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GC–MS IDENTIFICATION OF ENDOGENOUS GIBBERELLINS AND GIBBERELLIN CONJUGATES AS THEIR PERMETHYLATED DERIVATIVES

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Key Word Index—*Phaseolus coccineus*; Leguminosae; plant hormones; gibberellins; gibberellin conjugates; permethylation; GC–MS.

Abstract—A convenient method of preparing permethyl derivatives of GAs and GA-glucosides is described using NaH and MeI in DMF. The permethyl derivatives of the glucosides give sharp GC peaks and their MS provide information for the identification of the GA and carbohydrate moieties. The detection and characterization of endogenous GAs and GA-glycosides in pods of *Phaseolus coccineus* by GC–MS of permethylated extracts are described. Permethylation of carbohydrates, IAA, ABA and cytokinins by the same method is described.

INTRODUCTION

The analysis of water-soluble derivatives of GAs presents problems. These GA derivatives, present in the aqueous fraction of plant extracts after removal of the free GAs by extraction with EtOAc, can be hydrolysed [1], either enzymatically or with dilute acid, before or after extraction with *n*-BuOH. Hydrolysis gives either the free GAs, or the acidic hydrolysis products of the free GAs, which can be identified by GC–MS. The main draw-back of this procedure is the lack of information obtained on the nature of the carbohydrate and the position of the glycosidic linkage.

Unlike the Me ester TMSi ether (Me/TMSi) derivatives of the free GAs, the Me/TMSi derivatives of GA-glycosyl ethers or the TMSi derivatives of GA-glycosyl esters, are not very suitable for GC–MS analysis. Although GC conditions for these derivatives of GA-glycosides have been described [2, 3], decomposition occurs [4] at the high column temperature required for GC. Moreover these derivatives have high MWs which are often beyond the mass range of many commercial GC–MS instruments and their electron impact mass spectra (EIMS) are more characteristic of the carbohydrate portion than of the GA.

Methylated derivatives of cytokinins [5], and of free GAs [6], have been used in conjunction with GC–MS. Since methyl derivatives have lower MWs and greater thermal stability than TMSi derivatives, the preparation, GC and GC–MS of permethylated GA-glucosides were examined and their usefulness for the analysis of water-soluble constituents of pods of *Phaseolus coccineus* is reported. A preliminary account of this work has been presented [7].

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RESULTS AND DISCUSSION

Various methods of methylation were investigated using a selection of free GAs and carbohydrates as standards. The GAs contained one, two and three hydroxyl groups (GA_1, GA_4, GA_8 and GA_{13}); some contained ring A double bonds (GA_3, GA_5, GA_7); and one was an epoxide (GA_6). The carbohydrates were glucose, sucrose, mannitol and methyl-α-glucoside. Methylation of the GAs with Ag_2O–MeI in various solvents did not occur readily, and the procedure of Hakamori [8], successful for cytokinins [5], gave incomplete methylation of the GAs. However with NaH–MeI in DMF at room temperature under N_2 gas, complete methylation of the GAs was obtained after 2 hr. GC separation of the mixture of permethylated GAs on an OV-1 WCOT capillary column is shown in Fig. 1 and the prominent ions in the MS, obtained by GC–MS, are listed in Table 1. Some epimerization (*ca* 30%) of the 3β-hydroxy GAs, GA_1 and GA_4, occurred to give the perMe derivatives of the 3α-epimers. The carbohydrates were also completely methylated under these conditions although glucose gave a mixture of the permethylated anomers, containing mainly the β-anomer which had the shorter GC retention time. Permethylation of zeatin, dihydrozeatin, zeatin riboside, isopentenyladenine, adenosine, kinetin and benzyladenine gave the same results as the Hakamori procedure [8]. IAA gave a mixture of methyl *N*-methylindole-3-acetate (M^+ 203, 50% and $M^+ - 59$, 100%) and a derivative containing a third methyl group (M^+ 217, 28% and $M^+ - 59$, 100%); they had close retention times on an SE-33 column. Neither was the method suitable for ABA which gave MeABA plus mono- and di-Me derivatives of MeABA and these derivatives were not completely separated by packed column GC.

