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Short Communication

Identification of Ten Gibberellins from Sporophytes of the Tree fern, *Cyathea australis*[1]

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ABSTRACT

Ten gibberellins (GAs) have been identified by Kovats retention indices and full mass spectra from GC-MS analysis of purified extracts of sporophytes of the tree-fern, *Cyathea australis*. These include the known GA_1, GA_4, GA_9, GA_{15}, GA_{24}, GA_{35}, and GA_{58} and three new GAs, 12β-hydroxyGA_9 (GA_{69}), 12α-hydroxyGA_9 (GA_{70}) and 12β-hydroxyGA_4 (GA_{71}). The structure of GA_{71} was established by the preparation and characterization of its methyl ester (as a metabolite of GA_4 methyl ester in a culture of prothallia of *Lygodium japonicum*).

The distribution of GAs is relevant to the evolutionary development of hormonal systems in the plant kingdom. Continuing our previous studies on lower vascular plants (9, 12, 14) we now report the first identification of GAs from the sporophytes of ferns. Of the 10 GAs identified by GC-MS from the young leaves (croziers) of *Cyathea australis* L. (*Alsophila australis* L.), three are new GAs which have now been chemically characterized and are allocated (7) the GA-numbers: GA_{69} (12β-hydroxyGA_9); GA_{70} (12α-hydroxyGA_9); and GA_{71} (12β-hydroxyGA_4) (see Fig. 2 for chemical structures).

MATERIALS AND METHODS

Endogenous GAs in *Cyathea australis* (Fig. 1). *Plant Material and Extraction.* Croziers, 3 to 10 cm in length, (2.5 kg) of *C. australis* were collected from cultivated plants on the campus of the University of California, Los Angeles, in February 1984. They were immediately frozen and extracted three times with acetone (3 L). The acetone was evaporated and the aqueous residue was partitioned twice against *n*-hexane (2 L) at pH 5. The aqueous layer was then adjusted to pH 3 with 6 N HCl and extracted three times with EtOAc[2] (1.5 L). The EtOAc extract was extracted three times with 5% aqueous $NaHCO_3$ (1 L). The combined 5% aqueous $NaHCO_3$ extracts were adjusted to pH 7 and stirred for 30 min with PVP (150 g). The PVP was filtered off and washed twice with H_2O (pH 7.0; 1 L). The combined filtrate and washings were adjusted to pH 3 and extracted three times with EtOAc (1.5 L). The combined EtOAc fractions were dried over anhydrous sodium sulfate and evaporated to give the EtOAc-soluble acidic fraction (4.5 g).

Silicic Acid Partition Chromatography. The acidic fraction, adsorbed on Celite (10 g) was placed on a column of silica gel (64 g; Baker Analyzed Reagent, 60–200 mesh), impregnated (10) with 0.5 N aqueous formic acid (40 ml). The column was eluted stepwise in 200-ml fractions with increasing percentages of EtOAc in n-hexane (Fig. 1).

Sep-Pak Cartridge Treatment. Fractions from the silicic acid partition column were dissolved in MeOH: H_2O (v/v; 1:1; 2 ml) and loaded on to a Sep-Pak C_{19} cartridge (Waters, Inc.). The cartridge was eluted three times with MeOH:H_2O (v/v; 1:1; 2 ml) and then twice with MeOH:H_2O (v/v; 4:1; 3 ml). The eluates from each solvent mixture were combined and evaporated to dryness.

TLC. The Sep-Pak eluate (17.3 mg) with MeOH:H_2O (v/v; 1:1) from combined fractions 5 and 6 (see Fig. 1) was chromatographed on a Silica gel F_{254} plate (0.5 mm thick, Merck), developed with EtOAc:*n*-hexane:acetic acid (v/v/v; 24:16:1) (Fig. 1).

