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DETERMINATION OF ABSCISIC ACID IN *EUCALYPTUS HAEMASTOMA* LEAVES USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY AND DEUTERATED INTERNAL STANDARDS

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Key Word Index—*Eucalyptus haemastoma*; Myrtaceae; GC/MS; selected ion monitoring; HPLC; abscisic acid; [3-*Me*-$^2H_3$]abscisic acid; *trans*-abscisic acid.

Abstract—Abscisic acid and 2-*trans*-abscisic acid each with three deuterium atoms in the C-3 methyl group, have been synthesized chemically and used as internal standards in selected ion monitoring experiments to establish the endogeneous concentrations of these compounds and their conjugates in turgid and wilted *Eucalyptus haemastoma* leaves. The analytical procedure used GC/CIMS(methane) to detect the methyl esters of abscisic acid, 2-*trans*-abscisic acid and their deuterated internal standards. A three-fold increase in the concentration of abscisic acid occurred on wilting and the amounts of 2-*trans*-abscisic acid and conjugates of both compounds were determined.

INTRODUCTION

Selected ion monitoring (SIM) in GC/MS is a specific and sensitive technique for measuring low concentrations of natural products when an isotopically labelled internal standard is available. Because the internal standard is added at the beginning of the isolation procedure losses due to lengthy purification steps do not affect the accuracy of the method. This is particularly true for plants such as eucalypts that are rich in terpenoids and phenolics. Thus Summons *et al.* [1] were able to determine concentrations of cytokinins in radish seeds by using deuterated cytokinins as carriers and internal standards. To determine the endogeneous concentrations of abscisic acid (ABA, **1a**) using SIM, we therefore required a chemical synthesis of deuterated ABA in which the isotopic label would be stable to the procedures used for the isolation of endogenous ABA. Although [$^2H_6$] ABA is readily synthesized by exchange [2], its use is restricted since extractions must be carried out below pH 8 [3] and complete retention of the label during storage is in doubt. Consequently, we adopted a procedure based on Walton *et al.* [4] which introduced three deuterium atoms into the C-3 methyl group of ABA (**1b**) and this compound was shown to be stable to saponification conditions using alcoholic KOH.

Other methods are available for measuring endogenous concentrations of ABA but they are less sensitive or less precise than SIM. Thus, in the racemic dilution method [5] great care must be taken to ensure the purity of the ABA isolated for ORD measurements and at least 1 μg of (+)-ABA is required. Similarly, the procedure involving the dilution of radioactive ABA [6] becomes increasingly imprecise as a quantitative method as the amount of labelled ABA added exceeds the amount of endogenous material because the ratio between the two is obtained after subtraction of one measurement from the total amount present. This source of error becomes increasingly important when the amount of endogeneous ABA is less than 1 μg. Electron capture detection in GLC is without doubt the most sensitive applicable method, but its precision is limited since internal standards are not available.† However, as little as 100 pg can be precisely measured by SIM.

It has been inferred that in some species of eucalypts ABA is not the major growth inhibitor [7], although its presence has been demonstrated (Loveys, B. R., unpublished). We felt, therefore, that it was important to establish the concentrations, and the effect of wilting on these concentrations, of ABA in eucalypts. Also, we took the opportunity of determining the concentrations of the 2-*trans* isomer (*t*-ABA, **2a**) and the conjugates of *t*-ABA and ABA in both turgid and wilted leaves.

RESULTS AND DISCUSSION

The deuterated ABA (**1b**) and *t*-ABA (**2b**) synthesized from deuterated hydroxy keto-α-ionone (**3a**) by a Wittig reaction [8] had deuterium contents of

†The recent availability of high sp. act. [G-^{3}H]ABA (Radiochemical Centre, Amersham, U.K.) removes one criticism of the method that relies on labelled internal standards. However, the EC part of the method depends on the GLC peaks being composed entirely of methyl ABA.

73% and 68% [$^2H_3$] respectively and were virtualty free of [$^2H_0$] content.

