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GIBBERELLINS IN IMMATURE SEED OF *PRUNUS CERASUS*: STRUCTURE DETERMINATION AND SYNTHESIS OF GIBBERELLIN, GA_{95} (1,2-DIDEHYDRO-GA_{20})

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Key Word Index—*Prunus cerasus*; Rosaceae; sour cherry; seed; gibberellin; biosynthesis of gibberellin A_{32}; gibberellin A_{95}; synthesis of GA_{95}.

Abstract—Ten C-13-hydroxylated gibberellins (GA_3, GA_{17}, GA_{19}, GA_{20}, GA_{32}, GA_{44}, GA_{86}, GA_{87}, GA_{95} and GA_{95} isolactone) and two C-13-deoxy-GAs (GA_{25} and GA_{30}) were identified in immature seeds of sour cherry (*Prunus cerasus* L. cv. Montmorency) by GC-mass spectrometry. GA_{95} is a new GA whose structure was determined to be 1,2-didehydroGA_{20} by GC-mass spectral comparison with an authentic sample, synthesized from GA_3. In addition, six unknown GA-like compounds were detected by GC-mass spectrometry. It remains unclear whether GA_{95} isolactone was an artefact. On the basis of the structures of the endogenous GAs and the absence of GA_5, the sequence of $GA_{20} \rightarrow GA_{95} \rightarrow GA_3 \rightarrow GA_{87} \rightarrow GA_{32}$ could be conceived of as a possible biosynthetic pathway.

INTRODUCTION

Gibberellin A_{32} (GA_{32}) is a water-soluble GA containing four hydroxyl groups at C3β, C12α, C13 and C15β that has been identified in immature seeds of peach (*Prunus persica* L.) [1], apricot (*P. armeniaca* L.) [2] and sour cherry (*P. cerasus* L.) [3]. GA_{32} exhibits high biological activity despite its highly oxidized structure [4] and, interestingly, is more effective than GA_3 in inducing parthenocarpy in apricot [5], sour cherry [6], peach and Japanese apricot (H. M., unpublished). The florigenic activity of GA_{32} is two to three orders of magnitude greater than that of GA_1 in the long-day plant *Lolium temulentum* L. [7, 8]. GA_{32} may be biologically active in its own right, as in the case of GA_1 [9], GA_4 [10] and possibly GA_3 [11, 12]. Recently, another water-soluble GA, GA_{86} (1,2-dihydroGA_{32}) was isolated from immature seeds of peach [13].

Little is known about the biosynthesis of GA_{32} and GA_{86}, although GA_{87} (12α-hydroxyGA_3) has been suggested to be a possible intermediate in the biosynthesis of GA_{32} in the fruitlets of sweet cherry (*Prunus avium* L.) [14]. Other GAs known to be present in *Prunus* spp are: GA_5 in immature seeds of peach [15], and GA_1, GA_5 and GA_{29} in immature seeds of apricot [16]. In metabolic studies with apricot [17] and peach [18], no conversion of GA_5 to GA_{32} was observed. Because of the potential for regulating flowering and improving fruitset with GA and our interest in the biosynthetic origin of GA_{32} in sour cherry, we investigated the endogenous GAs in immature seeds of *P. cerasus* L. using GC-mass spectrometry.

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RESULTS

The polar gibberellins in immature sour cherry seeds were identified as GA_{32} and GA_{86} by GC-mass spectral analysis of an acidic butanol fraction following chromatographic purification. The mass spectra and Kovats retention indices (KRIs) are summarized in Table 1. The structures of GAs mentioned in Tables 1 and 2 are shown in Fig. 1. The ratio of the amounts of GA_{86} to GA_{32} was about 1:2 as calculated from the areas of the $[M]^+$ at m/z 592 and 590, respectively. A minor amount of GA_{32} was also identified in the acidic ethyl acetate fraction as described below.

The GAs less polar than GA_{32} and GA_{86}, that were extracted in an acidic ethyl acetate fraction were purified by reverse-phase HPLC, yielding a number of biologically active fractions. These fractions were analysed by GC-mass spectrometry leading to the identification of GA_3, GA_{17}, GA_{19}, GA_{20}, the isolactone corresponding to 1,2-didehydroGA_{20}, GA_{25},

Table 1. GC-mass spectral data of MeTMSi derivatives of GAs in HPLC fractions from the acidic butanol fraction of *Prunus cerasus* immature seeds

HPLC fraction no.	GA identified	KRI (programme 1)	Principal ion *m/z* (relative intensity in % base peak)
12–14	GA_{32}	2967	680([M]$^+$, 43), 665(39), 636(13), 590(100), 577(21), 546(16), 500(30)
	GA_{86}	2974	682([M]$^+$, 21), 667(15), 638(6), 592(100), 579(9), 548(4), 502(9)

GA_{30}, GA_{30} isolactone, GA_{32}, GA_{44} and GA_{87} (see Fig. 1 for the structures). The mass spectra and KRIs are given in Table 2. GA_{30} and its isolactone were identified simultaneously in fraction 21 and the isolactone was also detected in authentic GA_{30}, suggesting that the GA_{30} isolactone may have been an artefact derived from GA_{30} during GC. Based on bioassay, GA_{87} represented a major constituent of the acidic ethyl acetate fraction. In addition, compounds similar to GA_5, GA_{14}, GA_{20}, GA_{24} and GA_{30} were detected which, when compared with authentic GAs, exhibited similar mass spectra but had different KRIs. Such differences in KRIs were confirmed by coinjecting authentic GAs with the GA fractions in question. The structures of these GAs remain to be elucidated.

