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IDENTIFICATION OF THREE C_{20}-GIBBERELLINS: GA_{97} (2β-HYDROXY-GA_{53}), GA_{98} (2β-HYDROXY-GA_{44}) AND GA_{99} (2β-HYDROXY-GA_{19})

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Key Word Index—*Spinacea oleracea*; *Hordeum vulgare*; *Lycopersicon esculentum*; *Pisum sativum*; *Zea mays*; 2β,13-dihydroxy-C_{20}-gibberellins GA_{97}, GA_{98}, GA_{99}.

Abstract—Three new C_{20}-gibberellins, GA_{97} (2β-hydroxy-GA_{53}), GA_{98} (2β-hydroxy-GA_{44}) and GA_{99} (2β-hydroxy-GA_{19}), have all been isolated from spinach, GA_{97} also from tomato root cultures and pea pods, and GA_{98} from maize pollen. The structures of these compounds were established by GC–mass spectrometric comparisons of the trimethylsilylated methyl esters with authentic samples prepared from gibberellic acid (GA_3). Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Although more than 90 gibberellins (GAs) have now been isolated from natural sources and have been fully characterized, many more are known from their mass spectra and await full identification [1]. Because of the low natural abundance of most GAs, identification has, in most cases, entailed chemical synthesis of putative structures and comparison of the mass spectra and Kovats retention indices (KRIs) of synthetic and natural compounds following GC–mass spectrometry [1]. Several previously uncharacterized dihydroxy-C_{20}-GAs have been detected by GC–mass spectrometry in spinach [2] and other plant species [1, 3–7]. In some of these GAs, the hydroxyl groups were tentatively assigned to the 2β- and 13-positions; 13-hydroxylation was assumed on the basis of major peaks at m/z 207 or 208 in their mass spectra [1], while the second hydroxyl was assigned to the 2β-position from structural and biosynthetic considerations. Recently, the identity of one of these compounds with 2β-hydroxy-GA_{19} (**3**) was confirmed by the synthesis of its methyl ester from the fungal GA, gibberellic acid (GA_3) [8]. The same synthetic sequence also provides access to the related 2β-hydroxy-GA_{53} (**1**) and 2β-hydroxy-GA_{44} (**2**) analogues, enabling us to identify these compounds in a range of plant species.

In this paper, we describe the syntheses of the methyl esters of GAs **1** and **2** and their identification in spinach (*Spinacia oleracea*) and other plant species. The new GAs are assigned the trivial descriptors, GA_{97} (2β-hydroxy-GA_{53}), GA_{98} (2β-hydroxy-GA_{44}) and GA_{99} (2β-hydroxy-GA_{19}), respectively, according to established convention [9]. (GA_{96} = 12β-hydroxy-GA_{73} [10, 11].)

RESULTS AND DISCUSSION

The synthesis of the GA_{99} dimethyl ester (**5**) from GA_3 has been described elsewhere [8]. The preparation of the methyl esters of GA_{97} (2β-hydroxy-GA_{53}) (**7**) and GA_{98} (2β-hydroxy GA_{44}) (**9**) from bismethoxymethyl ether (**4**) (the immediate precursor to **5**) is outlined in Scheme 1. Thus, Wolff–Kishner reduction of the dicarboxylic acid derived from **4** [12, 13] followed by methylation yielded GA_{97} dimethyl ester bismethoxymethyl ether (**6**), which was converted into **7** by hydrolysis of the methoxymethyl groups with Dowex 50W-X2 resin (H^+ form) [14], while reduction of **4** with $NaBH_4$ yielded the lactone **8**, from which GA_{98} dimethyl ester (**9**) was obtained as described for **7**. The NMR spectra of **7** and **9** (Tables 1 and 2), and of the intermediates leading to them, were fully consistent with the structural assignments. Of particular note are the resonances at δ 4.11 (*d*, J = 12.3 Hz) and 4.37 (*dd*,