GA_3-*O*-3-β-D-glucopyranoside and GA_8-*O*-2-β-D-glucopyranoside were fully methylated by the new

Table 1. Principle ions in MS of permethylated GA standards

GA_1	390 (M^+, 100%), 331 ($M^+ - CO_2Me$, 19), 317 (36.5), 177 (19), 149 (42), 135 (13), 109 (18) and 71 (19)
GA_3	(M^+, 100%), 329 ($M^+ - CO_2Me$, 16), 312 ($M^+ - CO_2$/MeOH, 25), 253 ($M^+ - CO_2$/MeOH/CO_2Me, 16), 297 (M^+ – MeOH/CO_2Me, 11), 180 (39), 150 (82), 149 (30), 135 (43), 109 (36), 99 (32)
GA_4	360 (M^+, 7%) 342 ($M^+ - 18$, 2.5), 328 (M^+ – MeOH, 38), 300 ($M^+ - HCO_2Me$, 24), 289 ($M^+ - 71$, 28), 284 (M^+ – MeOH/CO_2, 70), 224 (M^+ – MeOH/CO_2, 70), 224 (M^+ – MeOH/CO_2Me, 70), 71 (100)
GA_5	358 (M^+, 100%), 299 ($M^+ - CO_2Me$, 20), 150 (54), 149 (33), 135 (32), 109 (24)
GA_6	374 (M^+, 100%), 315 ($M^+ - CO_2Me$, 34), 245 (80), 177 (60), 150 (29), 149 (77), 135 (24), and 109 (30)
GA_7	358 (M^+, 5%), 326 (M^+ – MeOH, 22), 298 ($M^+ - HCO_2Me$, 31), 281 (M^+ – MeOH/CO_2/H, 51), 222 ($M^+ - HCO_2Me/CO_2/HCO_2Me$, 100)
GA_8	420 (M^+, 100%), 361 ($M^+ - CO_2Me$, 15), 317 (37), 180 (18), 177 (19), 150 (16), 149 (33), 135 (11), 109 (13)
GA_{13}	434 (M^+, 2%), 402 (15), 374 (6), 342 (74), 314 (24), 310 (38), 282 (48), 71 (100)
GA_3-*O*-3-*β*-D-glucopyranoside—ref. [12]	
GA_8-*O*-2-*β*-D-glucopyranoside—Fig. 2	
GA_{35}-*O*-11-*β*-D-glucopyranoside	594 (M^+, 0%), 563 (M^+ – OMe, 0.1), 359 (M^+ – tetra $MeC_6H_{11}O_6$, 17), 283 (12.5), 223 (5), 101 (92), 88 (100)
GA_1-*β*-D-glucopyranosyl ester	594 (M^+, 3%), 562 E(M^+ – OMe, 0.1), 359 (M^+ – tetra $MeC_6H_{11}O_6$, 17), 149 (8.5), 101 (100), 71 (86)
GA_4-*β*-D-glucopyranosyl ester	564 (M^+, 0%), 532 (M^+ – MeOH, 2), 329 (M^+ – tetra $MeC_6H_{11}O_6$, 15.5), 225 (25), 101 (64), 88 (100)

procedure. Permethylation of the glucosyl esters of GA_1 and GA_4 caused some (less than 5%) hydrolysis of the ester function.

The MS of the permethylated GA standards (Table 1 and Fig. 2), showed fragmentations which closely paralleled those of the Me/TMSi derivatives [9]. For example the perMe and Me/TMSi derivatives of the 13-hydroxy GAs showed M^+ ions which were the base peaks, and the ions at *m/z* 208/207 in the Me/TMSi derivatives, representing the ring C/D fragment, appear at *m/z* 150/149 in the perMe derivatives. An ion at *m/z* 238 in the MS of the Me/TMSi derivatives, corresponding to the ring C/D fragment plus transfer of OMe from CO_2Me, appeared in the MS of the perMe derivatives at *m/z* 180. Also ions at *m/z* 129 and $M^+ - 129$ in the MS of 3-OTMSi GAs correspond to the ions at *m/z* 71 and $M^+ - 71$ in the MS of the 3-OMe GAs. Thus many of the ions in the MS of the Me/TMSi derivatives are represented by analogous ions of 58 mass units less in the MS of the perMe derivatives.