The relative intensity of the parent ion, and high mass fragment ions, is low in the EIMS of methyl abscisate. The base peak at *m/z* 190 has been used for the detection of ABA [9]. As this fragment incorporates the C-3 methyl group [10], it could be used for SIM using EIMS. However, CIMS (methane) yields more intense high mass ions for methyl abscisate and consequently it is the method of choice for a sensitive and specific assay for endogenous ABA. The MH^+ ion for methyl ABA is a relatively minor peak (10% relative abundance, Fig. 1), while the base peak occurs at *m/z* 261 $[MH - H_2O]^+$, probably from loss of the *t*-OH group at C-1'. Using this ion of mass 261 in conjunction with [3-*Me*-$^2H_3$]ABA (**1b**) as an internal standard (*m/z* 264), detection limits with a signal-to-noise ratio of >10:1, of 100 pg injected onto the GC column, can be obtained. The CIMS (methane) of methyl *t*-ABA contains MH^+ as the base peak (Fig.

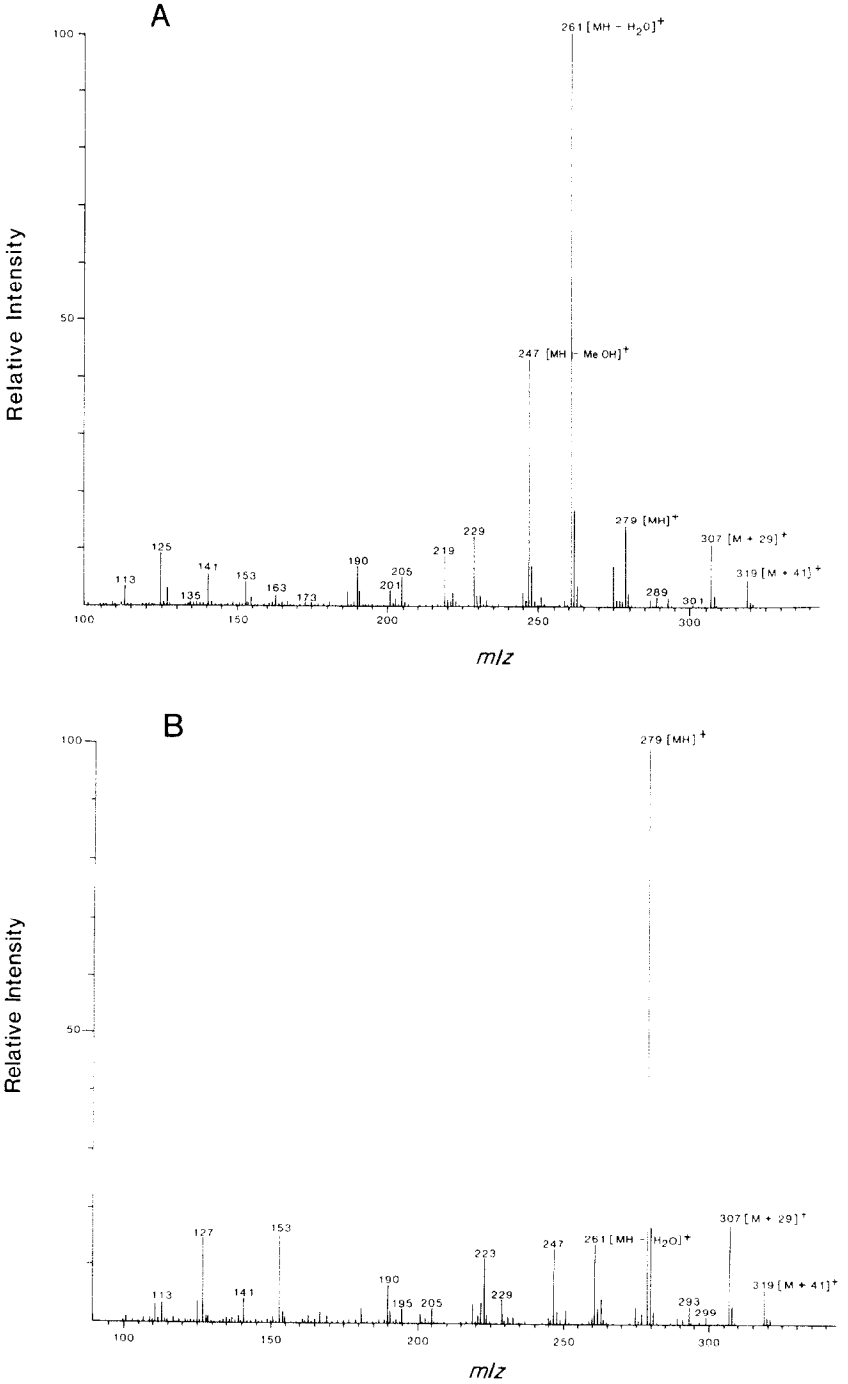


Fig. 1. CIMS (CH_4) of A: ABA (**1a**) and B: *t*-ABA (**2a**).

1). Consequently, this ion (*m/z* 279) and that at *m/z* 282 in the internal standard, were monitored for quantitative purposes.

The only previous attempt to use deuterated ABA for SIM analysis of endogenous ABA was carried out by Rivier *et al.* [11]. These authors used [$^2H_6$]ABA which had been prepared by base-catalysed exchange. Besides the disadvantage that this material cannot be used to estimate conjugates released by saponification, considerable effort must be expended to process the standards by the same procedure as the samples to ensure that there is no differential loss of exchangeable deuterium [12]. Treatment of a standard deuterated mixture with strong alkali for 2.25 hr or for 3 days failed to release a significant proportion of deuterium, thereby showing that **1b** and **2b** were suitable internal standards for the measurement of ABA and *t*-ABA released from conjugates by alkaline hydrolysis. However, storage of such a standard mixture for 6 months in MeOH at −2° led to some chemical degradation and some randomization of the label.

