_1 and GA_8 were lowered by BX-112 while the earlier precursors (GA_{53} , GA_{44} , GA_{19} , and GA_{20}) increased (Table 2). Thus, as was found in studies with rice (13, 17, 18), wheat (12), pea (19), and *Salix* (20), the main effect of BX-112 and other acylcyclohexanediones is inhibition of the 3β -hydroxylation step of GA_{20} to GA_1 . This step is also blocked in dwarf mutants of several species: *dl* in maize (1), *le* in pea (10), *dy* in rice (11), and *ga4* in *Arabidopsis* (21).

Besides 3β -hydroxylation of GA_{20} to GA_1 , four other hydroxylations of GA_{20} were observed in spinach: 1β -hydroxylation to GA_{60} , 2β -hydroxylation to GA_{29} , 2α -hydroxylation to GA_{81} , and 3α -hydroxylation to 3-epi- GA_1 (Table 5 and Fig. 3). Inhibition of 2β -hydroxylation by acylcyclohexanediones has been reported (13, 22, 23). In the present experiments, GA_{29} decreased relative to GA_{81} after the plants were treated with BX-112 (Table 4), but it does not necessarily follow that the absolute level of GA_{29} was decreased by BX-112, as it was in the case of GA_1 (Tables 2 and 5). Both the levels of GA_{60} and 3-epi- GA_1 were increased by BX-112 relative to GA_{81} (Table 4 and Fig. 3), indicating that 1β - and 3α -hydroxylation were not inhibited by BX-112. The observation that 3-epi- GA_1 and GA_{60} accumulate in spinach treated with BX-112 while stem growth is suppressed suggests that both these GAs have little or no activity in spinach.

During the first days of LD, the levels of GA_{53} and GA_{19} decrease (8). However, in the longer term, there is an overall increase in GA levels (Table 6). It appears that a certain threshold level of GA_1 is required for stem elongation to take place, considering that plants inhibited by BX-112 in LD (Table 2), as well as plants in SD (Table 6), had a GA content that was 4–5 times lower than that of elongating plants. However, the present GA analyses were performed on whole plants, not on meristematic tissue where the active compounds may be concentrated. In *Silene*, much larger differences in GA_1 content were detected in the subapical meristem (7), the site of GA action for stem elongation. The observation that petiole growth was less inhibited by BX-112 than stem growth (Table 1) and that petiole elongation occurs before initiation of stem elongation (4) may indicate that lower GA_1 levels are required for petiole elongation than for stem growth.

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