In anticipation of the likelihood that 1,2-didehydroGA_{20} isolactone (**5**) was likely to have been formed as an artefact from 1,2-didehydroGA_{20} (**4**) we undertook the syntheses of both compounds by a common route, as shown in Fig. 2; see also [19]. Upon GC-mass spectral analysis, the synthetic sample of compound **5** exhibited data identical to those of the natural substance (Table 2). Thus, we concluded that the unknown compound was 1,2-didehydroGA_{20} isolactone. In the synthesis, the methyl ester of gibberellic acid (GA_3) was converted into its 3β,13-bis(methoxymethyl) derivative and reduced with lithium and liquid ammonia to yield acid **1**, as reported previously [20]. Iodolactonization of **1** yielded **2**, in which the 13-hydroxyl was first deprotected, then elimination of HI was effected with diazabicycloundecane (DBU), furnishing the 1,2-didehydro GA_{20} methyl ester (**3**) [21]. Simple hydrolysis of **3** in aqueous base led to several products, but thiolate-induced demethylation [22] proceeded smoothly to the parent acid (**4**: 1,2-didehydroGA_{20}). GA_3 and its derivatives are well known to be degraded readily in acidic media [23], but **4** is appreciably more labile towards acidic reagents and was very easily rearranged to the isolactone **5** by brief exposure to Dowex resin (H^+ form) [24]. The order of reactions in the sequence **1**→**4** is therefore critical. Protection of the 13-hydroxyl is essential to prevent

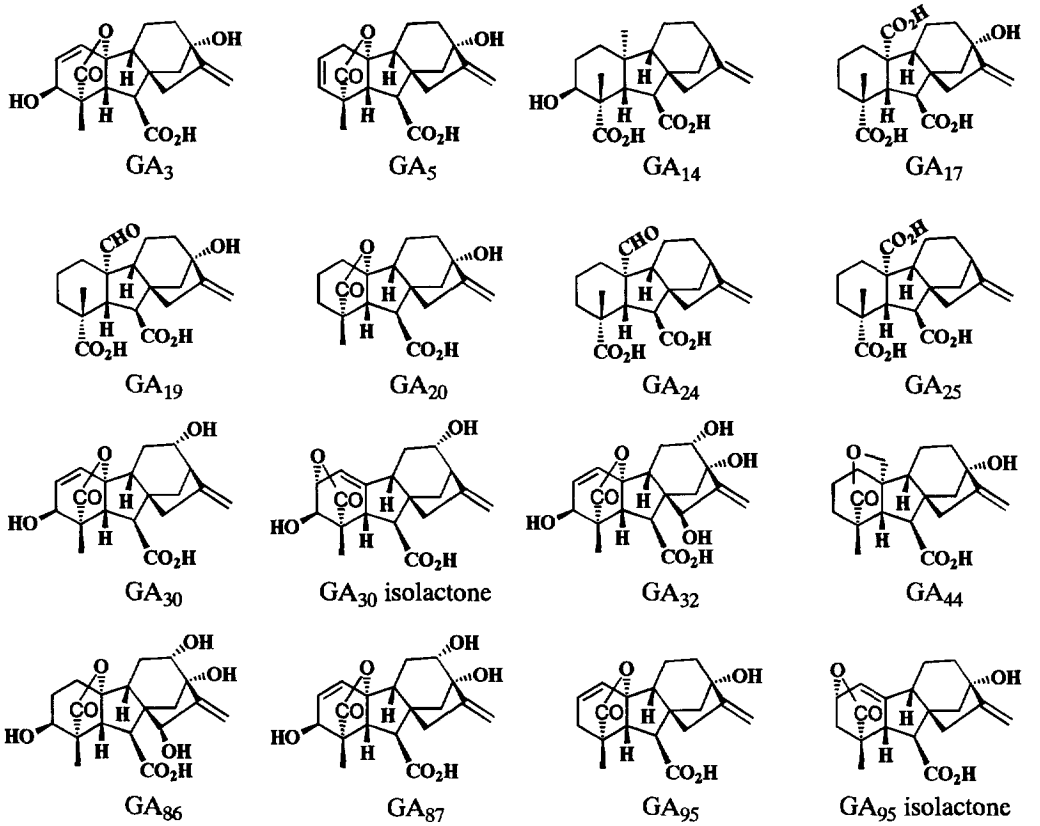


Fig. 1. Structures of GAs.

