

THE OCCURRENCE OF RAPHANATIN AS AN ENDOGENOUS CYTOKININ IN RADISH SEED

Identification and quantitation by gas chromatographic–mass spectrometric analysis using deuterium-labelled standards

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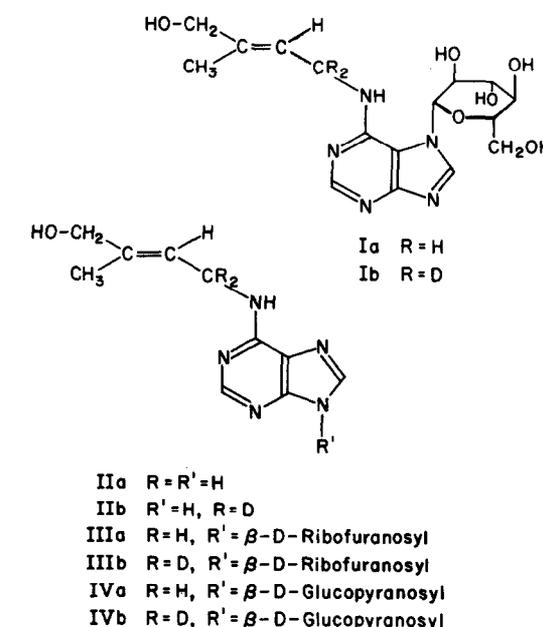
1. Introduction

When the phytohormone zeatin IIa [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine] is supplied exogenously to plant tissues, it is converted to a diversity of metabolites including a number of glucosides, namely, 7- β -D-glucopyranosylzeatin (Ia, termed raphanatin), 9- β -D-glucopyranosylzeatin IVa, O- β -D-glucopyranosylzeatin and its riboside, and O- β -D-glucopyranosyldihydrozeatin and its riboside [1–3]. The 7-glucoside Ia is of particular interest because of its very unusual structure. The only known endogenous purines in which a sugar (ribose) is linked to the 7 position are analogs of vitamin B₁₂ [4]. In this communication the occurrence of raphanatin as an endogenous cytokinin is established and quantitated using [²H₂]raphanatin Ib as an internal standard.

2. Materials and methods

2.1. Preparation of labelled compounds

The ²H₂-labelled zeatin IIb, zeatin-9- β -ribofuranoside IIIb, zeatin-9- β -glucopyranoside IVb and zeatin-7- β -glucopyranoside Ib used as internal standards/carriers were prepared by condensation of 6-chloropurine, or the appropriate 6-chloropurine glycoside, with *trans*-4-[4-²H₂]amino-2-methyl-but-2-enol. Detailed synthetic procedures will be published elsewhere.



2.2. Preparation and fractionation of radish seed extract

Commercially available dry radish seed (cv. long Scarlet, 25 g) was allowed to imbibe in stirred sterile water (70 ml) for 3 h. The drained seed was then extracted with 80% methanol, to which the deuterated compounds Ib \rightarrow IVb (100 μ g of each) had been added. The resulting extract was concentrated (< 40°C) to a few ml, mixed with the remainder of the water

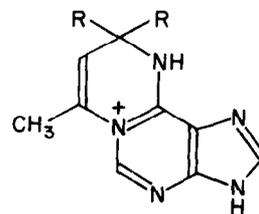
in which the seeds had imbibed, diluted to 100 ml with water, adjusted to pH 3.1 and finally passed through a cellulose phosphate column (Whatman P1, 140 ml, equilibrated to pH 3.1 in the NH_4^+ form). The column was washed with water (pH to 3.1 with acetic acid) and then eluted with 700 ml ammonium acetate buffer (0.3 M, pH 5.05). The evaporated eluate was dissolved in water (60 ml), adjusted to pH 6.5, percolated through a DEAE-cellulose column (Whatman DH1, 80 ml, HCO_3^- form) and washed from the column with water (700 ml). The column effluent was concentrated to 100 ml, adjusted to pH 3.0 and extracted with three 100 ml volumes of ethyl acetate, the extracts being discarded. The aqueous phase (pH adjusted to 7.0) was then extracted with four 100 ml volumes of water-saturated *n*-butanol. The residue obtained by evaporation of the combined extracts was subjected to preparative thin-layer chromatography (TLC) on silica gel (solvent: *n*-butanol/14 N $\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 6:1:2, v/v/v, upper phase). Three zones with R_F values of 0.55, 0.33 and 0.17, which contained the [$^2\text{H}_2$]zeatin (fraction 1), [$^2\text{H}_2$]zeatin riboside (fraction 2) and the two [$^2\text{H}_2$]zeatin glucosides (fraction 3; not separated) respectively, were eluted for gas chromatographic-mass spectrographic (GC-MS) analysis.