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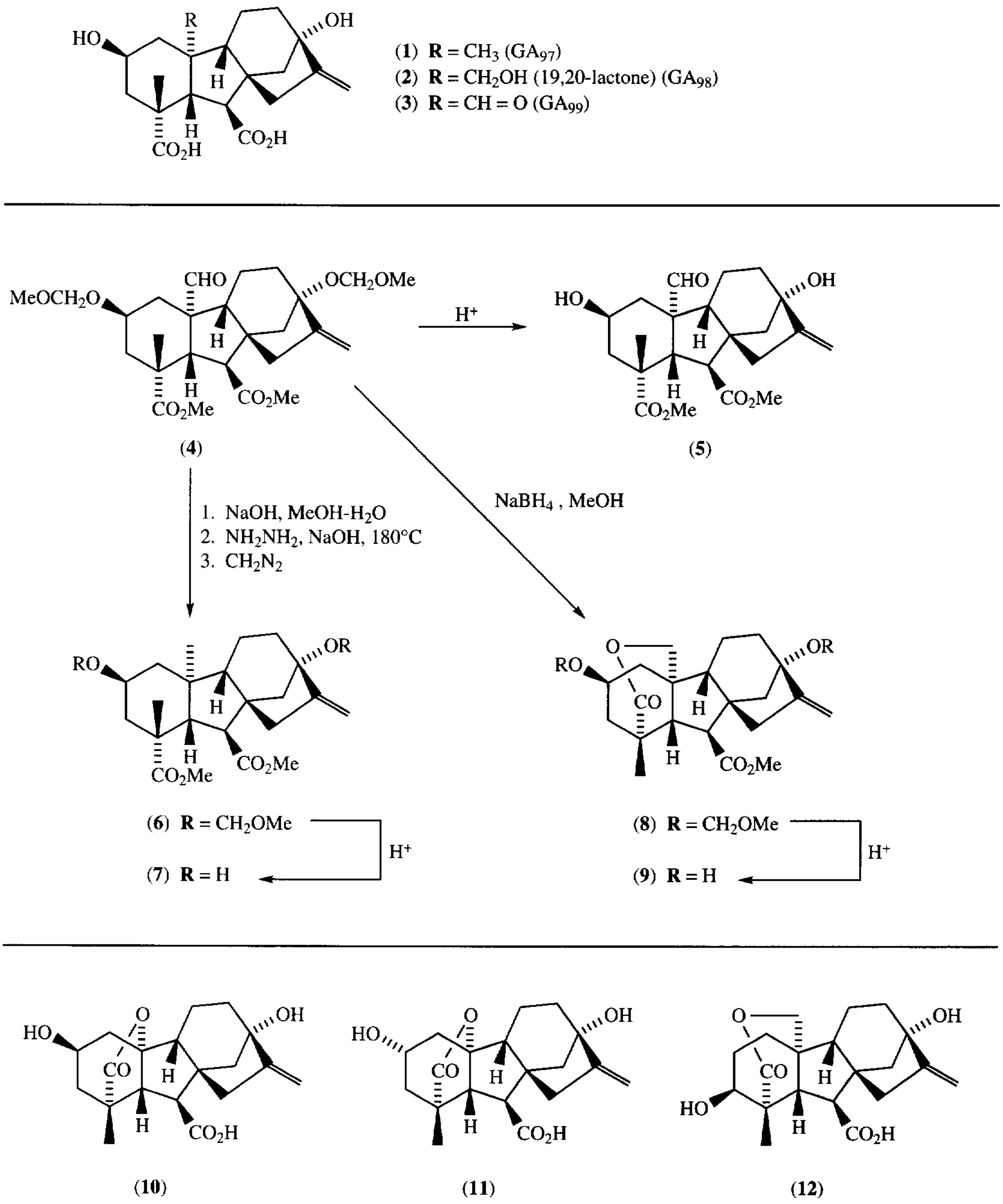

Scheme 1. Preparation of 2β,13-dihydroxy C_{20}-gibberellins.

Table 1. ^1H NMR spectral data for methyl esters of GA_{97} (**7**), GA_{98} (**9**) and GA_{99} (**5**) (300 MHz, $CDCl_3$)

H	GA_{97} (**7**)	GA_{98} (**9**)	GA_{99} (**5**)*
5	1.99 *d* (12.5)	2.20 *d* (12.6)	2.31 *d* (12.5)
6	3.32 *d* (12.5)	2.76 *d* (12.6)	3.83 *d* (12.5)
2	4.16 *m*	4.04 *m*	4.00 *m*
17	4.93 *br s*	4.92 *br s*	4.95 *br s*
	5.15 *br s*	5.23 *br s*	5.19 *br s*
18	1.14 *s*	1.14 *s*	1.17 *s*
20	0.71 *s*	4.11 *d* (12.3) (pro-*S*)	9.66 *s*
		4.37 *dd* (12.3, 2.4) (pro-*R*)	
CO_2Me	3.67, 3.71 *s*	3.71 *s*	3.65, 3.75 *s*

*From ref. [8].