The MS of permethylated carbohydrates have been discussed [13, 14]. The fragmentation patterns though similar were characteristic of the individual carbohydrates.

The MS of the GA-glucosides (Table 1 and Fig. 2) contained M^+ ions of reasonable intensity. Fragmentation was dominated by the fragmentation of the perMe glucosyl portion but information on the GA-portion can be obtained from the M^+ – tetramethyl glucose and M^+ – tetramethyl glucosyl ions. The comparison of the GC traces (Fig. 4B, C) and the MS (Figs. 2 and 3A) for the perMe and Me/TMSi derivatives of GA_8-*O*-2-*β*-D-glucopyranoside illustrates the superiority of the perMe derivative. The MS of the Me/TMSi derivative does not show an M^+ ion (*m/z* 972) and the M^+ – tetraTMSi glucosyl ion (*m/z* 505) is very weak.

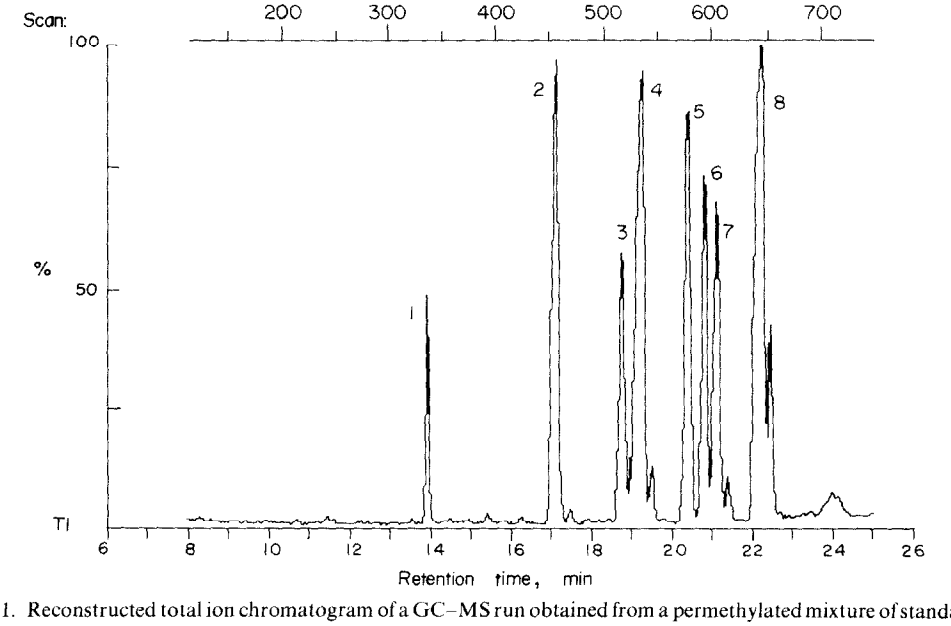

Fig. 1. Reconstructed total ion chromatogram of a GC–MS run obtained from a permethylated mixture of standard GAs on a OV-1 20 m WCOT glass capillary column. Peaks: 1, GA_9; 2, GA_5; 3, GA_4; 4, GA_7; 5, GA_{13}; 6, GA_6; 7, GA_3; 8, GA_8.