HPLC. Each fraction for HPLC (Fig. 1) was dissolved in MeOH containing 0.1% acetic acid (200 μl), and injected onto a Nucleosil-5 NMe_2 column (15 cm × 4.6 mm i.d.) (13). Fractions were eluted at room temperature with MeOH containing 0.1% acetic acid at a flow rate of 1.5 ml/min. Following injection, fractions were collected every 2 min. Each fraction was evaporated to dryness *in vacuo* below 30°C.

Bioassay. The dwarf rice (*Oryza sativa* L. cv Tan-ginbozu and Waito-C) micro-drop method (8) was used to assay each fraction. After SiO_2 partition chromatography, 12 g fresh weight/plant was assayed; after TLC or HPLC, 40 g fresh weight/plant was assayed.

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[2] Abbreviations: EtOAc, ethyl acetate; GA, gibberellin; MeOH, methanol; Me, methyl ester; MeTMSi, methylester trimethylsilyl ether; Rt, retention time; TMSi, trimethylsilyl ether.

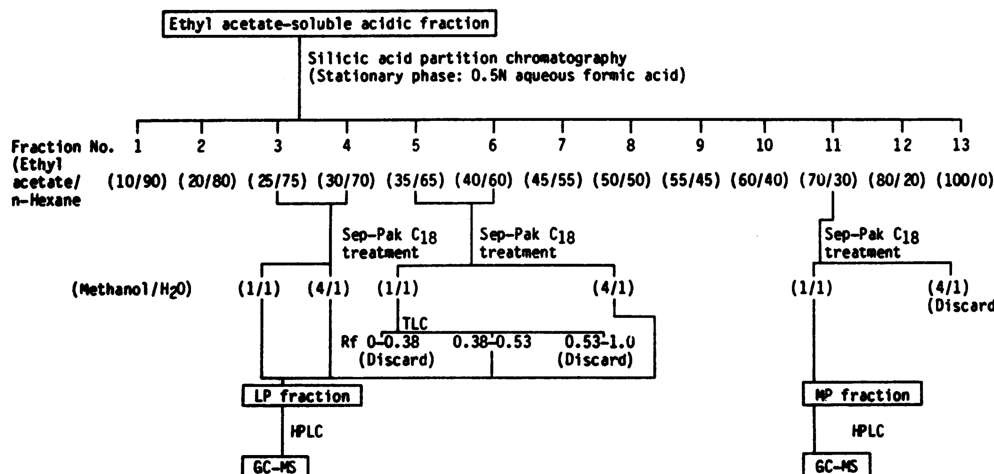

FIG. 1. Purification procedure for the EtOAc-soluble acidic fraction from acetone extracts of croziers of *C. australis*.

Table I. *GAs Identified by GC-MS Analyses (of their MeTMSi Derivatives) in Croziers of C. australis*

Retention time of GA_{40} used as standard. Full Spectra of GA_{69} and GA_{70} are given in the data of Sato *et al.* (11).

Fraction (see Fig. 1)	Retention Time on HPLC	GA Activity/ 2.5 kg Fresh Wt	GA	Kovats Retention Index	Principal Ions and Relative Abundance (base peak)
	min	*ng GA_3 equivalent*			*m/z*
LP-1	10–14[a]	500	GA_4	2497	418 (M^+, 19), 386 (15), 328 (19), 289 (88) 284 (86), 233 (42), 225 (65), and 129 (70)
			GA_9	2298	330 (M^+, 4), 298 (100), 286 (13), 270 (99), 243 (41), 227 (61), 226 (65), and 217 (30)
			GA_{15}	2601	344 (M^+, 10), 312 (17), 298 (8), 284 (44), 239 (100), and 195 (16)
LP-2	14–18[a]	100	GA_9	2298	See above
			GA_{69}	2492	418 (M^+, 15), 386 (14), 358 (20), 328 (34), 296 (86), 282 (44), 268 (100), and 223 (71)
			GA_{70}	2542	418 (M^+, 15), 403 (4), 358 (20), 328 (34), 296 (89), 282 (46), 268 (100), and 223 (76)
LP-3	18–26[a]	350	GA_{24}	2434	374 (M^+, 4), 342 (30), 314 (80), 310 (26) 286 (79), 254 (29), and 226 (100)
MP-1	10–14[b]	40	GA_1	2664	506 (M^+, 100), 491 (13), 447 (19), 377 (19), 375 (16), 235 (11), and 207 (44)
			GA_{35}	2642	506 (26), 416 (34), 390 (6), 287 (27), 282 (42) 223 (25), 221 (41), and 129 (59)
			GA_{58}	2730	506 (M^+ 37), 491 (10), 416 (100) 384 (90), 356 (91), 282 (85), 266 (56), and 223 (75)
MP-2	14–20[b]	200	GA_{58}	2730	See above
			GA_{71}	2687	506 (M^+, 44), 416 (98), 384 (95), 356 (100), 326 (39), 282 (77), 266 (61), 223 (63)