Table 2. ^{13}C NMR spectral data for methyl esters of GA_{97} (**7**), GA_{98} (**9**) and GA_{99} (**5**) (75 MHz, $CDCl_3$)

C	GA_{97} (**7**)	GA_{98} (**9**)	GA_{99} (**5**)
1	47.5	41.7	41.3
2	65.2	65.5	64.9
3	48.2	46.6	44.2
4	45.0	46.7	46.7
5	56.9	52.6	55.5
6	50.1	50.8	49.3
7	175.2	174.4	174.4
8	48.6	47.8	48.9
9	55.8	54.8	55.9
10	44.2	42.5	60.7
11	18.1	16.8	18.7
12	38.7	38.0	38.3
13	78.6	78.5	78.2
44	44.7	44.7	44.3
15	46.7	48.5	46.1
16	156.4	156.8	155.7
17	106.0	106.6	106.6
18	28.5	23.0	27.7
19	177.0	173.0	175.9
20	15.8	74.0	205.6
OMe	51.6	52.0	51.9
	51.7		51.9

$J = 12.3$ and 2.4 Hz) for H-20*S* and H-20*R*, respectively, observed for lactone **9**, the smaller coupling for H-20*R* arising from a 4-bond coupling to H-1β, while in **7** the 10α-methyl group gave rise to a resonance at δ 0.71. This high field location is due to shielding by the C-19 carboxyl function and falls in the same range as that observed for other 10α-methyl GAs [15], including the parent GA_{53} [12].

The identities of all three 2β,13-dihydroxy C_{20}-GAs in spinach extracts were confirmed by comparison of their KRIs and mass spectra with those of the synthetic compounds after GC–mass spectrometry of the derived methyl ester trimethylsilyl ethers (Table 3). The GA_{97} (**1**) in spinach extracts was present in HPLC fractions 11–13 and, in common with other C_{20}-GAs, was present in higher concentrations in plants grown in short days [2]. GA_{98} (**2**) and GA_{99} (**3**) eluted earlier than GA_{97} from the HPLC column, together with GA_{29} (**10**) and GA_{81} (**11**), and were more abundant in material grown in long days, than in material from short days (Talon, M., Gage, D. A. and Zeevaart, J. A. D., unpublished data). The abundance of these GAs parallels that of the corresponding non-2-hydroxylated compounds. Thus, 2β-hydroxylation may limit the concentration of the C_{20}-GA intermediates in the early 13-hydroxylation pathway, particularly under conditions (short days) when this pathway operates at a low rate and the intermediates accumulate. It is also possible that there is a 2β-hydroxylation pathway in spinach, i.e. GA_{97}, GA_{98} and GA_{99} may form part of a biosynthetic sequence, although there is presently no evidence for this.

Mass spectra similar to that of GA_{97} (**1**) have been observed for GAs in extracts from many plant species and it is possible that this GA is ubiquitous in higher plants. The current investigation shows that is present in the developing pods of garden peas and in cultured roots of tomato, as shown by GC–mass spectrometry (Table 4), and confirms that it also occurs in developing tomato fruit [7] and in both the floral apices [3] and seven-day-old leaf sheaths [4] of barley; GA_{97}-like spectra have also been obtained from extracts of *Silene armeria* [5] and *Arabidopsis thaliana* [6]. In common with other C_{20}-GAs, GA_{97} has been found to be present at higher concentrations in the slender mutant of barley than in wild-type plants, leading to the proposal that the accumulation of C_{20}-GAs combined with the low levels of C_{19}-GAs in the mutant were the result of severe down-regulation of 20-oxidase activity in this genotype [4].

Gibberellin A_{98} (**2**) was detected in pollen from the *d1* mutant of *Zea mays* (Table 4). This compound was first observed in pea embryos by Frydman *et al.* [16], who mistakenly identified it as GA_{38} (**12**). However, by comparison of the original mass spectral data with

Table 3. Comparison of KRIs and relative intensities of significant ions for MeTMSi derivatives of putative GA_{97}, GA_{98} and GA_{99} extracted from spinach leaves with those of synthetic compounds