Any error in estimating the amount of ABA or *t*-ABA released from conjugates would be introduced by losses of the conjugates during extraction [13, 14] rather than by loss of deuterium from [3-*Me*-$^2H_3$]ABA. The amounts of the conjugates appear to be high but the recent discovery of ABA-glucoside [15], and the inappropriateness of many extraction procedures, indicate that earlier values will have to be reassessed.

The proposed structure (**3b**) for the compound formed during the treatment of **3a** with base is derived from the following evidence. The CIMS (methane) showed a protonated molecular ion of mass 239, 16 mu greater than **3a**. The loss of 86 mu was also observed in the spectrum of **3b** and this can be accommodated by the presence of an epoxide in the side-chain of **3b**. This was confirmed by ^{1}H NMR; the protons at C-3 and C-4 in **3b** are at δ 3.63 and 3.31, while the equivalent protons in **3a** are at δ 6.86 and 6.46. The IR spectrum of **3b** lacks one of the two conjugated carbonyl groups present in **3a** and absorption bands between 800 and 1310 cm^{-1} are consistent with the presence of an epoxide function in **3b**. The IR spectrum of **3b**, unlike **3a**, shows intramolecular hydrogen bonding.

The concentrations of ABA, *t*-ABA and their conjugates in *E. haemastoma* leaves are given in Table 1. The concentration of ABA is higher in wilted leaves than in turgid leaves; consequently, the concentration of ABA in *E. haemastoma* is affected by water stress. However, the increase on wilting is only three- to four-fold while glasshouse-grown plants such as tomatoes and wheat may show an increase by as much as 40-fold [16]. The unresponsiveness of *E. haemastoma* may reflect the rigours of the environment rather than any fundamental difference in ABA metabolism. Kriedemann and Loveys [17] found increases of similar degree in several Australian arid zone plants. They found concentrations about one-fiftieth of those reported here. The difference may be attributed, at least in part, to losses during isolation. These true endogenous concentrations of ABA in a plant growing under natural conditions are difficult to assess because of the absence of comparable data.