[a] 13.9 min. [b] 18.7 min.

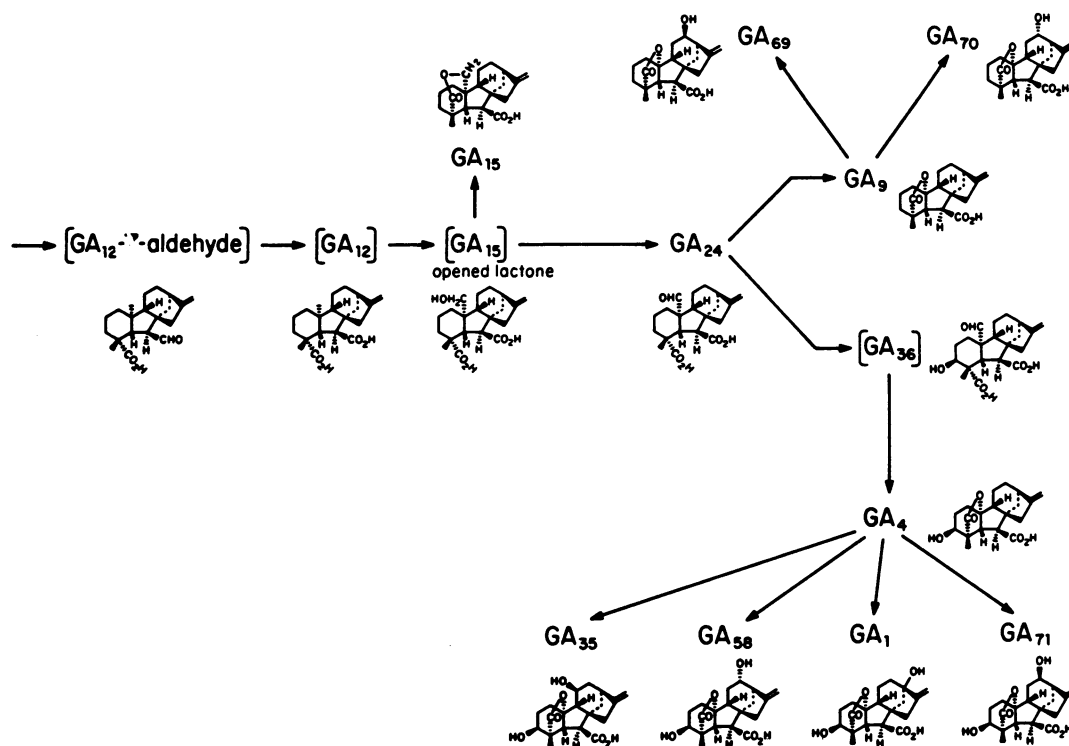

FIG. 2. Hypothetical biosynthetic pathway for the GAs in *C. australis* ([ ]: not identified by GC-MS).

