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Note

Separation of modified bases and ribonucleosides with cytokinin activity using fused silica capillary gas chromatography

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Derivatives of adenine and adenosine containing various moieties at the 6-amino nitrogen exhibit cytokinin or cell division activity in plant tissue[1,2]. Many of these compounds, both bases and ribonucleosides, have been found free or unbound as natural constituents of plant tissues and microorganisms. Cytokinin ribonucleosides have also been identified as constituents of tRNA from animals, plants, and microorganisms. The closely related structures and sub-micromolar concentrations of these compounds in tissue extracts and tRNA hydrolysates have made them difficult to separate and identify. Among the primary methods used to separate cytokinins have been gas chromatography (GC) using packed columns[3–5] and high-performance liquid chromatography[6–8]. Glass capillary column GC permits high resolution of most classes of volatile compounds; however, there has been very little work on the separation of volatile derivatives of cytokinins with this technique. In a recent study, Claeys *et al.*[9] used a glass capillary column to resolve permethyl derivatives of *cis*- and *trans*-zeatin and isopentenyladenine isolated from bacterial culture media. In the present investigation, we have employed high-resolution GC using a recently developed inert fused silica type capillary column to separate trimethylsilyl (TMS) derivatives of reference compounds representing a range of cytokinin bases and ribonucleosides known to occur as natural products.

EXPERIMENTAL

Reference compounds

trans-Zeatin, isopentenyladenine, isopentenyladenosine and phloretin were purchased from Sigma (St. Louis, MO, U.S.A.)*. Dihydrozeatin was purchased from Calbiochem (La Jolla, CA, U.S.A.). The remaining cytokinins were provided by Dr.

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Preparation of samples

Prior to silylation, compounds (25 μg each) were dried for 24 to 72 h over phosphorus pentoxide at 10^{-2} mmHg, or methylene chloride was added to the sample and then evaporated under a stream of nitrogen to remove water. Both procedures gave satisfactory results. Silylation was carried out at 60°C for 30 to 45 min in sealed reaction vials after adding 10 μl of dry pyridine (Supelco, Bellefonte, PA, U.S.A.) to dissolve the sample and 40 μl of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Regis, Chicago, IL, U.S.A.). Typically, 2-μl samples were analyzed.

Gas chromatography

Silylated compounds were analyzed using a microprocessor-controlled Hewlett-Packard Series 5880A instrument with a flame ionization detector. A 30 m × 0.25 mm I.D. fused silica capillary column coated with SE-54 (J & W Scientific, Rancho Cordova, CA, U.S.A.) was used. The injection port and detector temperatures were 220 and 280°C, respectively, and the column was temperature-programmed at the outset from 200 to 265°C at 4°C/min. Helium was used as carrier gas at a split ratio of 60:1 and a flow-rate through the column of 0.70 ml/min.

Gas chromatography–mass spectrometry

GC–mass spectrometry (MS) was carried out with a Hewlett-Packard Model 5985 instrument and a 20 m × 0.5 mm I.D. glass capillary column coated with SE-54 (Supelco). The mass spectrometer was operated at an ionization voltage of 70 eV and the source temperature was maintained at 150°C.

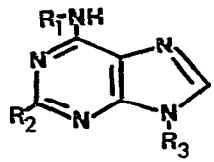
RESULTS AND DISCUSSION

Fused silica capillary columns are virtually inert due to lack of metal oxides and contaminants frequently present in glass. These columns have been found to be especially useful with nitrogen and sulfur compounds, such as amines and sulfides, which may degrade on other types of glass[10]. Since cytokinins contain amino nitrogen and in some cases sulfide groups a fused silica capillary column was selected for use with this class of compounds.

Cytokinins were chromatographed on an SE-54 fused silica capillary column with a single set of programmed temperature conditions following the evaluation of several programs and conditions. The structures and relative retention data for the cytokinins are given in Table I. All of the compounds, with the exception of two ribonucleosides, were resolved under the conditions used. A representative chromatogram obtained with compounds closely related structurally is shown in Fig. 1. Among the cytokinins resolved by this technique were dihydrozeatin, *trans*-zeatin and corresponding ribonucleosides, dihydroribosylzeatin and *trans*-ribosylzeatin. Separation of these pairs of compounds is frequently difficult to achieve using conventional packed column GC[11], especially on columns with stationary phases having low enough bleed to be used successfully in conjunction with MS. Bleed from capil-

TABLE I

STRUCTURES AND RETENTION DATA FOR CYTOKININS CHROMATOGRAPHED AS TMS DERIVATIVES ON SE-54 FUSED SILICA CAPILLARY COLUMN



R_1	R_2	R_3	*Common name*	*Relative retention**
$-CH_2-CH=C(CH_3)_2$	H	H	Isopentenyladenine	0.44
$-CH_2-CH_2-CH(CH_3)(CH_2OH)$	H	H	Dihydrozeatin	0.71
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	H	H	*cis*-Zeatin	0.75
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	H	H	*trans*-Zeatin	0.80
$-CH_2-CH=C(CH_3)_2$	H	Ribose**	Isopentenyladenosine	1.28
$-CH_2-CH_2-CH(CH_3)(CH_2OH)$	H	Ribose	Dihydroribosylzeatin	1.72
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	H	Ribose	*cis*-Ribosylzeatin	1.81
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	H	Ribose	*trans*-Ribosylzeatin	1.96
$-CH_2-C_6H_4(OH)(CH_3)$	H	Ribose	*o*-Hydroxybenzyladenosine	2.71
$-CH_2-CH=C(CH_3)_2$	CH_3S-	Ribose	2-Methylthioisopentenyladenosine	1.96
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	CH_3S-	Ribose	*cis*-2-Methylthioribosylzeatin	2.87
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	CH_3S-	Ribose	*trans*-2-Methylthioribosylzeatin	3.18

* Internal standard TMS-phloretin [2′,4′,6′-trihydroxy-3-(*p*-hydroxyphenyl) propiophenone] = 1.00.

