Phytochemistry, 1980, Vol. 19, pp. 223–225. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0201-0223 $02.00/0

GC–MS IDENTIFICATION OF ABSCISIC ACID AND ABSCISIC ACID METABOLITES IN SEED OF *VIGNA UNGUICULATA*

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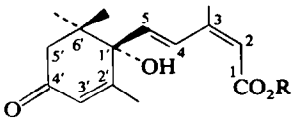
(*Revised received* 12 *April* 1979)

Key Word Index—*Vigna unguiculata*; Leguminosae; cowpea; GC–MS; abscisic acid; phaseic acid; dihydrophaseic acid; 6′-hydroxymethylabscisic acid.

Abstract—Abscisic acid, phaseic acid and 4′-dihydrophaseic acid were identified by GC–MS of derivatized (Me, MeTMSi) extracts from immature fruits of *Vigna unguiculata*. The fruits also contained some other ABA-related compounds, one of which might be *epi*-4′-dihydrophaseic acid while another was tentatively identified as 6′-hydroxymethylabscisic acid.

INTRODUCTION

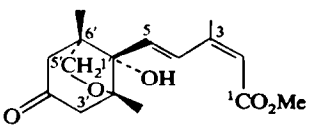
Vigna unguiculata (L. Walp), cowpea, exhibits an excessive abscission of immature fruits [1]. This limits the grain yield of this important food crop in tropical Africa. It was decided to investigate a possible hormonal basis for the abscission problem of cowpea. In this paper we report the presence of ABA (**1a**), an abscission accelerating substance [2], and some of its metabolites in immature fruits of cowpea. We report evidence for the natural occurrence as well as MS characteristics of 6-hydroxymethylabscisic acid (MeTMSi).



1a R = H
1b R = Me

RESULTS AND DISCUSSION

The acidic EtOAc fraction obtained from the fractionation of an aqueous MeOH extract from immature fruits of cowpea was purified by column chromatography. The column fractions were further purified with PVP before derivatization (Me, MeTMSi) and GC–MS. The presence of MeABA (**1b**) (fraction 5), methylphaseic acid (MePA) (**2**) (fractions 4 and 5) and



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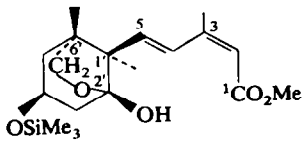
the methyl ester trimethylsilyl ether of 4′-dihydrophaseic acid (MeDPATMSi) (**3**) (fraction 4) was established by comparison of their MS with reference spectra. The R_t of the compounds were MeABA 7.1, MePA 8.0 min, and MeDPATMSi 9.2 min, respectively. A compound which might be the MeTMSi of *epi*-DPA was also detected in fraction 4. This compound had a slightly longer R_t (9.8 min) than MeDPATMSi but the MS (see Experimental) was very similar to the MS [3] of MeDPATMS.



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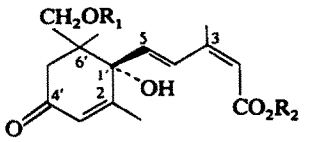
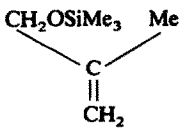
The essential features of the MS characteristics of a compound (detected in fractions 4 and 5, R_t 11.9 min), which was tentatively identified as the MeTMSi of 6′-hydroxymethylabscisic acid (**4b**) on the basis of MS fragmentation pattern are given in the Experimental. The MS was similar, in some features, to the MS of MeABA [4]. The presence of the TMSi ether group in **4b** compared with MeABA (**1b**) accounted for the observed M^+ of *m/e* 366 for **4b** as opposed to M^+ of *m/e* 278 (336–88) for MeABA. The loss of Me_3Si from the M^+ gave rise to the ion at *m/e* 294. The presence of the *m/e* 73 and 75 ions, associated with the TMSi group, indicated that the compound was silylated; the tertiary hydroxyl group of MeABA is not usually silylated [5] in crude extracts with the Sweeley [6] silylating reagent. The presence of ions at *m/e* 103 ($-CH_2OSiMe_3$) and $M^+ - 103$ indicated the presence of a methylenehydroxy group in the underivatized compound (**4a**). The presence of the



4a $R_1 = R_2 = H$

4b $R_1 = SiMe_3$, $R_2 = Me$

$M^+ - 31$ ion in the MS of the Me ester of **4a** supported this conclusion. The prominent *m/e* 125 ion in the MS of **4b** indicated the presence of the methyl-3-methylpentadienoate side-chain of MeABA which is associated with this fragment ion. Therefore one of the three methyl groups attached to the cyclohexenone ring of ABA had been hydroxylated. That this was the case was further supported as follows. A $M^+ - 56$ ion, characteristic [7] of the MS of MeABA, was assigned [7] to the loss of isobutylene from the M^+ of MeABA by a cleavage of the cyclohexenone ring. This ion was not present in the MS of compound **4b**. This ion was also absent in the reported [8] MS of the Me ester of an isolated conjugate of 6'-hydroxymethyl ABA. Instead, an $M^+ - 144$ ion, attributable to the loss of the silylated group (**5**) from the M^+ of compound **4b**, was present from which it is deduced that one of the *gem*-dimethyl groups attached to C-6' of the cyclohexenone ring of ABA had been hydroxylated. In the MS of MeABA, the base peak (*m/e* 190) was reported [7, 9] to be due to the loss of isobutylene and MeOH from the M^+. Similarly in the MS of this compound, the loss of the silylated group **5** and MeOH from the M^+ would give the observed base peak (*m/e* 190).