Table II. *Full Mass Spectrum of* GA_{71} *MeTMSi*

Ions (Relative Abundance)					
M/Z (% base peak)					
73 (43)	197 (32)	241 (12)	280 (34)	327 (11)	385 (29)
75 (44)	207 (11)	242 (10)	281 (28)	328 (11)	388 (13)
103 (10)	209 (10)	248 (14)	282 (77)	339 (15)	395 (29)
117 (12)	213 (15)	249 (16)	283 (20)	341 (11)	398 (28)
129 (27)	220 (11)	250 (19)	287 (32)	343 (15)	399 (20)
143 (14)	221 (51)	252 (10)	294 (39)	356 (100)	416 (98)
169 (10)	222 (49)	253 (26)	295 (11)	357 (46)	417 (31)
179 (10)	223 (63)	254 (18)	298 (14)	358 (16)	418 (12)
181 (17)	224 (17)	255 (10)	308 (13)	369 (12)	446 (13)
182 (14)	227 (44)	256 (12)	311 (13)	370 (38)	460 (10)
183 (11)	228 (11)	259 (15)	312 (14)	371 (29)	474 (13)
191 (20)	231 (14)	266 (61)	313 (34)	372 (46)	478 (16)
193 (16)	238 (13)	267 (46)	314 (11)	373 (10)	491 (24)
194 (10)	239 (14)	268 (18)	317 (31)	377 (14)	506 (44)
195 (20)	240 (21)	270 (10)	326 (39)	384 (93)	507 (17)
196 (16)					

GC-MS. Bioactive fractions from HPLC were analyzed at the Department of Chemistry, University of Bristol. Each sample was methylated with ethereal diazomethane and trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (5). A computerized VG7050 mass spectrometer (VG Analytical, Manchester, U.K.) was used with a DANI 3800 GC (Kontron Instruments, St. Albans, U.K.) fitted with a WCOT vitreous silica column (25 m × 0.2 mm i.d.) coated with a 0.25 μm layer of bonded OV-1. The conditions were the same as those previously published (3). The Kovats retention indices are not absolute values and may vary under different GC conditions (3, 6).

Metabolism of GA_4 Methyl Ester by *Lygodium japonicum*. *Plant Material, Feeds, and Extraction.* At the University of Tokyo, spores (4 mg) of *Lygodium japonicum* (Thunb.) Sw. were aseptically inoculated and cultured in 20 Petri dishes (3 cm i.d.) each containing 7.5 ml Murashige and Skoog's mineral salts solution (0.1×) solidified with 0.3% agar. The prothallia were grown under continuous white light of 5 w m^{-2} (Toshiba 40 SD/NL; Tokyo Shibaura Electric Co., Japan) at 25°C for 3 weeks (11). The prothallia were then transferred to five 100-ml Erlenmeyer flasks, each containing 20 ml of the above medium without agar; GA_4 Me (0.5 mg in 50 μl of ethanol/flask) was added to the culture media. Three weeks later the culture was filtered (11) and the filtrate was extracted three times at pH 3 with EtOAc (50 ml). The combined EtOAc fractions (150 ml) were washed with 5% aqueous $NaHCO_3$ (20 ml), dried over anhydrous sodium sulfate, and evaporated to give the EtOAc-soluble neutral fraction (3.6 mg).

HPLC. The neutral EtOAc fraction was dissolved in acetonitrile (100 μl) and injected onto a Senshu PAK ODS column (Senshu Scientific Co., Tokyo, Japan; 30 cm × 6 mm i.d.). The column was eluted with acetonitrile:hH_2O (v/v; 30:70) at a flow rate of 1.5 ml/min to give GA_1 Me (about 3 μg; Rt 6.6 min), 12α-hydroxyGA_4 Me (about 120 μg; Rt 7.6 min), and 12β-hydroxyGA_4 Me (about 40 Rt 8.0 min). These three metabolites were further purified by HPLC using the same Senshu PAK ODS column, eluted with acetonitrile:H_2O (v/v; 27:73) at a flow rate of 1.5 ml/min (Rts of GA_1 Me and 12α- and 12β-hydroxyGA_4 Me; 10.2, 12.1, and 13.4 min, respectively) for the following GC-MS and H-NMR analysis. (H-NMR spectra were determined in deuterochloroform using a JEOL FX-400 FT NMR spectrometer.)