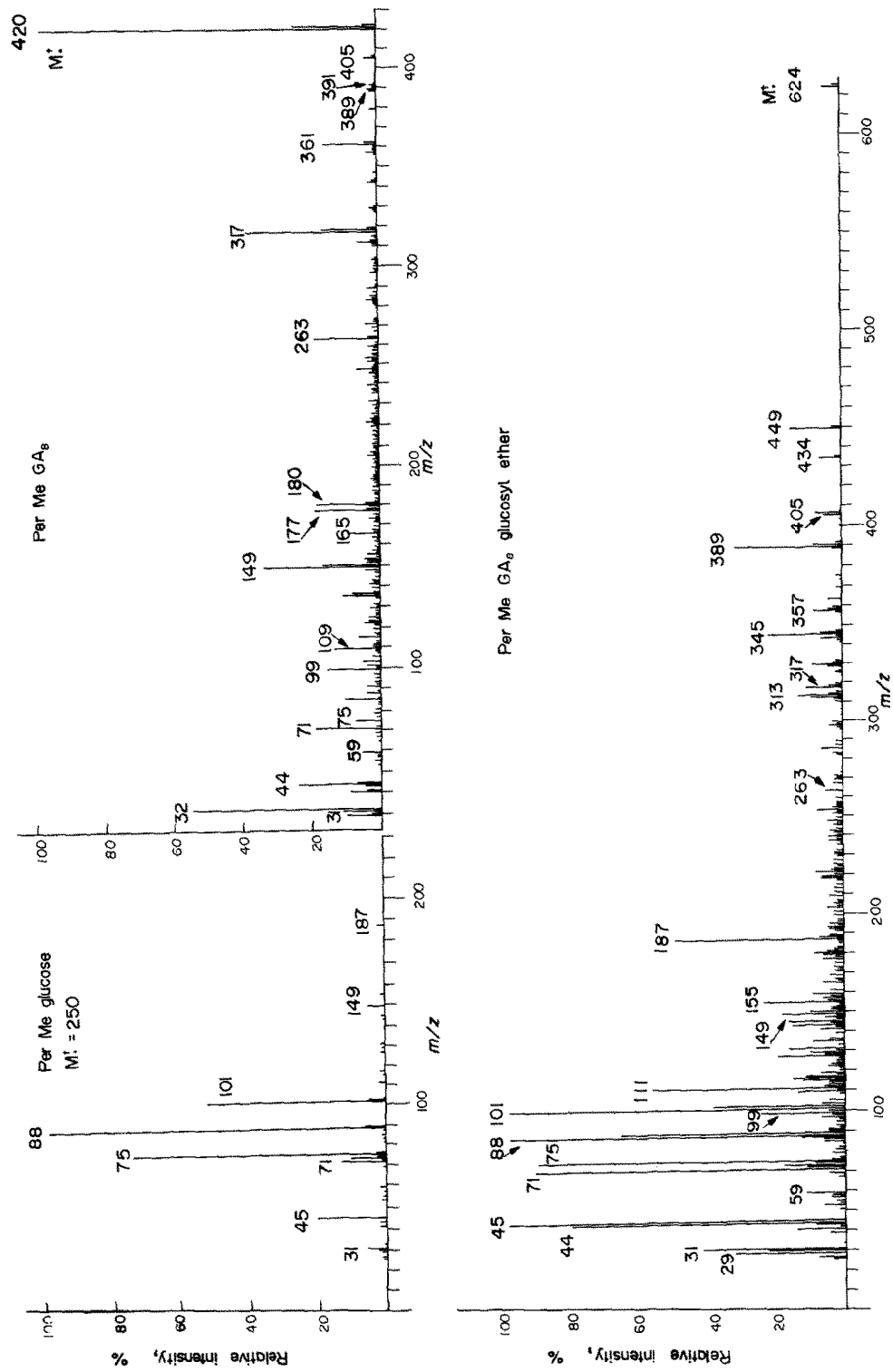

Fig. 2. EIMS (24 eV) of glucose, GA_8 and GA_8-*O*-2-β-D-glucopyranoside standards after permethylation.