A portion of fraction 3 was subjected to two additional purification steps. Firstly, chromatography on a polyamide column (5 ml) using ethanol/water (1:4, v/v) and secondly, TLC of the resulting fraction containing the 7- and 9-glucosides on DEAE-cellulose (solvent: methanol/water, 1:1, v/v). The TLC fraction in which both deuterium-labelled glucosides occurred was designated fraction 4.

2.3. Combined gas chromatographic-mass spectro-metric analysis of seed extracts

In a recent publication [5], we reported the GC-MS characteristics of the pertrimethylsilyl (TMS) derivatives of cytokinin glucosides (including Ia and IVa) and on their potential utility in cytokinin analysis. The chemical derivatisation methods used in the present investigation were essentially as given there. The fractions 1-3 were derivatised and analysed separately using a Varian MAT 111 instrument. The GC-MS conditions employed were the same as those given previously [5].

Selected ion monitoring [6] for the purpose of



a R = H, m/e 188

b R = D, m/e 190

precise quantitation was carried out using a mixture of the derivatised fractions 1, 2 and the further purified glucoside fraction 4. Duplicate injections, interposed by an injection of a standard mixture of the corresponding TMS-deuterated compounds, were analysed with a Finnigan 3200 GC - MS-6110 Computer System using essentially the same conditions as those employed on the MAT 111. The ion at m/e 188 (a), which is common to and intense in the TMS derivatives of Ia to IVa, together with its $^2\text{H}_2$ counterpart at m/e 190 (b) were monitored continuously throughout the GC-MS runs of the combined TLC fractions 1, 2 and 4.

3. Results and discussion

The likely occurrence of raphanatin Ia in radish seed was indicated in initial experiments in which radish seed was extracted and worked up as in 2.2. without the addition of the deuterium-labelled analogs. Each stage was monitored by GC-MS of a silylated aliquot of the total extract. At the stage of preparative TLC on SiO_2 , the GC trace corresponding to fraction 3 was extremely complex. There was, however, a minute peak at the correct retention time for penta-TMS-raphanatin which showed a mass spectrum containing some of the diagnostic ions expected for this compound but from which an unequivocal assignment could not be made. Attempts to obtain a spectrum freeze from background contamination, either by further purification steps or by scaling up the experiment were unsuccessful.

Subsequently, the deuterated compounds Ib \rightarrow IVb were synthesised and the extraction procedure repeated in their presence. The use of stable isotope-labelled derivatives as carriers for the identification and quanti-

tation of small amounts of the corresponding unlabelled compound by mass spectrometry and GC-MS is a well established technique [7], particularly in metabolism studies. Nevertheless, we believe this to be the first reported example of the application of this technique to the identification and quantitation of endogenous phytohormones.

Preparative TLC fractions 1-3, when trimethylsilylated and analysed by GC-MS all showed total ion current (TIC) monitor peaks with retention times identical to the respective deuterated carriers. The TIC chromatogram of fraction 1 was quite complex with several components eluting close to the carrier compound and contributing to the background contamination which showed up at the lower mass end of the mass spectra scanned over the TMS-Iib peak. The molecular ion (m/e 365) and $[M-CH_3]^+$ (m/e 350) regions were however free from interference and showed no contributions at m/e 363 and 348 due to unlabelled TMS-zeatin. From this it could be concluded that, within detection limits, zeatin was not present in the seed.

The GC-MS of fraction 2 showed no other compounds eluting in the region of the TMS- $[^2H_2]$ zeatin riboside peak and the mass spectral peak groups for its molecular ion (m/e 641), $[M-CH_3]^+$ (m/e 626), $[M-CH_2OTMS]^+$ and the low mass ions at m/e 190 and m/e 158 showed no significant enhancement of the ions at m/e 639, 624, 536, 188 or 156 which would have resulted if unlabelled zeatin-9- β -riboside had been present in the seed.

Fraction 3 showed two gas chromatograph peaks with retention times identical to the per-TMS derivatives of the respective $[^2H_2]$ zeatin glucosides Ib and IVb. The mass spectral scans taken during elution of the TMS-IVb peak again showed no measurable presence of unlabelled TMS-zeatin-9-glucoside. Mass spectra recorded during elution of the TMS- $[^2H_2]$ raphanatin peak, however, showed a significant contribution (approx. 20%) from unlabelled TMS-raphanatin to all the diagnostic ions. The molecular ion and $[M-CH_2OTMS]^+$ peak groups in the extract are compared with those of the carrier Ib alone in fig.1. This result confirmed the earlier indication that raphanatin was present in the radish seed.