GC-MS. Fractions showing UV absorption at 210 nm were derivatized to their MeTMSi with pyridine : *N,O-bis*-trimethylsilylacetamide:trimethylsilyl chloride (v/v/v; 10:20:1). A Hitachi M-80A mass spectrometer was used, equipped with a glass column (1 m × 3 mm i.d.) packed with 2% OV-1 on Chromosorb W-DMCS; column temperature, 200°C; carrier gas, He at a flow of 35 ml/min.

RESULTS

Fractions 1, 2, 7, 8, 9, 10, 12, and 13 (Fig. 1) of the EtOAc-soluble acids from an acetone extract of croziers from *Cyathea*

Table III. *Assignments of H-NMR Spectra of 12-HydroxyGAs Identified from C. australis (δ-Value in $CDCl_3$ Downfield from TMS Internal Standard)*

	C-3 H (OH)	C-4 CH_3	C-5 H	C-6 H	C-7 OCH_3	C-12 H (OH)	C-13 H	C-17 H_2
GA_{70} methyl[a] ester (12α-OH)		1.08 (3H, s)	2.57 (1H, d)	2.64 (1H, d)	3.65 (3H, s)	3.7	2.64 (1H, d)	4.99, 5.10
GA_{69} methyl[a] ester (12β-OH)		1.10 (3H, s)	2.56 (1H, d)	2.70 (1H, d)	3.72 (3H, s)	4.08 (1H, m)	2.83 (1H, t)	5.11, 5.16
GA_{58} methyl ester (12α-OH)	3.85 (1H, bt) J = 3 Hz	1.15 (3H, s)	3.22 (1H, d) J = 10 Hz	2.73 (1H, d) J = 10 Hz	3.71 (3H, s)	3.75 (1H, q) J = 7.2, 17 Hz	2.63 (1H, d) J = 7.2 Hz	5.00, 5.10
GA_{71} methyl ester (12β-OH)	3.86 (1H, bt) J = 3 Hz	1.16 (3H, s)	3.21 (1H, d) J = 10 Hz	2.69 (1H, d) J = 10 Hz	3.72 (3H, s)	4.10 (1H, sext.) J = 7, 7, 3.5 Hz	2.84 (br) $W_{1/2}$ = 14 Hz	5.10, 5.17

[a] Sato *et al.* (11).

australis were inactive in the dwarf rice assay.

The bioactive fractions 3 and 4 were combined and passed through a Sep-Pak column (Fig. 1). The eluates (Fig. 1) both showed GA-like activity. Fractions 5 and 6 from the SiO_2 column were inhibitory in the rice assay. They were combined, passed through a Sep-Pak column (Fig. 1); one eluate was inhibitory, the other showed GA-like activity. The inhibitory eluate was chromatographed by preparative TLC and the resulting fractions bioassayed. Zones R_F 0 to 0.38 were inactive; zones R_F 0.38 to 0.53 were bioactive; inhibition was observed in zones R_F 0.53 to 1.0. The bioactive fraction from TLC and the bioactive Sep-Pak eluates were combined and designated the low polarity (LP) fraction.

Fraction 11 from the SiO_2 column was bioactive as was the MeOH:H_2O (v/v; 1:1) eluate from a Sep-Pak column (Fig. 1) of this fraction: it was designated the moderate polarity (MP) fraction. (The MeOH:H:$_2$O [v/v; 4:1] eluate was inactive).