Identified compound	Kovats retention index* (KRI)	Significant ions (*m*/*z*) with % abundance in reference and sample
GA_{97}-Me-TMSi	2743	536($[M]^+$, 55), 521(12), 504(10), 477(14), 446(4), 387(11), 371(7), 327(10), 297(6), 239(42), 208(64), 207(100), 193(11), 179(18)
Sample (spinach)	2742	536($[M]^+$, 61), 521(13), 504(11), 477(16), 446(4), 387(11), 371(8), 327(12), 297(5), 239(46), 208(67), 207(100), 193(11), 179(17)
GA_{98}-Me-TMSi	2973	520($[M]^+$, 52), 505(8), 461(6), 371(7), 343(5), 304(5), 294(6), 281(6), 238(23), 223(4), 207(100), 180(12)
Sample (spinach)	2970	520($[M]^+$, 42), 505(8), 461(6), 371(8), 343(5), 304(5), 294(6), 281(4), 238(24), 223(5), 207(100), 180(13)
GA_{99}-Me-TMSi	2771	550($[M]^+$, 11), 535(17), 522(100), 490(22), 462(26), 432(30), 400(30), 373(33), 372(35), 343(21), 313(23), 263(19), 239(38), 208(52), 193(35), 180(18), 167(29)
Sample (spinach)	2767	550($[M]^+$, 8), 535(19), 522(100), 490(29), 462(29), 432(52), 400(35), 373(45), 372(50), 343(27), 313(26), 263(25), 239(45), 208(49), 193(42), 180(13), 167(31)

*DB-5MS capillary column.

Table 4. Comparison of KRIs and relative intensities of significant ions for MeTMSi derivatives of synthetic GA_{97} and GA_{98} with those of GAs detected in cultured tomato roots and maize pollen, respectively

Identified compound	Kovats retention index (KRI)	Significant ions (*m/z*) with % abundance in reference and sample
GA_{97}-Me-TMSi	2693*	536($[M]^+$, 24), 521(5), 504(5), 477(8), 387(6), 327(8), 268(6), 239(35), 207(100), 193(12), 179(19), 147(10), 119(19)
Sample (tomato roots)	2695*	536($[M]^+$, 26), 521(5), 504(8), 477(9), 387(7), 327(8), 268(6), 239(41), 207(100), 193(2), 179(19), 147(5), 119(9)
Sample (pea pods)	2694†	536($[M]^+$, 14), 521(5), 477(8), 387(7), 327(8), 268(7), 239(36), 207(100), 193(2), 179(20), 147(12), 119(21)
GA_{98}-Me-TMSi	2951*	520($[M]^+$, 48), 505(7), 461(5), 371(7), 343(5), 294(5), 238(28), 207(100), 193(10), 180(14), 167(9)
Sample (maize pollen)	2963*	520($[M]^+$, 20), 505(3), 461(3), 371(4), 343(3), 294(4), 238(16), 207(100), 193(4), 180(11), 167(5)

*OV-1 column [1].
†DB1-15N column.

those for the synthetic compound, the GA detected is now thought to be GA_{98} (Gaskin, P., unpublished results). This result is consistent with the observation that 3-hydroxylation does not occur in pea embryos at the developmental stage at which they were analysed, whereas 2β-hydroxylation activity is relatively strong [17].