Table 2. GC-mass spectral data of MeTMSi derivatives of GAs in HPLC fractions from the acidic ethyl acetate fraction of *Prunus cerasus* immature seeds

Fraction no. in first HPLC (second HPLC)*	GA identified	Kovats retention index (KRI: programme 1)	Principal ion *m/z* (relative intensity in % base peak)
3–5 (8)	GA_{32}	2967	680([M]$^+$, 36), 665(26), 636(11), 590(100), 577(11), 546(9), 500(13)
3–5 (14, 15)	GA_{87}	2848	592([M]$^+$, 100), 577(9), 502(32), 489(69), 476(26), 458(11), 193(22)
3–5 (21)	GA_{30}	2760	504([M]$^+$, 37), 414(34), 382(23), 369(40), 280(51), 221(100)
	GA_{30}-like	2688	504([M]$^+$, 100), 489(19), 414(61), 361(32), 324(17), 280(11), 265(17), 221(26)
	GA_{30} isolactone	2640	504([M]$^+$, 53), 414(50), 382(58), 369(100), 280(53), 221(89)
3–5 (26)	GA_3	2689	504([M]$^+$, 100), 489(11), 461(6), 371(9), 347(11), 317(26), 208(24)
9	GA_{95}(1,2-didehydroGA_{20}) isolactone	2505	416([M]$^+$, 100), 401(13), 387(24), 371(4), 357(20), 343(17), 299(6), 238(36)
10	GA_5-like	2460	416([M]$^+$, 100), 401(20), 372(7)
13	GA_{30}-like	2640	504([M]$^+$, 54), 489(4), 414(43), 382(57), 369(100), 221(43)
15	GA_{20}	2490	418([M]$^+$, 100), 403(17), 389(7), 375(32), 359(13), 301(10)
17	GA_{14}-like	2449	448([M]$^+$, 9), 433(15), 416(100), 388(22), 298(72), 272(72)
18, 19	GA_{20}-like	2486	418([M]$^+$, 77), 403(23), 375(55), 358(15), 328(100), 301(15), 223(28)
22	GA_{44}	2784	432([M]$^+$, 100), 417(10), 403(2), 373(14), 238(28), 208(17)
24	GA_{19}	2595	462([M]$^+$, 17), 434(100), 402(22), 374(34), 359(6), 345(15), 207(21)
26	GA_{17}	2578	492([M]$^+$, 100), 477(4), 463(9), 460(15), 433(26), 401(11), 373(27), 208 (66)
28	GA_{24}-like†	2464	374([M]$^+$, 23), 342 (40), 314(100), 284(28), 254(17), 225(43)
29	GA_{25}†	2449	404([M]$^+$, 3), 372(48), 312(76), 284(100), 253(13), 225(52)
Authentic GAs‡	GA_5	2482	416([M]$^+$, 100), 401(6), 372(4), 357(9), 343(4), 299(15), 275(4), 208(9)
	GA_{14}	2497	448([M]$^+$, 17), 433(18), 416(100), 388(45), 298(86), 287(79), 239(47), 231(70)
	GA_{24}†	2453	374([M]$^+$, 12), 342(39), 314(100), 286(84), 254(57), 225(82)

*Different HPLC conditions except the HPLC column were employed in the 1st and 2nd HPLC.

†Analysed as the methyl ester.

‡Data of authentic GAs with which naturally occurring GAs were identified are not shown.

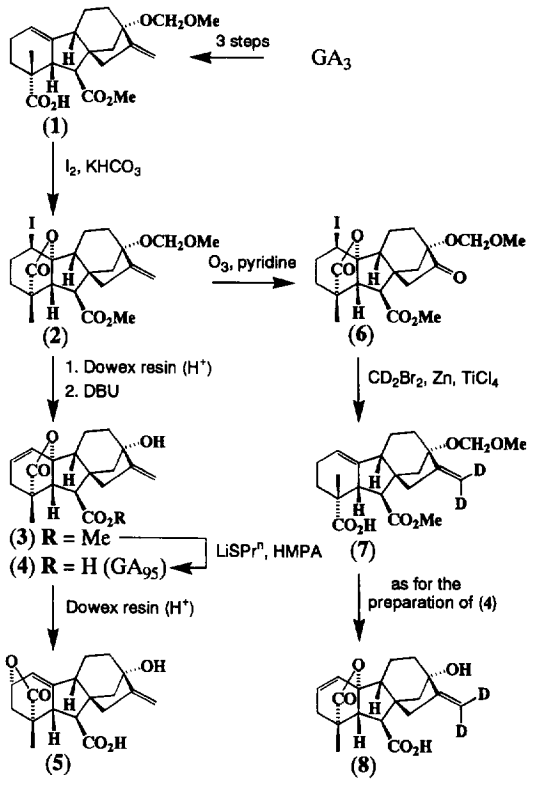


Fig. 2. Synthesis of GA_{95} (1,2-didehydroGA_{20}), GA_{95} isolactone and [$^2H_2$]GA_{95}.

rearrangement of the D-ring system [25], but then removal of the protecting group must be carried out prior to the introduction of the Δ^1-double bond.