Table 1. Endogenous concentration (μg/g fr. wt) of ABA, *t*-ABA and these compounds as conjugates in turgid and wilted *E. haemastoma* leaves

	Wilted		Turgid	
	Non-conjugated	Conjugated	Non-conjugated	Conjugated
ABA	4.83*	5.1*†	1.30*	4.42
t-ABA	0.22	0.48*	0.25	0.87

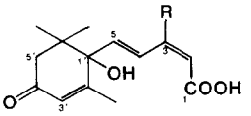
*Mean of two determinations on the GC/MS.

†It appears that most of the ABA was lost during extraction and thus this value is based on small peak heights.

E. haemastoma leaves contain conjugates of ABA at high concentrations and also *t*-ABA, but the concentration of neither of these fractions is increased on wilting. The effects reported for the 'G' growth regulators of *E. grandis* [7] may be non-specific, inhibitory effects.

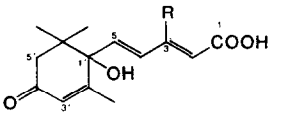
EXPERIMENTAL

Exchange of 1 - [1' - *hydroxy* - 4' - *oxo* - 2',6',6' - *trimethylcyclohex* - 2' - *enyl*] - 3 - *oxobut* - 1 - *ene* (**3a**) *and isolation of the deuterated products.* The **3a** used was a gift from Hoffman–La Roche. Its synthesis has been documented [8]. The vol. of 0.1 M NaOD required to exchange fully the hydrogens of the side-chain methyl of **3a** was determined by ^{1}H NMR titration in 0.4 ml *p*-[$^2H_8$]-dioxane plus 0.1 ml D_2O. When the side-chain Me was completely exchanged the ring methylene was about half exchanged and the solution became pink. After dissolving **3a** (100 mg) in dry dioxane



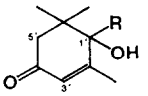
1a R = $-CH_3$

1b R = $-CD_3$

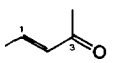


2a R = $-CH_3$

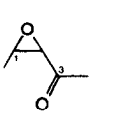
2b R = $-CD_3$



3a R =



3b R =



(40 ml) and D_2O (10 ml), Na (43 mg), dissolved in 10.0 ml D_2O, was added and the reaction mixture stirred (20°, 2 hr). The soln was acidified with dry $(COOH)_2$ to pH 4. H_2O was added and the mixture extracted (×3) with Et_2O and the organic phase evaporated. The residue was dried by passage through a small column containing dry $CaSO_4$, using dry Et_2O as eluent which was then evaporated. The sample was dissolved in C_6H_6 and applied to a Si column (Merck Lobar A, 240 mm × 10 mm) and eluted with 5% *iso*-PrOH in hexane at 38 ml/hr. The two major peaks monitored at 264 nm, which were not well resolved, were collected in one fraction. These two peaks were resolved by HPLC on a 7.8 mm × 300 mm μ-Porasil column using 5% *iso*-PrOH in hexane at 4 ml/min (*ca* 50 kg/cm^2) and monitoring at 254 nm. All HPLC equipment was from Waters Associates. Deuterated **3b**, RR_t 0.80, yield *ca* 28 mg. Deuterated **3a**, RR_t 1.00, yield 18.87 mg.