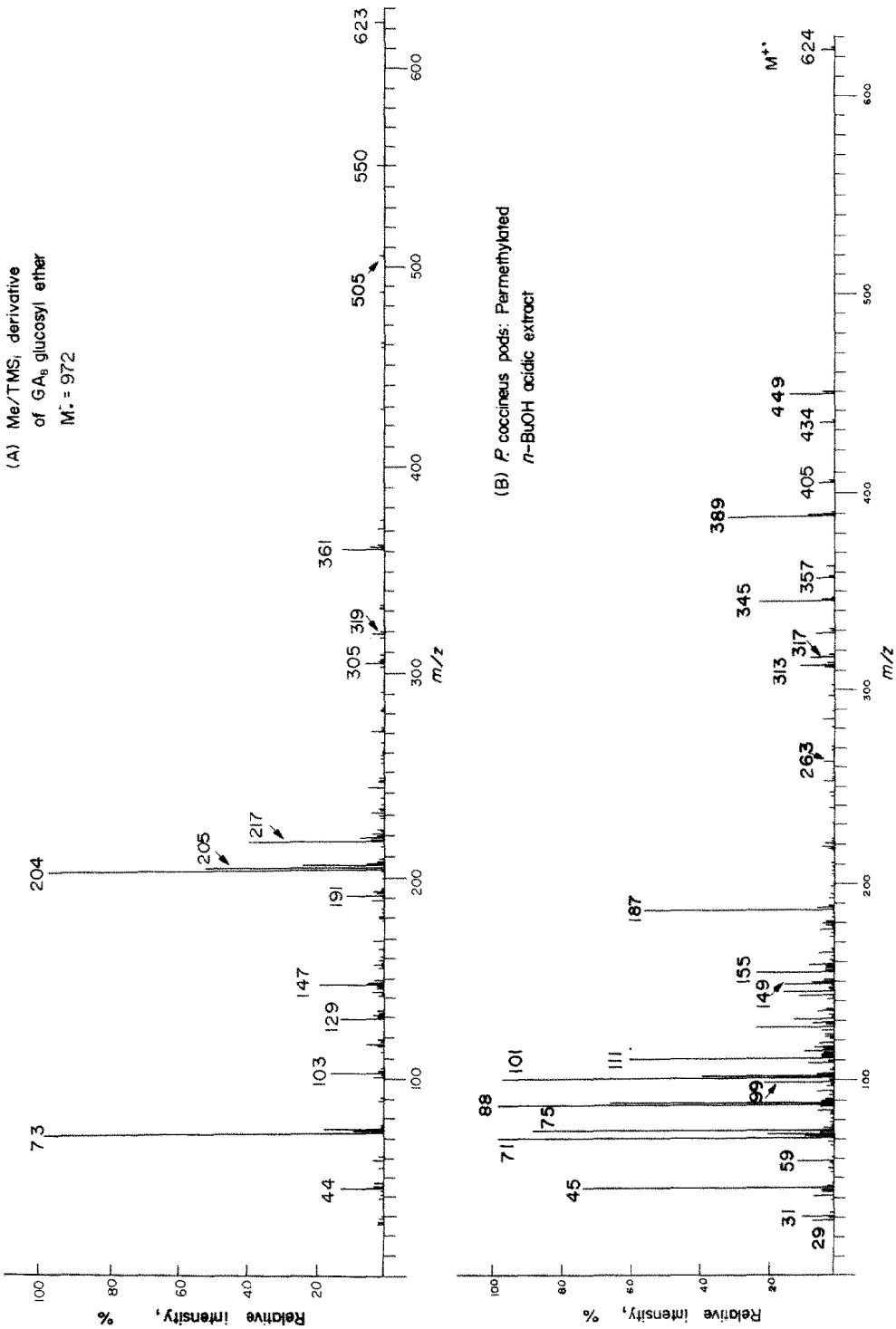

Fig. 3. A. EIMS (24 eV) of the Me/TMSi derivative of GA_8-*O*-2-β-D-glucopyranoside. The relative abundances of the ions have been normalized to the *m/z* 73 fragment. B. EIMS (24 eV) of the compound emerging from the GC run of the permethylated acidic *n*-BuOH extract of *P. coccineus* pods, having a retention time corresponding to that of the permethylated GA_8-*O*-2-β-D-glucopyranoside standard.