1,2-DidehydroGA_{20} isolactone (**5**) in sour cherry may be considered to be derived by isomerization of 1,2-didehydroGA_{20}(**4**) during purification. Thus, [$^2H_2$]1,2-didehydroGA_{20} (**8**) for use as an internal reference was synthesized as outlined in Fig. 2. For the introduction of deuterium, the 16-ene function in **2** was oxidatively cleaved and then replaced, using the Lombardo modification [26] of the Nozaki–Hayashe procedure. Again, protection of the 13-hydroxyl is important: the use of the methoxymethyl ether function avoids more extensive oxidation during the ozonization and is the preferred group for optimal yields from the methylenation reaction. A new extract was prepared from immature sour cherry seeds and, using compound **8** as an internal standard, the presence of 1,2-didehydroGA_{20} was investigated. Because, in preliminary experiments, 1,2-didehydroGA_{20} was found to be isomerized in part to isolactone during purification and GC-mass spectral analysis, milder experimental conditions were employed as described in Experimental. As a result, [$^2H_2$]-1,2-didehydroGA_{20} (**8**) (KRI 2489) and its isolactone (KRI 2504) were detected, as expected, in fractions 19 and 13 (**8**, *m/z* (rel. int) 418 $[M]^+$ (100), 403 (14), 389 (24), 373 (8), 359 (18), 345 (18), 301 (8), 240 (28); isolactone of **8**, *m/z* 418 $[M]^+$ (100), 403 (12), 389 (13), 374 (12), 359 (13), 345 (12), 301 (32), 240 (12)). The ratio of **8** to its isolactone was *ca* 2:1 on the basis of the peak areas of their molecular ions, indicating that *ca* one-third of **8** was isomerized even when using relatively mild experimental conditions. However, no endogenous 1,2-didehydroGA_{20} could be reliably quantified because its level was extremely low compared with the amount (10 μg) of the deuterated standard added to the extract.

In order to reinvestigate the endogenous levels of 1,2-didehydroGA_{20} (**4**) and its isolactone (**5**) as well as GA_5, we again purified carefully a newly prepared extract. In this experiment, the deuterated internal standard was eliminated for fear of diluting the endogenous GAs with the non-deuterated contaminant. GC-mass spectral analysis of HPLC fractions using two column temperature programmes confirmed the presence of 1,2-didehydroGA_{20} isolactone in fraction 14, while 1,2-didehydroGA_{20} was found in fraction 21, as expected (Table 3). Thus, it was conclusively shown that a new compound, 1,2-didehydroGA_{20} was endogenous to sour cherry seed and subsequently has been allocated the GA_{95} descriptor according to the MacMillan–Takahashi Protocol [27]. GA_{93} and GA_{94} correspond to 1β-hydroxy-2β,3β-epoxy-GA_4 and 1β-hydroxy-2β,3β-epoxy - GA_9, respectively (P. Harrison, S. Findlow, M. Penny, C. L. Willis, P. Gaskin and J. R. Lenton, *J. Chem. Soc., Perkin Trans* **1**, submitted). The amounts of GA_{95} and its isolactone were calculated on

Table 3. Identification of MeTMSi derivatives of GA_{95}, GA_{95} isolactone and GA_{20} in GC-mass spectral re-investigation of HPLC fractions from *Prunus cerasus* immature seeds

HPLC fraction no.	GA identified	Kovats retention index		Principal ion *m/z* (relative intensity in % base peak)
		Programme 1	Programme 2	
14	GA_{95} isolactone	2507	2489	416([M]$^+$, 100), 401(13), 387(27), 371(9), 357(23), 343(21), 299(7), 238(33)
21	GA_{95}	2490	2478	416([M]$^+$, 100), 401(25), 387(12), 372(10), 357(20), 343(11), 299(40), 238(9)
23, 24	GA_{20}	2487	2475	418([M]$^+$, 100), 403(13), 389(4), 375(43), 359(13), 301(11)
Authentic GAs	GA_{95} isolactone	2505	2490	416([M]$^+$, 100), 401(14), 387(25), 371(9), 357(20), 343(18), 299(10), 238(32)
	GA_{95}	2490	2478	416([M]$^+$, 100), 401(11), 387(13), 372(13), 357(17), 343(15), 299(39), 238(13)

the basis of the areas of the molecular ions (*m/z* 416) to be 30 and 180 ng kg^{-1} fresh wt, respectively. The six-fold higher level of GA_{95} isolactone compared with GA_{95} suggests that the former may also be endogenous, since it has already been shown that only one-third of the amount of GA_{95} was isomerized during purification. However, further decisive evidence will be required in order to be conclusive. Conversely, GA_5, which, if endogenous, was expected to be present in fraction 20, could not be detected by either GC-mass spectrometry or GC-SIM in any of the fractions from 19 to 24. GA_{20} was found mainly in the expected fractions 23 and 24 (Table 3).

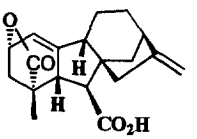
DISCUSSION

Ten C-13-hydroxylated GAs (GA_3, GA_{17}, GA_{19}, GA_{20}, GA_{32}, GA_{44}, GA_{86}, GA_{87}, GA_{95} and GA_{95} isolactone) and two C13-deoxy GAs (GA_{25} and GA_{30}) were identified in immature sour cherry seed. The present findings indicate that the early C-13 hydroxylation pathway, rather than the early nonhydroxylation pathway is predominant in GA synthesis in sour cherry seed. Recently, it was found that GA_{86} obtained from sour cherry by reverse-phase HPLC has similar activity to GA_{32} in promoting parthenocarpic development of sour cherry (H. M., unpublished), indicating that both GA_{32} and GA_{86} may play an important physiological role in fruit growth.