Identification of compound **3b**. 8.4 mg **3a** were exchanged in dioxane/1 M NaOH as described for the preparation of deuterated **3a** using 1/10th quantities. Non-deuterated **3b** was isolated using the μ-Porasil column as described above. Deuterated **3b** (8.3 mg) obtained in the preparation of deuterated **3a**, was treated as described for the 8.4 mg of **3a** above. **3b** was the only significant product isolated on the μ-Porasil column. ^{1}H NMR and GCMS showed that the deuterium incorporated into **3b** (*ca* $^2H_5$) by exchange, remained exchangeable. UV $\lambda_{max}^{iso\text{-PrOH}}$ nm (log ϵ): **3b**: 232 (3.97); **3a**: 236 (4.12); ^{1}H NMR (A Jeol FX-100 operating at 2.35 T in the Fourier Transform mode was used with TMS as the int. standard. The peaks from **3a** were identified using a non-gated double irradiation technique). **3b** ($CDCl_3$): δ 5.98 (1H, *s*, 3′-H), 3.63 (1H, *d*, $J_{3,4}$ = 2 Hz, 3-H), 3.31 (1H, *d*, 4-H), 2.64 (1H, *d*, $J_{5',5'}$ = 18 Hz, 5′-H), 2.37 (1H, *d*, 5′-H), 2.16 (3H, *s*, 1-Me), 2.01 (3H, *s*, 2′-Me), 1.17 (3H, *s*, 6′-Me), 1.10 (3H, *s*, 6′-Me); **3a** ($CDCl_3$): δ 6.86 (1H, *d*, $J_{3,4}$ = 16 Hz, 3-H), 6.46 (1H, *d*, 4-H), 5.98 (1H, *s*, 3′-H), 2.54 (1H, *d*, $J_{5',5'}$ = 16 Hz, 5′-H), 2.32 (1H, *d*, 5′-H), 2.32 (3H, *s*, 1-Me), 1.90 (3H, *s*, 2′-Me), 1.12 (3H, s, 6′-Me), 1.04 (3H, *s*, 6′-Me); GC/CIMS(CH_4) *m/z*: **3b**: 239 $[MH]^+$, 221 $[MH-H_2O]^+$, 165 $[MH-74]^+$ (base peak), 153 $[MH-86]^+$, **3a**: 223 $[MH]^+$, (base peak), 205 $[MH-H_2O]^+$; IR ν_{max}^{KBr} cm^{-1}: **3b**: 3460 (*br*, H bonded-OH), 1740 (> C=O), 1660 (> C=C-C=O), 1310, 1140, 900, 800 (unidentified, differ significantly from **3a**); **3a**: 3490 (sharp, -OH), 1670 (> C=C-C=O), 1630 (> C=C <).

Synthesis and isolation of [3-Me-$^2H_3$]*ABA* (**1b**) *and* t-[3-Me-$^2H_3$]*ABA* (**2b**). Carboxyethyltriphenylphosphonium bromide (mp 159°, lit. 158°) was synthesized in quantitative yield from triphenylphosphine and ethylbromoacetate [18] using 1/10th quantities. Carboethoxymethylenetriphenylphosphorane was then prepared [18] [mp (recryst) 124°, lit. (not recryst) 116–117°]. Phosphorane (23 mg) was added to deuterated **3a** (18.87 mg) in 1.59 ml toluene and the mixture refluxed for 4 hr [8, method A]. The products of the Wittig reaction were separated by HPLC using the μ-Porasil column with the conditions given above. **3a**: RR_t 1.00, recovered 9.64 mg (51%). Triphenylphosphine oxide, RR_t 0.91 (tailing). UV, $\lambda_{max}^{iso\text{-PrOH}}$ nm: 257, 263, 269; GC/CIMS (CH_4) *m/z*: 279 $[MH]^+$, 201 $[MH-C_6H_6]^+$. Et-*t*-ABA, RR_t 0.41, yield 7.15 mg (29%). UV $\lambda_{max}^{5\%\ iso\text{-PrOH/hexane}}$ nm (log ϵ): 259 (4.328). Et-ABA, RR_t 0.31, yield 4.71 mg (19%). UV $\lambda_{max}^{5\%\ iso\text{-PrOH/hexane}}$ nm (log ϵ): 267 (4.326).

Et-ABA and Et-*t*-ABA were saponified [95% EtOH–60% KOH (2:1), 2 hr, 40°] separately, acidified, extracted (×3) into Et_2O, dried and evaporated.

1b; ^{1}H NMR ($CDCl_3$): δ 7.83 (1H, *d*, $J_{5,4}$ = 16 Hz, 4-H), 6.18 (1H, *d*, 5-H), 5.98 (1H, *s*, 3′-H), 5.78 (1H, *s*, 2-H), 2.54 (1H, *d*, $J_{5',5'}$ = 18 Hz, 5′-H), 2.28 (1H, *d*, 5′-H), 2.03 (trace, *s*, 3-Me), 1.94 (3H, *s*, 2′-Me), 1.13 (3H, *s*, 6′-Me), 1.04 (3H, *s*, 6′-Me).