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Successive losses of CO from the base peak would give rise to the *m/e* 162 and *m/e* 134 ions. Loss of Me from the *m/e* 162 ion would give rise to the *m/e* 147 ion while cleavage of the side-chain would give rise to the *m/e* 125 ion. The fragmentation pattern of the MS of this compound thus agreed very well with the expected fragmentation pattern of the MeTMSi of 6'-hydroxymethylabscisic acid. Another possibility, the enol TMSi ether of MePA (M^+ *m/e* 366) shows a different MS fragmentation. The compound was therefore tentatively identified as the MeTMSi of 6'-hydroxymethylabscisic acid.

6'-Hydroxymethylabscisic acid (**4a**, 'Metabolite C') was first identified by Milborrow [10] as a metabolite of labelled ABA fed to tomato shoots. It was reported [11] to be unstable rearranging easily to phaseic acid and the MS could not be obtained. Subsequent attempts [11] to isolate 6'-hydroxymethylabscisic acid were unsuccessful. The isolation of a 'Metabolite C' conjugate has recently been reported [8].

EXPERIMENTAL

Plant material. The seeds of cowpea cv New Era were grown in the field at spacings 91 × 30 cm. Flowers were labelled on the day they opened in order to determine the age of each fruit. The fruits (6 days old) were frozen in liquid N_2 and then freeze-dried.

Extraction procedure and fractionation. The freeze-dried 6-day-old fruits (110 g dry wt) were ground in a Waring blender in 80% aq. MeOH (*ca* 15 × ml/g dry wt of fruit). The ground fruits were extracted 3 × (24 hr each time) with 80% aq. MeOH. The combined filtrates were concd to aq. phase *in vacuo*. The pH of the aq. phase was adjusted to 8.0–8.1 with 2 M aq. NaOH. The aq. phase was extracted with petrol (60–80°, 3 × 1/3 vol. of aq. phase), then EtOAc (3 × 1/3 vol. of aq. phase). The pH of the aq. phase was adjusted to 3.0 with 2 M HCl and the aq. phase extracted with EtOAc (4 × 1/3 vol, of aq. layer). The combined EtOAc layer was washed with H_2O and evapd *in vacuo* giving the acidic EtOAc fraction as a gum (700 mg).

Purification of extract. The acidic EtOAc fraction was put on a column of activated charcoal and celite (1:2). The column was eluted with a gradient of increasing Me_2CO concn in H_2O. Twenty 150 ml fractions were collected. An aliquot of each column fraction was dissolved in phosphate buffer (0.2M, pH 8.0) using *ca* 0.5 ml buffer per extract from 1 g dry wt of fruits. Prewashed PVP (50–100 mg per ml of buffer) was added and each mixture was shaken for *ca* 30 min. The PVP was filtered off, fresh PVP was added to each filtrate and the shaking and filtration procedures repeated twice. The combined phosphate buffer filtrates (for each fraction) were washed with petrol (3 × 1/3 vol. of buffer). The pH of the buffer was adjusted to 3.0 with 2 M HCl and the buffer was extracted with EtOAc (4 × 1/3 vol of buffer). The EtOAc was washed with H_2O and evapd *in vacuo* to give the purified extract for each column fraction.

Derivatization and GC–MS. Aliquots of extracts from column fractions were methylated with CH_2N_2. The dried methylated extracts were dissolved in dry Py and silylating reagent (Py–$(Me_3Si)_2$ NH–Me_3SiCl, 2:2:1) was added. Aliquots were examined by GC–MS, using a Pye 104 GLC coupled to an A.E.I. MS 30 dual beam mass spectrometer through a silicone-membrane helium separator. A silanized glass column (171 × 0.2 cm) packed with 2% SE 33 coated on Gaschrom Q (80–100) mesh was used. Column temp. was programmed from 180° to 280° at 3°/min with He flow rate of 25 ml/min. MS were obtained at 24 eV with a source temp. of 210° and a separator temp. of 190°. The MS were processed on-line by a DEC Linc-8 computer.

MS of presumed epi-Me-*DPA-TMSi*, *m/e* (rel. int.): 368(M^+; 1), 350($M^+ - 18$; 3), 335(6), 318(3), 278(6), 246(11), 245(9), 220(16), 199(15), 189(16), 188(27), 177(17), 163(31), 161(25), 159(75), 154(33), 153(36), 146(18), 135(21), 125(51), 122(47), 121(45), 117(39), 109(33), 95(21), 75(53), 73(88), 69(25), 43(100).

MS of presumed Me-6'-*hydroxymethylABA-TMSi*, *m/e* (rel. int.): 366(M^+; <1), 348($M^+ - 18$; 1), 335($M^+ - 31$; 5), 334($M^+ - 32$; 6) 319(6), 318(9), 294($M^+ - 72$; 8), 263($M^+ - 103$; 5), 246(8), 245(13), 231(15), 227(15), 222($M^+ - 144$, 11), 221(26), 217(14), 216(11), 203(10), 193(11), 191(19), 190(100), 189(19), 187(13), 177(17), 162(18), 161(34), 145(14), 143(14), 135(14), 134(14), 131(23), 125(44), 115(10), 112(13), 105(11), 103(21), 75(42), 73(> 100), 69(12).

Acknowledgements—We thank Prof. Jake MacMillan for his interest and suggestions, the I.U.C. for a nine-month sponsorship (to A.A.A.) and the Rockefeller Foundation for a research grant (to D.E.E.)

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