** Ribose attached as β-D-ribofuranosyl group.

lary columns is usually minimal and does not interfere with GC–MS analyses. In addition, the *cis* and *trans* isomers of zeatin, ribosylzeatin, and 2-methylthioribosylzeatin were all resolved on the capillary column. Recent work on the development of high-performance liquid chromatographic techniques for the separation of geometrical isomers of cytokinins[12,13] showed that reversed-phase columns resolved *cis* and *trans* isomers of zeatin and ribosylzeatin, but *cis*- and *trans*-2-methylthioribosylzeatin were not separated[13]. Conventional packed column GC was also used to separate the TMS derivatives of the latter compounds[5], but there was undesirable peak broadening during packed column analysis of higher-molecular-weight cytokinin ribonucleosides, especially methylthio derivatives. Generally, narrow band widths and symmetry characterize capillary column peaks and extend the sensitivity of compound detection beyond that obtained with packed columns[14], although no direct comparisons were made in the present work. Two compounds, *trans*-ribosylzeatin and 2-methylthioisopentenyladenosine, could not be separated on the SE-54 capillary column. Coinjection of these compounds using either programmed temperature or isothermal conditions resulted in a single peak in each case.

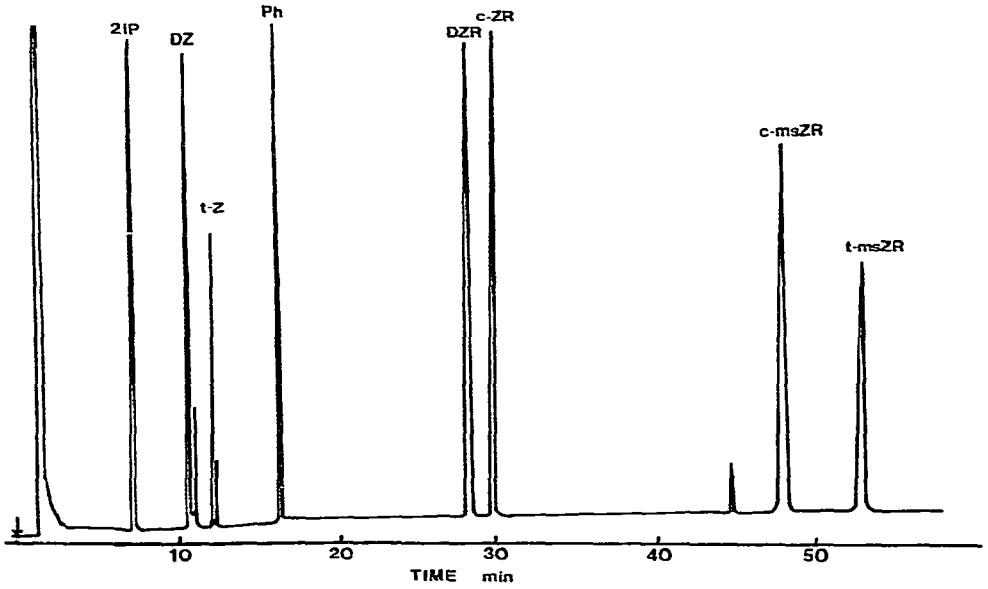


Fig. 1. Chromatogram of a mixture of silylated cytokinins including pairs of closely related compounds on a 30 m × 0.25 mm I.D. SE-54 fused silica capillary column programmed from 200 to 265°C at 4°C/min. Abbreviations: 2iP = isopentenyladenine; DZ = dihydrozeatin; t-Z = *trans*-zeatin (contaminant in commercial dihydrozeatin); Ph = phloretin (internal standard); DZR = dihydroribosylzeatin; c-ZR = *cis*-ribosylzeatin; c-msZR = *cis*-2-methylthioribosylzeatin; t-msZR = *trans*-2-methylthioribosylzeatin.

Silylation of cytokinin ribonucleosides usually gave sharp singlet peaks during GC analysis. However, silylation of the zeatins, namely *trans*-zeatin, *cis*-zeatin, and dihydrozeatin, frequently resulted in the formation of doublet or multiplet peaks even after rigorous drying of the samples prior to the derivatization reaction. In order to obtain information on the principal derivative formed from these bases, freshly silyl-

ated *trans*-zeatin was analyzed by GC–MS. The major peak yielded a molecular ion at *m/e* 363 and prominent ions at *m/e* 349, 348, 274, 273, 272, 261, 260, 258, 257, and 232 which corresponded with data reported by Purse *et al.*[4] for $(TMS)_2$-*trans*-zeatin. The secondary peaks were not identified. Composition of the silylated zeatin-type bases changed upon prolonged storage at $-20°$ as indicated by GC retention data.

The derivative formed upon silylation of *trans*-ribosylzeatin was also examined by GC–MS. The spectrum revealed a molecular ion at *m/e* 639 and characteristic ions at *m/e* 624, 550, 549, 537, 536, 406, 321, 320, 292 and 290. The fragmentation pattern of this compound was consistent with $(TMS)_4$-*trans*-ribosylzeatin and was very similar to that recently published[15] for $(TMS)_4$-*cis*-ribosylzeatin.

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