This first demonstration of the simultaneous occurrence of GA_{32} with GA_3 and GA_{87} in sour cherry seeds suggests that GA_3 may be converted to GA_{32} via GA_{87}. A functional pathway from GA_3 to GA_{32} appears to be suggested by the fact that both GA_3 and GA_{87} can induce parthenocarpy of *Prunus* species, although to a lesser extent when compared with GA_{32} [5] (H. M., unpublished). An alternative route to GA_{32} may involve GA_{30} rather than GA_3, with GA_{87} acting as an intermediate. No candidate for the precursor to GA_{30} in sour cherry seed was detected. It seems less likely that GA_{86} is converted to GA_{32} by dehydrogenation, as no analogous process such as the conversion of GA_1 to GA_3 has been observed in plants [11]. It is most likely that GA_{86} is synthesized from GA_{20}, although the relevant intermediates were not detected.

GA_3 can be formed from GA_{20} via GA_5 in shoots of *Zea mays* [11]. The cell-free conversion of GA_{20} to GA_5 has been observed in *Phaseolus vulgaris* L. [28, 29] and *P. coccineus* L. [30], but not in rice [29], while the *in vitro* metabolism of GA_5 to GA_3 has been demonstrated in *Marah macrocarpus* [31] and rice [29]. GA_5 has been detected in immature seeds of peach [15] and apricot [16]—species closely related to sour cherry. Convertibility of GA_5 to GA_3, GA_1 and other GAs has been demonstrated in cell suspension cultures of peach leaf [18] and less conclusively in immature seeds of apricot [17]. However, as far as the present work shows, GA_3 co-occurs with GA_{95}, but not with GA_5 in sour cherry seeds, raising the possibility that GA_3 may be biosynthesized from GA_{20} via GA_{95} by 3β-hydroxylation. The potential involvement of GA_{95} in the biosynthesis of GA_3 is an intriguing subject for future studies.

GA_{95} (**5**) is the first GA to be isolated that incorporates a Δ^1-double bond in the absence of a 3-hydroxyl and as such is a new and interesting structural type. In reporting the isolation of several GAs from *Gibberella fujikuroi* Brown *et al.* [32], described the isolation of a substance corresponding to the isolactone of 13-deoxy-GA_{95}, namely **9** and there is excellent agreement between the [^{1}H] NMR data reported by them (100 MHz, $CDCl_3$) [δ 1.23 (4-Me), 2.52 (1H, *d*, J = 6.5 Hz, H-6), 3.15 (H-5), 4.80 (H-2), 5.94 (1H, *br s*, H-1)] for the methyl ester of this substance and those observed by us for the methyl ester of GA_{95} isolactone (see Experimental). It was surmised by them that GA_{11} (**10**) was formed through auto-oxidation of the isolactone and that the natural endogenous GA was probably 1,2-didehydroGA_9 (13-deoxyGA_{95}) (**11**). The results of our present work are consistent with the assumed presence of **11** in *G. fujikuroi*.



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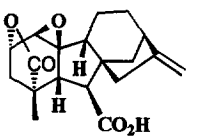
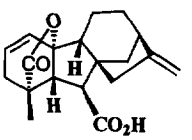


(10) GA_{11}



(11)

EXPERIMENTAL

Plant materials. Fruit of sour cherry (*Prunus cerasus* cv. Montmorency) were collected 4 weeks after anthesis from trees growing at the Horticultural Research Center at Michigan State University in 1993 or 1994. The fruit were frozen in the field with dry ice and held at −20° until the immature seeds were removed.

Bioassay. The dwarf rice (*Oryza sativa* L. cv. Tanginbozu) micro-drop bioassay [33] was used to test for biological activity.

ent-1-Iodo-13-methoxymethoxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone (**2**). A soln of acid (**1**) (223 mg) in THF (6 ml) and H_2O (10 ml) containing Na_2SO_4 (180 mg) was stirred at room temp. while iodine (70 mg) dissolved in CH_2Cl_2 (6 ml) was added slowly over a 20-min period. The mixture was diluted with EtOAc washed with brine then 0.1 M $Na_2S_2O_3$, followed by brine again, and dried over Na_2SO_4. Removal of the solvent yielded iodolactone (**2**) (240 mg). Crystallization from EtOAc–pentane yielded pure material (180 mg), mp. 187

decomp. ^{1}H NMR (300 MHz, $CDCl_3$)g; δ 1.11 (3H, *s*, H-18), 2.65 (1H, *d*, J = 9.8 Hz, H-6), 3.37 (3H, *s*, OCH_2OCH_3), 3.43 (1H, *d*, J = 9.8 Hz, H-5), 3.74 (3H, *s*, CO_2CH_3), 4.53 (1H, *d*, J = 4.3 Hz, H-1), 4.54, 4.76 (2 × 1H, AB*d*, J = 7.1 Hz, OCH_2OCH_3), 5.06 (1H, *br s*, H-17), 5.16 (1H, *br s*, H′-17).