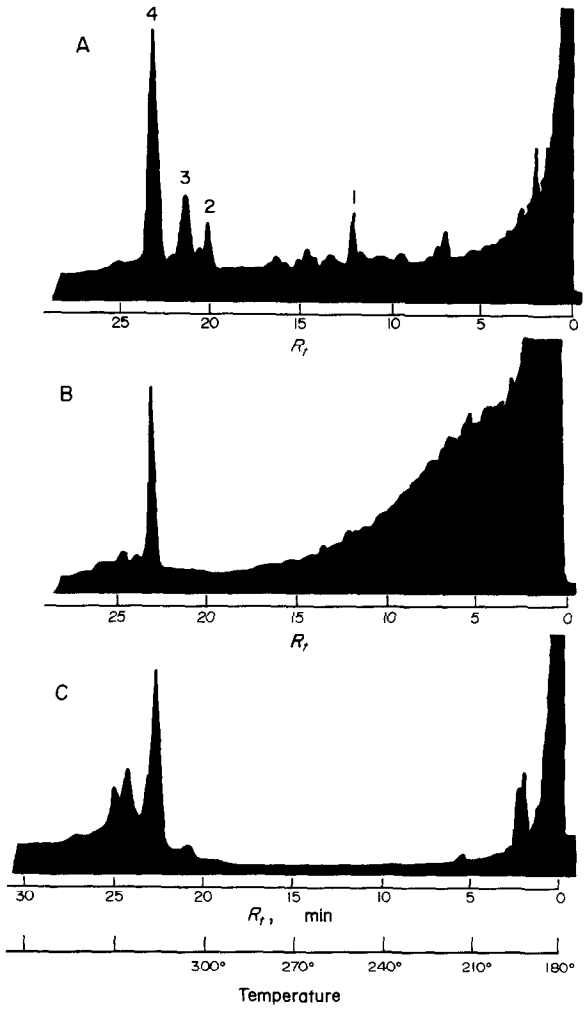

Fig. 4. GCs obtained using FID for: A. the perMe *n*-BuOH-soluble fraction of *P. coccineus* pods. Peaks 1 to 4 were identified by GC–MS as indicated in the text; B. the perMe derivative of GA_8-*O*-2-*β*-D-glucopyranoside; C. the Me/TMSi derivative of GA_8-*O*-2-*β*-D-glucopyranoside. GC conditions were the same for all three runs (see text).

The usefulness of permethylation, combined with GC–MS, for crude plant extracts was evaluated, using the pods of *Phaseolus coccineus*. The seeds of *P. coccineus* have been shown to contain GA_1, GA_5, GA_6, GA_8, GA_{17}, GA_{19} and GA_{20} [10], GA_8-*O*-2-*β*-D-glucopyranoside [11], and unidentified glucosides of GA_{17}, GA_{20}, and GA_{28} [1].

After extraction of the pod in the usual way, the acidic EtOAc and *n*-BuOH soluble fractions were permethylated and examined by GC–MS. For completeness, the fractions were also hydrolysed with a pectinase preparation and the Me/TMSi-derivatised hydrolysates were analysed by GC–MS. The GLC trace (Fig. 4A) for the permethylated BuOH-soluble fraction showed one major component (peak 4) the MS of which (Fig. 3B) was identical in all important respects with that of the reference spectrum of GA_8-*O*-2-*β*-D-glucopyranoside (Fig. 2). Also present in the permethylated BuOH-soluble fraction were perMe GA_8 (peak 1) and two perMe GA-glycosides (peaks 2 and 3) which, from the M^+ and $M^+ - 31$ perMe glucosyl ions could be the derivatives of GA_1 and GA_{34}. However, conclusive identification of these two GA-glycosides requires reference spectra.

Table 2. GAs and GA-glucosides identified in *P. coccineus* pods (51 days old) by GC–MS as perMe or Me/TMSi derivatives

Fraction	Compound
n-BuOH acids perMe	GA_8 perMe
	GA_8 glucosyl-ether perMe
	GA_{34} glucoside perMe*
	GA_1 glucoside perMe*
n-BuOH acids/enzyme hydrolysed Me/TMSi	GA_5 Me/TMSi
	GA_6 Me/TMSi
	GA_8 Me/TMSi
	GA_{17} Me/TMSi
	GA_{20} Me/TMSi
	GA_{34} Me/TMSi
EtOAc acids perMe	GA_1 perMe + 3-*epi*-GA_1 perMe
	GA_6 perMe
	GA_8 perMe
	GA_{34} perMe* + 3-*epi* GA_{34} perMe*
	Three perMe glycosides
EtOAc acids/enzyme hydrolysed Me/TMSi	GA_1 Me/TMSi
	GA_4 Me/TMSi
	GA_5 Me/TMSi
	GA_6 Me/TMSi
	GA_8 Me/TMSi
	GA_{20} Me/TMSi
	GA_{34} Me/TMSi
	GA_{38} Me/TMSi

*Tentative.

