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Note

High-performance liquid chromatographic analysis of permethylated cytokinins*

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Cytokinins exist in most plant tissues in minute amounts. The isolation of material of sufficient purity for structural determination by physical techniques requires extensive use of chromatographic methods. The polar nature of most of the naturally occuring cytokinins places limits on the types of HPLC systems that can be utilized for their isolation and analysis. The latter necessitates formation of volatile derivatives for GLC and GLC–MS. The most widely used derivatives for cytokinins have been TMS[1,2]. With the advent of permethylation[3] more recent use of these cytokinin derivatives have been made[4,5]. Advantages of permethyl over TMS derivatives include their chemical stability and lower molecular weight. In addition, the permethylated cytokinin is non-polar which expands the chromatographic potential for alternate purification procedures.

The purpose of this work was to modify the permethylation procedure and to devise HPLC purification techniques for permethylated cytokinins.

EXPERIMENTAL

Reagents

Potassium *tert.*-butoxide from Aldrich and reagent-grade DMSO from Pierce were stored in a drying cabinet. After redistillation methyl iodide was dispersed into small vials over molecular sieve and a drop of mercury, and the vials held in a plastic bottle containing silica gel and stored at 4°C.

The DMSO anion was prepared by mixing DMSO plus potassium *tert.*-butoxide for 1 h at 45°C with nitrogen flowing through the system at 30 l/h. The DMSO anion at a final molarity of 0.1 to 0.2 was the easiest to handle with respect to viscosity

* Abbreviations: TMS = Trimethylsilate; Z = zeatin; ZR = zeatin riboside; ZROG = zeatin riboside-O-glucoside; Z9G = zeatin-9-glucoside; Me = methyl; GA = gibberellins; DMSO = dimethylsulphoxide; GLC = gas–liquid chromatography; HPLC = high-performance liquid chromatography; MS = mass spectrometry; 2iP = N^6-isopentenyl adenine; 2iPA = N^6-isopentenyl adenosine; diHZR = dihydrozeatin riboside; diHZROG = dihydrozeatin riboside-O-glucoside; diHZ = dihydrozeatin; diHZOG = dihydrozeatin-O-glucoside.

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and can be achieved by mixing 6 ml DMSO with about 180 mg potassium *tert.*-butoxide. After preparation the DMSO anion was dispensed into small vials and stored over silica gel in a plastic container at −20°C.

Permethylation procedure

Cytokinins were placed in Reactivials (Pierce) and dried with a stream of nitrogen. Experiments with and without vacuum drying indicated the vacuum step was not essential. Reactivials were positioned on a warming plate at 40°C; 50 μl DMSO anion were added (*ca.* 10 × excess of the cytokinin) and the vial shaken; 10 μl methyl iodide then added and shaken; allowed to stand; 50 μl water added, and finally partitioned with 100 μl chloroform to remove the Me cytokinin from the reagents. *Note:* the relatively non-polar cytokinins 2iP and 2iPA will partition into chloroform even when not permethylated.

HPLC

Two separate purification systems for methylated cytokinins were developed. One using an adsorption column of 150 × 4.5 mm I.D. Spherisorb eluted with hexane–isopropanol–triethylamine (85:15:0.1) at 2 ml/min and the other a polar column of 250 × 4.5 mm I.D. Partisil 10PAC eluted with hexane–ethanol–triethylamine (90:10:0.1) at 2 ml/min.

UV spectrometry

UV spectra were obtained using a Beckman DU-8 spectrophotometer. Samples of Me cytokinins were run in methanol.

Mass spectrometry

MS spectra of Me cytokinins were obtained with a Pye 104 GC connected to a Kratos MS 30. The GLC conditions were: column, 3% OV-1 on Gas-Chrom Q (100–120 mesh); helium flow-rate, 35 ml/min; column temperature, for Z 220–280°C at 8°C/min and for ZR isothermal at 300°C. For Z and ZR the MS 30 was set at 70 eV, 300 μA, 3 sec/decade scan speed, source temperature at 190°C and 200°C, respectively, and jet temperature of 280°C.

Scintillation counting

[^{14}C]Cytokinins were permethylated and assayed in 5 ml of a solution containing 1.5 *M* toluene, 6 g PPO and 750 ml Triton X-100. Counting was conducted in a Phillips PW 4540 liquid scintillation analyzer.