ent-13-*Hydroxy*-1-*iodo*-20-*norgibberell*-16-*ene*-7,19-*dioic acid* 7-*methyl ester* 19,10-*lactone*. A soln of iodolactone (**2**) (245 mg) in MeOH (10 ml) and H_2O (2 ml) containing Dowex 50W resin (250 mg) was heated under reflux for 3 hr. After filtration through Celite, the solvent was removed under red. pres. and the residue taken up in EtOAc, washed with 20% KH_2PO_4, brine, and dried over Na_2SO_4. After removal of solvent the product was chromatographed on silica gel and the 13-carbinol (200 mg, 89%) eluted with EtOAc–hexane (1:1.5) and obtained as a foam. ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.05 (3H, *s*, H-18), 2.59 (1H, *d*, J = 9.9 Hz, H-6), 3.38 (1H, *d*, J = 9.9 Hz, H-5), 3.68 (3H, *s*, CO_2CH_3), 4.49 (1H, *d*, J = 4.3 Hz, H-1), 4.92 (1H, *br s*, H-17), 5.20 (1H, *br s*, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$); 16.8 (C-18), 16.9 (C-11), 27.5 (C-1), 30.8 (C-2), 31.6 (C-3), 38.0 (C-12), 42.2 (C-15), 44.8 (C-14), 49.0 (C-4), 49.4 (C-8), 50.7 (C-6), 51.2 (C-9), 52.0 (OCH_3), 54.8 (C-5), 78.0 (C-13), 94.0 (C-10), 107.6 (C-17), 156.5 (C-16), 172.3 (C-7), 178.3 (C-19).

ent-13-*Hydroxy*-20-*norgibberell*-1,16-*diene*-7,19-*dioic acid* 7-*methyl ester* 19,10-*lactone* (**3**). A soln of the iodide prepared above (100 mg) in dry CH_2Cl_2 (6 ml) was treated with DBU (560 μl) and heated at reflux overnight. The mixture was diluted in EtOAc, washed with 1 M HCl (2×), H_2O, brine, and dried over Na_2SO_4. After removal of solvent the residue was chromatographed on silica gel and the product eluted with EtOAc–pentane (1:1.5). Diene (**3**) was obtained as a white foam (55 mg). ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.19 (3H, *s*, H-18), 2.75 (1H, *d*, J = 10.5 Hz, H-6), 2.95 (1H, *d*, J = 10.5 Hz, H-5), 3.73 (3H, *s*, CO_2CH_3), 4.96 (1H, *br s*, H-17), 5.27 (1H, *br s*, H′-17), 5.88 (1H, *dt*, J = 9.0, 3.3 Hz, H-2), 6.16 (1H, *dt*, J = 9.0, 2.4 Hz, H-1). ^{13}C NMR (75 MHz, $CDCl_3$); 17.0 (C-18), 17.5 (C-11), 37.8 (C-3), 38.3 (C-12), 42.9 (C-15), 44.7 (C-14), 47.8 (C-4), 50.4 (C-8), 50.9 (C-6), 51.1 (C-9), 52.0 (OMe), 56.4 (C-5), 78.0 (C-13), 89.9 (C-10), 106.7 (C-17), 129.7 (C-2), 130.8 (C-1), 156.7 (C-16), 172.7 (C-7), 179.4 (C-19).

ent-13-*Hydroxy*-20-*norgibberell*-1,16-*diene*-7,19-*dioic acid* 19,10-*lactone*(GA_{95}) (**4**). A soln of diene ester (**3**) (55 mg) in HMPA (1.5 ml) was treated with Li thiopropoxide (200 mg) and the mixt. stirred at room temp. under N_2 for 3 hr. The mixture was then diluted with EtOAc, washed with $CuSO_4$ soln, 3 M HCl (3×), brine, and dried over Na_2SO_4. After removal of solvent, the residue was chromatographed on silica gel and the acid (**4**) (GA_{95}) was eluted with a mixture of EtOAc–CH_2Cl_2–pentane–MeOH–HOAc (1:0.5:0.5:0.025:0.025) and obtained as a foam (32 mg). ^{1}H NMR (300 MHz, $CDCl_3/d_4$-MeOH); δ 1.19 (3H, *s*, H-18), 2.68 (1H, *d*, J = 10.7 Hz, H-6), 2.86 (1H, *d*, J = 10.7 Hz, H-5), 4.96 (1H, *br s*, H-17), 5.30 (1H, *br s*, H′-17), 5.82 (1H, *dt*, J = 9.0, 3.3 Hz, H-2), 6.13 (1H, *dt*, J = 9.0, 2.4 Hz, H-1).