EXPERIMENTAL

Synthesis of GA_{98} and GA_{99}

*Dimethyl-*ent*-2α,13-di(methoxymethoxy)gibberell-16-ene-7,19-dioate* (**6**). Aldehyde **4** (96 mg, 0.19 mmol) was dissolved in MeOH (1 ml) and 2 M NaOH (3 ml). The reaction mixt. was heated under reflux for 24 hr. After cooling, the mixt. was diluted with EtOAc containing 20% 2-BuOH (50 ml) and was acidified with H_3PO_4 (10%, 10 ml). The phases were sepd and the aq. phase was extracted with EtOAc–2-BuOH (2 × 20 ml). The combined organic phases were washed with brine to pH 4. The organic phase was dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. Purification on silica gel (hexane–EtOAc–HOAc, 2:1:0.1) provided the parent acid (80 mg, 88%) as an oil. Dry N_2H_4 (0.25 ml) was added to a portion of this material (30 mg, 0.081 mmol) dissolved in ethanediol (2 ml) and the reaction mixt. was heated at 100° for 30 min. Half a pellet of NaOH (*ca* 200 mg) was added and the temp. was raised to 116° for 1 hr. Finally, the temp. was raised to 180° and the reaction continued overnight. After cooling, the mixt. was diluted with EtOAc–2-BuOH (4:1, 50 ml) and acidified with H_3PO_4 (10%, 10 ml). The phases were sepd and the aq. phase was extracted with the EtOAc–2-BuOH mixt. (2 × 20 ml). The combined organic phases were washed with brine to pH 4. The organic phase was dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The residue was dissolved in MeOH (10 ml), treated with an excess of Et_2O–CH_2N_2, the solvent removed under a stream of N_2 and finally purification on silica gel (hexane–EtOAc, 3:1) afforded **6** (10 mg, 25%) as an oil. IR ν_{max} cm^{-1}: 1730. ^1H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, *s*, H-20), 1.12 (3H, *s*, H-18), 1.98 (1H, *d*, $J = 12.3$ Hz, H-5), 2.51 (1H, *ddd*, $J = 13.1, 3.2, 1.4$ Hz, H-3α), 3.33 (1H, *d*, $J = 12.3$ Hz, H-6), 3.36, 3.37 (2 × 3H, *s*, OMe), 3.68, 3.76 (2 × 3H, *s*, -CO_2Me), 4.20 (1H, *m*, H-2), 4.56, 4.76 (2 × 1H, AB*d*, $J = 7.1$ Hz, OCH_2OMe), 4.66, 4.70 (2 × 1H, AB*d*, $J = 6.8$ Hz, OCH_2OMe), 4.99 (1H, *br s*, H-17), 5.06 (1H, *br s*, H'-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.7 (C-20), 17.9 (C-11), 28.3 (C-18), 38.4 (C-12), 44.0, (C-10), 44.1 (C-14), 44.8 (C-4 and C-1 overlapped), 45.3, 45.7 (C-15, C-3), 48.7 (C-8), 50.1 (C-6), 51.6, 51.7 (CO_2Me), 55.3, 55.4 (OMe), 55.7 (C-9), 57.2 (C-5), 71.0 (C-2), 83.7 (C-13), 91.9, 95.2 (CH_2OMe), 107.1 (C-17), 152.5 (C-16), 175.3, 177.0 (C19, C7). EIMS *m/z* (rel. int.): 480 ($[M]^+$, 8), 448 (32), 418 (30), 389 (55), 358 (59), 343 (55), 327 (25), 313 (35), 299 (52), 239 (52), 179 (57), 149 (42), 85 (55), 71 (65), 59 (100). HREIMS *m/z* calc. for $[M]^+$, $C_{26}H_{40}O_8$: 480.2723; found 480.2723.

*Dimethyl-*ent*-2α,13-dihydroxygibberell-16-ene-7,19-dioate* (*GA_{97} dimethyl ester*) (**7**). Dowex 50W-X2 resin (80 mg wet resin) was added to a soln of **6** (8 mg, 0.017 mmol) in MeOH (4 ml) and H_2O (0.5 ml). The reaction mixt. was heated under reflux for 10 hr, cooled, then diluted with EtOAc (50 ml) and filtered through a pad of Celite. The filtrate was then washed with brine (10 ml), dried over Na_2SO_4 and the solvent removed *in vacuo*. Chromatography on silica gel (hexane–EtOAc, 3:1–1:2) afforded the 2-methoxymethoxy, 13-hydroxy derivative (2 mg, 30%), then diester **6** (3.0 mg 46%) as a foam. For NMR data see Tables 2 and 3. EIMS *m/z* (rel. int.): 360 $[M - MeOH]^+$ (42), 332 (19), 314 (100), 299 (51), 288 (16), 273 (24), 255 (45), 239 (25). HREIMS *m/z* calc. for $[M - MeOH]^+$, $C_{21}H_{28}O_5$: 360. 1937; found 360.1936.

ent*-2α,13-Di(methoxymethoxy)-20-hydroxygibberell-16-ene-7,19-dioic acid 7-methyl ester 19,20-lactone* (**8**). $NaBH_4$ (1.3 mg, 0.03 mmol) was added to a soln of **4** (12 mg, 0.03 mmol) in MeOH (3 ml) at 0°. After