2b: ^{1}H NMR ($CDCl_3$): δ 6.50 (1H, *d*, $J_{5,4}$ = 16 Hz, 4-H), 6.17 (1H, *d*, 5-H), 5.96 (1H, *s*, 3′-H), 5.88 (1H, *s*, 2-H), 2.52 (1H, *d*, $J_{5',5'}$ = 17 Hz, 5′-H), 2.31 (trace, *s*, 3-Me), 2.28 (1H, *d*, 5′-H), 1.91 (3H, *s*, 2′-Me), 1.12 (3H, *s*, 6′-Me), 1.03 (3H, *s*, 6′-Me). The assignments for **2b** were confirmed using homonuclear decoupling.

Preparation and quantitation of the internal standard for SIM. The **2b** obtained by saponification was isomerized in Me_2CO in sunlight for 5 days and **2b** was separated from **1b** by HPLC on a C_{18} reversed phase column (7.8 mm × 300 mm), 95% EtOH–0.2% HOAc (1:3), 4.0 ml/min, 254 nm (*ca* 130 kg/cm^2), RR_t: **1b** 1.00, **2b** 0.81. This separation afforded **2b** which was isomerized in [$^2H_6$] Me_2CO in sunlight for 2 weeks. The resulting mixture of **1b** and **2b** was used as the int. standard in the experiments reported here. An aliquot was methylated (CH_2N_2) and the deuterium content in the two peaks determined by GCMS (see below). Aliquots were also treated with saponification mixture (95% EtOH–60% KOH, 2:1) for 2.25 hr and 3 days at room temperature, acidified, extracted (×3) into Et_2O, dried and methylated and the resulting mixtures of Me esters were examined by GC/MS.

A methanolic soln of the mixture of **1b** and **2b** was prepared (300 μg/ml). An aliquot of the mixture was methylated (CH_2N_2) and analysed by GLC (Pye Unicam, XE-60 1.5 m × 4 mm column, N_2 carrier gas split in the detector oven; 1 part to EC and 24 parts to FID): 12.30 μg **1b**, 13.30 μg **2b**, 4.41 μg minor components. The EC gave the same proportions of **1b** to **2b** as the FID.

Plant material. Small leafy branches of *E. haemastoma* were collected from a tree in bushland at Gordon, N.S.W., Australia. They were brought to the laboratory in clear polythene bags and supported in conical flasks, with or without H_2O, in moving air for 6.5 hr. 'Turgid branches'; initial wt 138.2 g, final wt 146.4 g, % change +6%, wt of leaves 107.1 g; 'wilted branches': initial wt 96.8 g, final wt 89.7 g, % change −7%, wt of leaves 67.2 g.

Extraction. The leaves were detached from the branches and weighed and the int. standard was then added: 'turgid leaves', 0.246 μg [3-*Me*-$^2H_3$]ABA; 0.266 μg *t*-[3-*Me*-$^2H_3$]ABA/g leaves. 'Wilted leaves', 1.230 μg [3-*Me*-$^2H_3$]ABA; 1.330 μg *t*-[3-*Me*-$^2H_3$]ABA/g leaves. The leaves were then homogenized in 200 ml 1% HOAc in Me_2CO and 2,6-di-*t*-butyl-4-methylphenol (10 mg/l) added prior to storage overnight. The fibrous material was filtered off and rehomogenized and the combined extracts evaporated to the aq. phase. This was extracted (×5) with Et_2O and the aq. residue was retained for subsequent saponification. The combined Et_2O extracts were evaporated and extracted with satd $NaHCO_3$. After acidification (pH 3.0) the combined extracts were extracted (×3) with Et_2O and evaporated.