ent-13-*Hydroxy*-20-*norgibberell*-1(10),16-*diene*-7,19-*dioic acid* 19,2-*lactone* (**5**). A soln of diene acid (**4**) (27 mg) in MeOH (1 ml) and H_2O (0.2 ml) was treated with Dowex (H^+) resin (25 mg) and the mixt. heated under reflux for 2 hr. After filtration through Celite the soln was diluted with EtOAc, washed with brine, dried over Na_2SO_4 and the solvent removed to provide the isolactone (**5**) (16 mg) as a foam. ^{1}H NMR (Me ester) (300 MHz, $CDCl_3$); δ 1.24 (3H, *s*, H-18), 2.59 (1H, *d*, J = 6.2 Hz, H-6), 3.19 (1H, *m*, H-5), 3.73 (3H, *s*, CO_2CH_3), 4.85 (1H, *t*, J = 5.3 Hz, H-2), 5.05 (2H, *br s*, H-17), 5.94 (1H, *br s*, H-1).

ent-1-*Iodo*-13-*methoxymethoxy*-16-*oxo*-20-*norgibberellane*-7,19-*dioic acid* 7-*methyl ester* 19,10-*lactone* (**6**). A soln of iodide (**2**) (270 mg) in CH_2Cl_2 (1.5 ml) was added to a saturated soln of ozone in CH_2Cl_2 (20 ml) containing pyridine (1 ml) at −78°, then the mixt. immediately quenched with dimethyl sulphide (2 ml). The soln was allowed to warm to room temp, the solvent removed under red. pres., and the residue chromatographed on silica gel. Elution with EtOAc–pentane (1:2) afforded starting material (**2**) (100 mg) followed by ketone (**6**) (93 mg), which was obtained as a foam. ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.11 (3H, *s*, H-18), 2.68 (1H, *d*, J = 10.0 Hz, H-6), 3.31 (3H, *s*, OCH_2OCH_3), 3.43 (1H, *d*, J = 10 Hz, H-5), 3.74 (3H, *s*, CO_2CH_3), 4.50 (1H, *d*, J = 4.3 Hz, H-1), 4.59, 4.76 (2 × 1H, AB*d*, J = 7.1 Hz, OCH_2OCH_3).

17,17-[$^2H_2$]-*ent*-13-*Methoxymethoxy*-20-*norgibberell*-1(10),16-*diene*-7,19-*dioic acid* 7-*methyl ester* (**7**). A soln of ketone (**6**) (300 mg) in CH_2Cl_2 (6 ml) under an N_2 was treated with a suspension of Lombardo reagent (4.5 ml) prepared by stirring a mixt. of [$^2H_2$]-CH_2Br_2, titanium tetrachloride and zinc dust. After stirring for 30 min at room temp., the mixt. was poured into ice-cold 1M HCl and ice, then extracted with EtOAc. After washing with H_2O (3×), brine, and drying over Na_2SO_4, the solvent was removed and the residue chromatographed on silica gel. Diene acid (**7**) (158 mg, 69.5%) was eluted with EtOAc–pentane (1:2) containing a few drops of HOAc and obtained as a foam. ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.21 (3H, *s*, H-18), 2.16 (1H, *dd*, J = 15.9, 1.9 Hz, H-15), 2.53 (1H, *d*, J = 19.5 Hz, H′-15), 2.81 (1H, *m*, H-5), 3.05 (1H, *d*, J = 5.8 Hz, H-6), 3.32 (3H, *s*, OCH_2OCH_3), 3.67 (3H, *s*, CO_2Me), 4.56, 4.76 (2 × 1H, AB*d*, J = 6.9 Hz, CH_2OCH_3), 5.32 (1H, *m*, H-1). ^{13}C NMR (75 MHz, $CDCl_3$); 18.3 (C-11), 23.1 (C-2), 26.3 (C-18), 34.6 (C-3), 36.8 (C-12), 39.1 (C-4), 43.3 (C-15), 45.0 (C-14), 46.3 (C-5), 50.0 (C-8), 50.1 (C-6), 51.0 (C-9), 51.6 (OCH_3), 55.0 (CH_2OCH_3), 84.4 (C-13), 91.5 (CH_2OCH_3), 113.7 (C-1), 140.6 (C-10), 151.0 (16), 176.5 (CO), 181.4 (CO).

17,17-[$^2H_2$]-*ent*-13-*Hydroxy*-20-*norgibberell*-1,16-*diene*-7,19-*dioic acid* 19,10-*lactone* (GA_{95}) (**8**). This material was prepared from diene acid (**7**) as described for the parent GA_{95} (**4**). NMR spectra for all intermediates and for (**8**) itself were essentially identical to

those of the protio derivatives, except for the absence of signals from H,H'-17 and from C-17.

Extraction and Purification of the acidic-BuOH fraction. Immature seeds (900 g fresh wt) harvested in 1993 were extracted with 80% MeOH. The extract was red. to the aq. phase *in vacuo*, partitioned against EtOAc (pH 2.5) and H_2O-satd BuOH (pH 8.0 and then pH 2.5). The last acidic BuOH extract was applied onto a column of charcoal (100 g), and eluted stepwise with 10–50% aq. acetone. The eluates with 45% and 50% acetone were combined and purified on a Senshu-Pak ODS 4253-D column (25 cm × 10 mm). The mobile phase was supplied at 2 ml min^{-1} with the following gradient: 0–4 min, H_2O (0.1% HOAc); 4–32 min, H_2O (0.1% HOAc)–MeOH; 32–52 min, MeOH. Frs with R_t values of 11.0 to 14.0 min were dissolved in MeOH and loaded on a column of Bondesil diethylaminopropyl (DEA) silica (0.4 g, Analytichem International), which was successively eluted with 10-ml batches of 0.75, 1.0 and 1.5% HOAc in MeOH. The first two frs were combined prior to GC-MS.