Gibberellin A_{34}, recently reported [15] in seeds of *P. coccineus*, was also tentatively identified as the perMe derivative in the EtOAc-soluble acids and conclusively identified as the Me/TMSi derivative in the enzyme hydrolysates of both the EtOAc and BuOH-soluble acids. Gibberellin A_{38}, also recently reported to be present in these seeds, was detected only as the Me/TMSi derivative in the hydrolysed EtOAc-soluble fraction and may therefore be present as a glycosyl ester. Gibberellin A_{19}, previously found in extracts of these seeds, was not detected.

EXPERIMENTAL

Permethylation procedure. The standard (1 mg) or plant extract (1 mg, dried over P_2O_5 for 12 hr), dissolved in the minimum vol. of MeOH, was treated with excess CH_2N_2 in Et_2O for 30 min. After removal of solvent in a stream of dry N_2, the residue, in $HCONMe_2$ (0.5 ml, freshly distilled) and MeI (0.5 ml, freshly distilled over P_2O_5 and stored over molecular sieve), was treated with NaH (5 mg, washed with petrol). The reaction vial was flushed with dry N_2 gas for 5 sec and the H_2 gas allowed to escape freely. When H_2 evolution ceased the vial was closed and shaken for 15 min at room temp. After 2 hr without shaking, MeOH (0.5 ml) was added and the solvents were evapd at 50° by a stream of N_2 gas. Addition of H_2O (1 ml) then extraction with EtOAc (3 × 1 ml) yielded the crude permethylated derivatives.

PLC of the permethylated GA_3 on Si gel with EtOAc–petrol (1:1) gave 3,13-dimethyl GA_3 Me ester (R_f 0.55), mp 151–3° (crystallized from EtOAc–petrol, 1:1); correct elemental composition and reported [12] MS and NMR.

Quantitative permethylation of the standards as determined by GC, before and after trimethylsilylation, was obtained down to 10 μg per sample.

GC conditions. A Varian 2700 was used, fitted with a glass column (1.5 m × 2 mm i.d.), packed with 2% OV-1 on Gas Chrom Q (100–120 mesh) and temp. programmed from 180° to 300° at 6 or 8°/min. F.I.D. detected 10 ng 3,13-dimethyl GA_3Me ester with a signal:noise ratio of 10:1 when run isothermally at 250° with an attenuation of 32×10^{-11} AFS.

GC–MS. The conditions used routinely in this work have already been described and all MS were obtained at 24 eV.

Extraction of pods of Phaseolus coccineus. Pods (293 g, from 51-day-old fruits, grown in standard greenhouse conditions) which had been stored at −50° for 7 months, were extracted at 4° with MeOH–H_2O (4:1, v/v, 3 × 500 ml). The extract was reduced to small vol. on a rotary evaporator below 40° and the aq. soln added to a Dowex 1 × 1 column (150 g, 30 × 1.5 cm), in the formate form. The column was eluted with de-ionized H_2O (150 ml) which removed pigments. The GAs were then eluted with EtOH–1 M HCO_2H (4:1, 500 ml) and the eluate was reduced to small vol. The aq. residue to which a few ml toluene was added was adjusted to pH 8.0 with satd aq. $NaHCO_3$ then extracted with EtOAc (5 × 10 ml); this EtOAc extract was discarded. The aq. layer was adjusted to pH 2.5–3.0 with 3 M HCl and extracted successively with EtOAc (3 × 100 ml) and *n*-BuOH (3 × 100 ml). These extracts were evapd to give 173 mg from the EtOAc and 323 mg from the *n*-BuOH.

Aliquots were permethylated as described or methylated (CH_2N_2) then treated with HMDS–TMCS–C_5H_5N (2:1:3). Enzymatic hydrolyses were performed as described by Gaskin and MacMillan [1].

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