The LP and MP fractions were both purified further by HPLC to give bioactive fractions LP-1, LP-2, LP-3, MP-1, and MP-2 which were analyzed by GC-MS (Table I). The major GAs identified were GA9, GA_{15}, GA_{24}, GA_{58}, and GA_{71}; the minor ones, GA_1, GA_4, GA_{35}, GA_{69}, and GA_{70}. Gibberellin A_1, GA_4, GA_{35}, and GA_{58} (see Fig. 2 for chemical structures) were identified by comparison of the Kovats retention indices and mass spectra of their MeTMSi derivatives with those of authentic samples.

Gibberellin A_9, GA_{15}, and GA_{24} were similarly identified as their Me esters. Of the three GAs which are allocated new GA-numbers (7), GA_{59} (12β-hydroxyGA_9) was initially identified in extracts from developing grain of *Hordeum vulgare* L. cv Proctor (3), by comparison of the Kovats retention index and mass spectrum with authentic 12β-hydroxyGA_9 obtained from a feed of 12β-hydroxykaurenoic acid to the mutant B1-41a of *Gibberella fujikuroi* (Saw.) Wr. (4); this identification has recently been confirmed by the isolation of 12β-hydroxyGA_9 Me as a metabolite of GA_9 Me in cultures of prothallia of *Lygodium japonicum* (11). The identity of GA_{70} was similarly established by comparison with 12α-hydroxyGA_9, previously identified by GC-MS in the reduction products from the metabolites of 12-oxokaurenoic acid in cultures of *G. fujikuroi*, mutant B1-41a (4); 12α-hydroxyGA_9 Me was recently isolated as a metabolite of GA_9 Me in cultures of prothallia of *L. japonicum* (11). The structure of GA_{71} was established as 12β-hydroxyGA_4 by the preparation and characterization of the Me ester, together with Ga_{58} (12α-hydroxyGA_4) Me and GA_1 Me, as metabolites of GA_4 Me in cultures of the prothallia of *L. japonicum*. The characterization of GA_{71} Me was based on the GC-MS analysis of its TMSi derivative (Table II) and the similarity of the chemical shifts and multiplicities of characteristic signals of its H-NMR spectrum to those in structurally related GAs, as shown in Table III.

In addition to the identified GAs, shown in Table I, several unidentified compounds were detected including, possibly, 16,17-dihydroxy derivatives of GA_{12} and GA_{12}-7-aldehyde, a dihydrophaseic acid, an oxodihydrophaseic acid, and a trihydroxykaurenoic acid. But neither authentic samples nor authentic mass spectral data were available to confirm their chemical structures.

DISCUSSION

These results extend the original report (9) that GA-like substances are present in the sporophytes of the tree fern, *Cyathea australis*. Ten GAs have been identified from the croziers (Table I). On the basis of current knowledge of the biosynthesis of GAs, the structures of the identified GAs can be conveniently presented in a hypothetical pathway (Fig. 2) from GA_{12}-7-aldehyde. This hypothetical pathway includes the nonselective hydroxylation of GA_4 at positions 11, 12, and 13 (which has been observed in feeds of the methyl esters of GA_9 and GA_4 to prothallia of *Lygodium japonicum* [see Sato *et al.* 1985; this paper]).

With the identification of GA_{36} from the sporophytes of *Psilotum nudum L.* (12) and GA_9 Me from the prothallia of *L. japonicum* (14), GAs have now been shown to occur in one genus of the Psilotaceae in one genus of the Schizaeaceae and in one genus of the Cyatheaceae. The Psilotaceae are the most primitive living vascular plants. Our data suggest that GAs are of widespread occurrence in land plants and probably evolved early in the evolution of vascular plants. In fungi GAs are limited, as yet, to two Ascomycetes, *Gibberella fujikuroi* and *Sphaceloma manihoticola* (1). Gibberellins have yet to be identified from members of bryophytes, algae, and bacteria, although there are reports of the occurrence of Ga-like substances in these three groups (2).

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