Purification of the acidic EtOAc fraction to analyse less polar GAs. The acidic EtOAc fr. obtained from immature seeds (1 kg fresh wt), harvested in 1993, was dissolved in 0.1 M Pi buffer (pH 8.3), loaded on a column of PVP (30 g) and eluted with the same buffer (360 ml). The eluate was extracted with EtOAc at pH 2.5. The EtOAc phase was washed with H_2O, evapd to dryness, dissolved in 70% MeOH (0.1% HOAc) and subjected to CC in an ODS silica column (6.2 g). The eluate with 70% MeOH (0.1% HOAc) (62 ml) was redissolved in 15% acetone and applied to a charcoal column (3 g, Wako Pure Chemical Co.), which was eluted with aq. Me_2CO increasing the Me_2CO content. The eluates with 50 to 100% Me_2CO were dissolved in MeOH and passed through a column of DEA silica (0.3 g), eluted with MeOH, 0.75% HOAc in MeOH and 1% HOAc in MeOH. The last two frs were combined and subjected to HPLC using a Senshu-Pak ODS 2201-D column (20 cm × 6 mm). The mobile phase was supplied at a flow rate of 1 ml min^{-1} at 40° with the following program: 0–20 min, 45% MeOH (0.1% HOAc); 20–36 min, 45–95% MeOH (0.1% HOAc); 36–46 min, 95% MeOH (0.1% HOAc). Frs were collected every 1.5 min. Frs 3 to 5 were combined and rechromatographed on the same column at 1 ml min^{-1} using the following mobile phase gradient: 0–2 min, 20% MeOH (0.1% HOAc); 2–32 min, 20–50% MeOH (0.1% HOAc); 32–50 min, 50% MeOH (0.1% HOAc). Frs were collected every min.

Purification of the acidic EtOAc fraction following addition of $[^2H_2]$-1,2-*didehydroGA*$_{20}$. $[^2H_2]$-1,2-didehydroGA$_{20}$ (10 μg) was added to the MeOH extract obtained from freeze-dried immature seeds (0.5 kg fresh wt equivalents) harvested in 1994. The extract was red. to the aq. phase, mixed with 0.1 M Pi buffer (pH 7.0) and partitioned against EtOAc. The aq. phase was partitioned against EtOAc at pH 4 and then at pH 3.5, and these EtOAc extracts were combined, taken up in 0.1 M Pi buffer (pH 7), and applied to a column of PVP (50 g). The eluate, with the same buffer (400 ml), was adjusted to pH 3.5 and partitioned against EtOAc. The EtOAc extract was dissolved in MeOH and loaded on a column of DEA (3 g), which was eluted with each 120 ml of MeOH and MeOH containing 1% HOAc. The latter eluate was subjected to HPLC, using identical conditions as for the first HPLC in the preceding experiment. Frs were collected at 1-min intervals. Eluates were reduced to the aq. phase and partitioned against EtOAc. The EtOAc extracts were analysed by GC-MS using temp. program 1. Less stringent conditions were adopted: namely, trimethylsilylation was conducted under milder conditions (60°, 10 min) and the GC injection port temp. was lowered to 180°.

Purification of the acidic EtOAc fr. to analyse for 1,2-*didehydroGA*$_{20}$ *and congeners without internal standard.* Freeze-dried immature seeds (1.1 kg fresh wt equivalents) harvested in 1994 were treated as in the preceding experiment, except that extraction with EtOAc at pH 4 was eliminated, and that different HPLC conditions were employed. In HPLC, Senshu-Pak ODS 3251-D (250 × 8 mm) was used and the mobile phase consisted of isocratic elution with 45% MeOH (0.1% HOAc) for 30 min at a flow rate of 2 ml min^{-1} at 40°.

GC-MS and GC-SIM. Fractions to be analysed were methylated with ethereal CH_2CN_2 and trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide at 80° for 30 min unless otherwise stated. A JEOL model AX 505W GC-MS system fitted with a fused silica capillary column DB-1 (15 m × 0.258 mm) was operated at an ionization voltage (EI) of 70 eV. The He carrier gas flow rate was *ca* 1 ml min^{-1} with 30 kPa head pressure. Unless othewise stated, the injection port temp was 220° and the ionization chamber temp. was 250°. Column temp. was controlled by program 1 or 2. Unless otherwise stated, in program 1 the column temp. was successively programmed at 130° for 2 min, at 32° min^{-1} to 220°, then at 220° for 4 min, and finally at 8° min^{-1} to 270° with a 5-min isothermal hold at the end of the program. In programme 2, temp. was programmed at 130° for 2 min, at 32° min^{-1} to 200°, at 1° min^{-1} to 230°, and finally at 32° min^{-1} to 280° with a 5-min isothermal hold at the end of the programme. KRIs were obtained according to Kovats [34].

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