Preliminary studies with mixtures of synthetic TMS-Ia and Ib had shown that there was a slight gas-

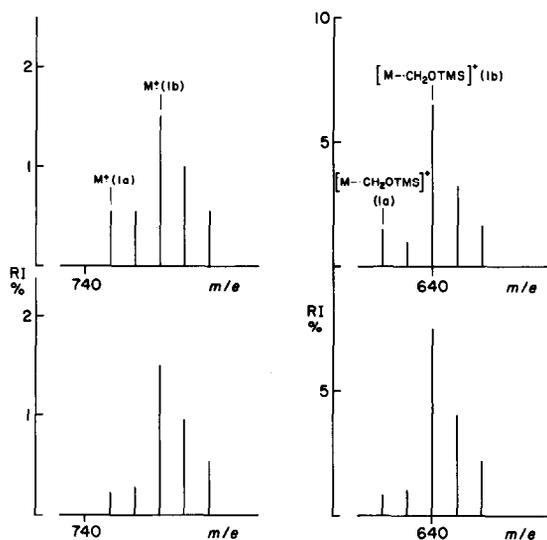


Fig.1. Molecular ion and $[M-CH_2OTMS]^+$ fragment ion peak groups for TMS-raphanatin from the radish seed extract (above) and the carrier alone (below).

chromatographic separation of deuterated from undeuterated molecules. Therefore, accurate quantitative results for the seed extract could not be obtained from the above relative intensity measurements alone and it was necessary to utilise the technique of selected ion monitoring to quantify the level of endogenous raphanatin. Figure 2 shows the ion current traces for m/e 188 and 190 monitored continuously during elution of the TMS-zeatin glucosides present in the extract (fraction 4) and in a 1:1 standard mixture of Ib and IVb. Although there is obviously a low recovery of $[^2H_2]$ raphanatin in the final purification steps (as evidenced by comparing the relative ion currents for the m/e 190 ion from IVb and Ib in the 1:1 mixture and the seed extract) this does not affect the calculations of the amount of unlabelled species originally present in the extract since the deuterated carrier acts as an internal calibrant. Averaged area measurements of the m/e 188 and 190 peaks showed that, after correction for the m/e 188 present in TMS-Ib, 17% of the raphanatin peak carried no deuterium label. This represents a concentration of endogenous raphanatin of 6.8×10^{-7} g/g seed. Similar area measurements calculated from the m/e 188 and 190 ions monitored during elution

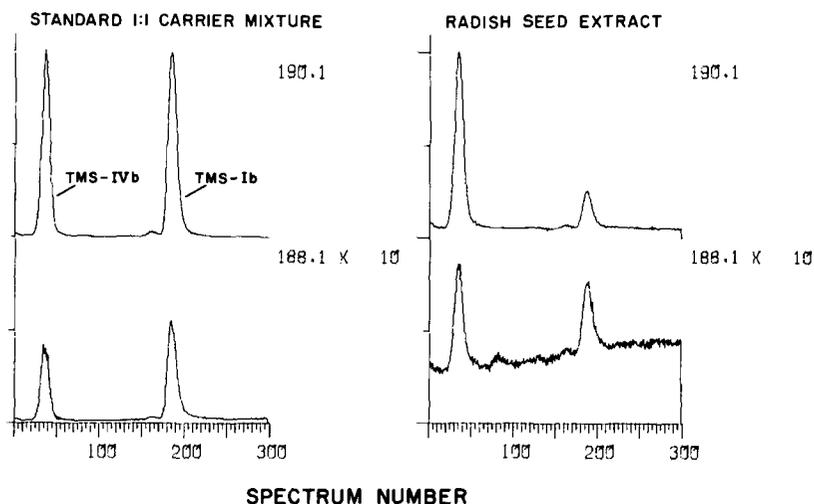


Fig.2. Ion current traces for m/e 190 and m/e 188 ($\times 10$) from selected ion monitoring experiment.

of TMS-IIb, IIIb and IVb, showed no measurable increase in unlabelled species. These confirmed the previous result that, within the limits of experimental error, no zeatin, zeatin-9- β -riboside or zeatin-9- β -glucoside were present in the radish seed.

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