15 min, TLC analysis showed that the reaction was complete. The soln was diluted with EtOAc (30 ml) and acidified with NaH_2PO_4 soln (20%, 5 ml). The phases were sepd and the aq. phase extracted with EtOAc (2 × 10 ml). The combined organic phases were washed with brine (2 × 5 ml), dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. Chromatography on silica gel (hexane–EtOAc, 2:1) afforded lactone **8** as a foam (10 mg). IR ν_{max} cm^{-1}: 1730. ^1H NMR (300 MHz, $CDCl_3$): δ 1.14 (3H, *s*, H-18), 2.28 (1H, *d*, $J = 12.6$ Hz, H-5), 2.75 (1H, *d*, $J = 12.6$ Hz, H-6), 3.32, 3.36 (2 × 3H, *s*, OCH_2OMe), 3.70 (3H, *s*, CO_2Me), 3.70 (1H, *m*, H-2), 4.12 (1H, *d*, $J_{gem} = 12.1$ Hz, 20-pro-*S*-H), 4.37 (1H, *dd*, $J_{gem} = 12.1$ Hz, $J_{20R,1\beta} = 2.0$ Hz, 20-pro-*R*-H), 4.53, 4.73 (2 × 1H, AB*d*, $J = 7.2$ Hz, OCH_2OMe), 4.62 (2H, *s*, OCH_2OMe), 4.98 (1H, *br s*, H-17), 5.11 (1H, *br s*, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.5 (C-11), 22.9 (C-18), 37.8 (C-12), 40.5 (C-1), 41.6 (C-3 and C-14 overlapped), 42.2 (C-10), 44.7 (C-15), 45.3 (C-4), 48.2 (C-8), 50.8 (C-6), 52.7 (CO_2Me), 54.7 (C-5), 55.3 (C-9), 55.3 (2 × OCH_2OMe), 71.2 (C-2), 73.9 (C-20), 83.5 (C-13), 91.9, 95.3 (2 × OCH_2OMe), 107.6 (C-17), 153.1 (C-16), 172.9 (C-19), 174.1 (C-7). EIMS *m/z* (rel. int.): 464 ($[M]^+$, 70), 449 (20), 433 (45), 419 (22), 402 (80), 391 (50), 375 (90), 361 (92), 342 (45), 330 (100), 312 (80), 298 (55), 179 (80). HREIMS *m/z* calc. for $[H]^+$, $C_{25}H_{36}O_8$: 464.2410; found 464.2412.

ent-2α, *13,20-Trihydroxygibberell-16-ene-7,19-dioic acid 7-methyl ester 19,20-lactone* (**9**). Dowex 50W-X2 resin (80 mg wet resin) was added to a soln of lactone **8** (11 mg, 0.023 mmol) in MeOH (3 ml) and H_2O (0.5 ml). The reaction mixt. was then heated under reflux for 14 hr, after which time TLC analysis indicated that the reaction was complete. The reaction mixt. was diluted with EtOAc (50 ml) and filtered through a pad of Celite. The filtrate was then washed with brine (10 ml), dried over Na_2SO_4 and the solvent removed *in vacuo*. Chromatography on silica gel (hexane–EtOAc, 2:1–1:2) afforded the desired deprotected lactone **9** (8.0 mg, 89%) as a foam. IR ν_{max} cm^{-1}: 1730. For NMR data see Tables 1 and 2. EIMS *m/z* (rel. int.): 376 $[M]^+$ (90), 344 (55), 316 (30), 298 (100), 280 (25), 271 (30), 253 (87), 135 (60). HRMS (EI) *m/z* calc. for $[M]^+$, $C_{21}H_{28}O_6$: 376.1886; found 376.1887.

Isolation and identification of endogenous gibberellins

Spinach plants (*S. oleracea* L. cv. Savoy Hybrid 612, Harris Seed Co., Rochester, NY) were grown and harvested as described previously [2]. Lyophilized material (25 g) of plants grown under short-day conditions or after exposure to 8 long days was analysed. Extraction, purification and analysis of GAs by GC-MS was as described in ref. [18], except that for identification of GA_{98} and GA_{99}, the GC was equipped with a DB-5MS capillary column (30 m × 0.32 mm × 0.25 μm film, J&W Scientific). GAs present in spinach extracts were identified by full-scan GC-MS by comparison with the KRIs and MS of authentic compounds.

Root cultures of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) were grown and analysed as described previously [19], except that GC-MS was carried out using a VG 7070 instrument [1].

Homozygous *dwarf-1* maize (*Z. mays* L.) seedlings were treated weekly with GA_3 (10 μg per plant) from emergence until pollination. Freshly harvested pollen (20 g) was extracted and the GAs purified using published procedures [4]. GAs in the extract were identified by full scan GC-MS [1].

Pea fruits (*Pisum sativum* L. cv. Alaska, I3) were harvested 5 days after anthesis. After removal of developing seeds, the pericarps (*ca* 50 g fr. wt of fruit walls) were ground in a polytron in precooled 80% MeOH. The first extraction was carried out at 4° for 2 hr, followed by two more extractions at 23°. Purification and isolation was then carried out as described previously [20]; GA_{97} (**1**) was located in a fr. with a R_t similar to that of GA_1 on C_{18} HPLC and identified by full-scan GC-MS.

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