A second int. standard (0.369 μg [3-*Me*-$^2H_3$]ABA; 0.399 μg *t*-[3-*Me*-$^2H_3$]ABA/g leaves) was added to each of the aq. residues obtained from the 'turgid' and 'wilted' leaves so that the concentrations of ABA and *t*-ABA conjugates could be estimated. The residues were then saponified (95% EtOH–60% KOH (2:1) 40°, 2.25 hr), and acidified with H_2SO_4. The solid material was filtered off and the filtrate extracted (×3) with Et_2O and the combined extracts evaporated.

Chromatography. All four samples (free ABA plus *t*-ABA from turgid and wilted leaves and from the corresponding saponificates) were firstly applied to small columns made by joining 4 'SepPaks' (10-mm dia. 20-mm plastic columns

containing C_{18} bonded Si–Waters Associates) end to end with capillary glass tubing. The column was eluted with 100 ml of each of: 0.2% HOAc then 95% EtOH–0.2% HOAc (7:13) and finally 95% EtOH. The second fraction was evaporated and the others discarded. The second fractions of the hydrolysed conjugates from turgid and wilted leaves were chromatographed on a Merck Lobar RP-8 column (240 mm × 10 mm) with 95% EtOH–0.2% HOAc (2:3). The fraction containing ABA and *t*-ABA was collected by reference to standards because the extracts contained large amounts of brown material.

All four samples were then separated into fractions containing ABA or *t*-ABA by HPLC on a C_{18} reversed phase column under the conditions given above. Finally, the fractions containing ABA or *t*-ABA from the reversed phase column were suspended in MeOH, and methylated with CH_2N_2. The solns were evaporated to dryness, taken up in CH_2Cl_2, filtered (0.5 μm Sweeney adaptor–Millipore) and evapd to dryness. These fractions were then chromatographed (HPLC) on the μ-Porasil column, as above, to afford samples for GC/MS that contained either Me-ABA or Me-*t*-ABA as the major component.

GC/CIMS (CH_4). Analyses were carried out with a Finnigan Model 3200 Chemical Ionization GC/MS system interfaced to the same manufacturer's Incos 2300 Data system. CH_4 (20 ml/min) was used as the GC carrier gas and CI reagent gas (source pressure 0.8 torr). The ion source temperature was maintained at 150° and the interface oven and GC injector at 260°. The column was 3% OV-101 on Gas Chrom Q, 100–120 mesh (1.8 m × 2 mm, glass) and was temp. programmed from 170° at 10°/min. The following deuterium contents were found for the internal standard: Me-ABA; $^2H_1$, 1.6%; $^2H_2$, 22.5%; $^2H_3$, 73.4%; $^2H_4$, 2.4%. Me-*t*-ABA; $^2H_1$, 5.3%; $^2H_2$, 23.1%; $^2H_3$, 68.2%; $^2H_4$, 3.5%. Deuterium contents were not significantly altered by treating the int. standard with saponification mixture at room temp. for 2.25 hr or for 3 days. For selected ion monitoring the following base peaks were used: Me-ABA, $m/z = 261$; Me-[3-*Me*-$^2H_3$]ABA, $m/z = 264$; Me-*t*-ABA, $m/z = 279$ and Me-*t*-[3-*Me*-$^2H_3$]ABA, $m/z = 282$.

Aliquots of the Me-ABA and the Me-*t*-ABA samples collected from the μ-Porasil column were injected into the GC/MS and the ratios of the peak heights of the appropriate ions recorded. Standards were prepared by mixing unlabelled ABA plus *t*-ABA with constant amounts of the int. standard and then methylating the mixture with CH_2N_2. The standard curves so prepared were linear over the ranges used [Me-ABA: peak height = 1.307 $^2H_0/^2H_3 + 0.529$ (correlation coeff. = 0.9986); Me-*t*-ABA: peak height = 1.913 $^2H_0/^2H_3 + 0.259$ (correlation coeff. = 0.9990)]. The concentrations and total amounts of ABA and *t*-ABA in *E. haemastoma* leaves were then calculated.

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