RESULTS AND DISCUSSION

The commonly used procedure for methylation of cytokinins has many steps and is time consuming[6]. Our modifications of the procedure result in favorable changes. The use of potassium *tert.*-butoxide in place of sodium hydride for the preparation of the DMSO anion has several advantages. The rate of reaction is rapid and no hydrogen is produced. The starting material does not need to be washed free of mineral oil, thus contamination of small samples prior to MS analysis is reduced. The DMSO anion solution is free of particulate matter which can cause syringe

blockage as when NaH is used. The time in DMSO anion prior to adding methyl iodide was checked at intervals of zero to 60 min using [^{14}C]ZOG. No greater than 3% difference of recovered activity could be found and the zero time period was chosen for all subsequent experiments. In practice this meant adding the DMSO anion to each vial, vigorously shaking each vial and then adding the methyl iodide. Similar results were found for the time in methyl iodide, however, in this instance 30 min was chosen for convenience.

The efficiency of derivatisation was estimated by the use of ^{14}C-labelled cytokinins together with HPLC and MS analysis of the products. In all cases the procedure outlined above gave greater than 90% permethylation with S.E. less than 2. An advantage of this procedure is that samples do not need to be exhaustively dried as they do for the production of TMS derivatives. Though samples were routinely dried over phosphorus pentoxide under vacuum overnight it was found that the same efficiency was obtained for samples dried by an stream of nitrogen immediately prior to derivatisation. Once permethylated the cytokinins are stable for long periods and can be used as laboratory standards.

An aspect worth emphasizing is the fresh preparation of DMSO anion on a regular basis. If prepared each 2 months, a point stressed by others[4], one can expect good permethylation results. We have had numerous permethylation failures traced to DMSO anion stored beyond 2 months at −20°C.

Hormone analysis from plant organs involves organic solvent extraction of large samples and subsequent separation of materials via solvent partitioning and chromatography. While these procedures are useful and important the putative compound must be further separated from contaminants before reliable identification and estimation of amounts present are possible. When purifying a putative cytokinin from plant extracts it is useful to have a wide range of separation procedures throughout the scheme. In the last stage of extract preparation for MS analysis it is important to attempt separation of the methylated putative cytokinin from remaining contam-

TABLE I

HPLC OF METHYLATED CYTOKININS ON SPHERISORB OR PARTISIL 10 PAC

Compound	*Retention time (min:sec)*	
	*Spherisorb**	*Partisil 10 PAC***
Me 2iPA	2:15	2:45
Me diHZR	2:45	3:15
Me ZR	3:00	3:30
Me diHZROG	3:30	4:00
Me Z9G	3:30	3:30
Me ZROG	4:00	4:15
Me 2iP	4:45	5:30
Me diHZ	6:45	6:30
Me Z	8:00	7:45
Me diHZOG	12:30	8:15
Me ZOG	13:45	9:30

* Isocratic. Hexane–isopropanol–triethylamine (85:15:0.1), 2 ml/min.

** Isocratic. Hexane–ethanol–triethylamine (90:10:0.1), 2 ml/min.

inates. Toward that end we investigated HPLC systems for permethylated cytokinins. Since permethylation reduces cytokinin polarity we chose adsorption columns and polar columns eluting both with non-polar solvents. The most useful results were obtained with adsorptive Spherisorb and polar Partisil 10 PAC (Table I). Each contrasted system allows opportunity for further purification of the permethylated cytokinin from extract contaminants prior to MS analysis. It is even possible on Spherisorb to separate mixtures of cytokinins (Fig. 1). While one does not usually examine multiples of cytokinins the added flexibility is useful. In each HPLC system it is necessary to use a basic solvent system to suppress ionization of the amino group. This was achieved by adding triethylamine to the eluant. Attempts to achieve good separation under neutral or acidic conditions were unsuccessful.

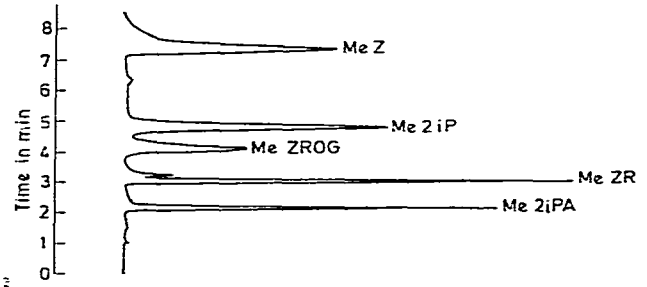

Fig. 1. Isocratic development of Me 2iPA, Me ZR, Me ZROG, Me 2iP and Me Z on Spherisorb with hexane–isopropanol–triethylamine (85:15:0.1) at 2 ml/min.

The Me cytokinins were collected after HPLC and UV spectrum obtained. Of the 11 analyzed all provided peaks at 275 nm. From each HPLC column one cytokinin was obtained for an MS spectrum. From Spherisorb, Me Z showed M^+ at *m/e* 261 with a base peak at *m/e* 230 (ref. 7), while Me ZR from Partisol 10 PAC showed M^+ at *m/e* 421 with a base peak at *m/e* 216 (ref. 7).

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