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Short communication

Derivatives of cytokinins for negative ion mass spectrometry

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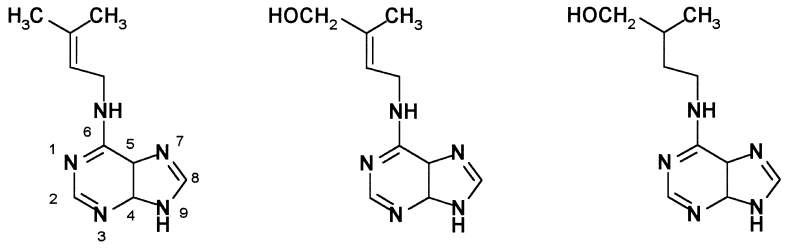
Abstract

It has been assumed that the pentafluorobenzylation reaction of the cytokinin bases, isopentenyladenine (iP), zeatin (Z) and dihydrozeatin (DZ) is selective for the 9-position of the purine ring. However, in the process of developing a HPLC separation for the putative *O-tert*.-butyldimethylsilyl-9-pentafluorobenzyl (*t*BuDMSi/pfBz) derivatives of Z and DZ, and 9-pfBz-iP, it was discovered that the minor product of the pentafluorobenzylation reaction (14–20% of the major derivatives) was pentafluorobenzylated at the 3-position. When the amount of cytokinin base was relatively low, the additional 3-pfBz derivatives were not detected.

Keywords: Derivatization, GC–MS; Negative ion mass spectrometry; Pentafluorobenzylation; Butyldimethylsilylation; Cytokinins

1. Introduction

The use of immunoassays in plant hormone analysis, in particular the cytokinins (**1**, **2** and **3**, see Scheme 1), has greatly increased the speed of analysis and allowed the processing of many samples at any one time. However, these assays, relying as they do upon the competitive interaction between antibody and ligand, may be subject to interference from cytokinin-like substances in crude extracts. Such interference can badly compromise the integrity of an analysis but the problem can be minimised by



1 **2** **3**

Scheme 1.

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rigorous purification of samples before analysis. Nevertheless, it is still advisable to validate the results of immunoassays using a definitive methodology such as isotope dilution mass spectrometry (MS). This may be most sensitively carried out using negative ion MS of the pentafluorobenzyl (pfBz) derivatives of the cytokinin bases, isopentenyladenine (iP, **1**), zeatin (Z, **2**) and dihydrozeatin (DZ, **3**) [1]. One of the attractive features of these derivatives is their stability in aqueous solvents so that they may be subject to chromatography (thin-layer chromatography, TLC and high-performance liquid chromatography, HPLC) prior to GC–MS. [1–3].

2. Experimental

2.1. HPLC

HPLC was carried out with equipment supplied by Waters Associates (Milford, MA, USA). Solvent was delivered by two constant flow-rate pumps (M-6000A) and samples introduced through a Model U6K universal injector. The absorbance of the column effluent was monitored at 269 and 280 nm, using a Waters 490 programmable multi-wavelength detector. The system was controlled, and data acquired, using Waters Maxima 820 software. All samples were prepared in the eluting solvents and filtered or centrifuged prior to injection. All solvents were HPLC grade; methanol (BDH), acetic acid (Unichrom, Ajax) and acetonitrile (E. Merck).

The derivatised cytokinins, pfBz-iP, *tert*.-butyldimethylsilyl (*t*BuDMSi)/pfBz-Z and *t*BuDMSi/pfBz-DZ were preparatively chromatographed on an analytical μBondapak phenyl column (Waters, 30 cm×3.9 mm) eluted with a linear gradient of 70% to 85% methanol in water (1.5 ml/min).

2.2. GC–MS

A fused-silica capillary column (SGE; 12 m×0.33 μm I.D.) coated with a methylsilicone bonded phase (BP1; thickness, 0.5 μm) was eluted with He (inlet pressure 5 p.s.i.) directly into the ion source of a Finnigan 4500 GC–MS system. Injections were made via a capillary on-column injector (SGE; OC1-3). The column was temperature programmed from 180°C (hold 30 s) to 260°C at 8°C/min and held there for 1 min. The mass spectrometer was operated in the chemical ionisation mode (ammonia, 0.2 Torr) with a source temperature of 100°C and ionisation energy 110 eV. Electron impact ionisation (EI) mass spectra were determined with an ionisation energy of 70 eV and a source temperature of 150°C. Spectra were also obtained using the direct inlet probe.

2.3. Derivatisation

Per-*t*BuDMSi derivatives were made according to Ref. [3] and pfBz derivatives were made as described in Ref. [1].

2.4. UV absorbance

UV spectra were recorded in 1-cm quartz cells on a Shimadzu UV-265 spectrophotometer over a range of 220 to 320 nm using 0.1 *M* NH_3 (aq.) in 95% aqueous ethanol, 0.1 *M* HCl in 95% aqueous ethanol or 95% aqueous ethanol.

3. Results and discussion

Previously, it had been assumed that the pentafluorobenzylation reaction was selective for the 9-position of the purine ring [1]. However, in the process of developing a HPLC separation for pfBz-iP, *t*BuDMSi/pfBz-Z and *t*BuDMSi/pfBz-DZ it was discovered that the pentafluorobenzylation reaction yielded two products which could be readily separated. These products were then preparatively chromatographed and spectroscopically examined (Fig. 1, Table 1). When the amount of cytokinin base was relatively low, the additional derivatives were not detected.

The mass spectra of each of the major products proved to be identical with previously published spectra [1] and from the UV spectra obtained in acidic, basic and neutral solvents (Table 1) it was confirmed that the purine ring was N^6,9-disubstituted [4]. The pattern of UV absorption of the minor products of pentafluorobenzylation (14–20% of major derivatives) proved typical of N^6,3-disubstituted purines.

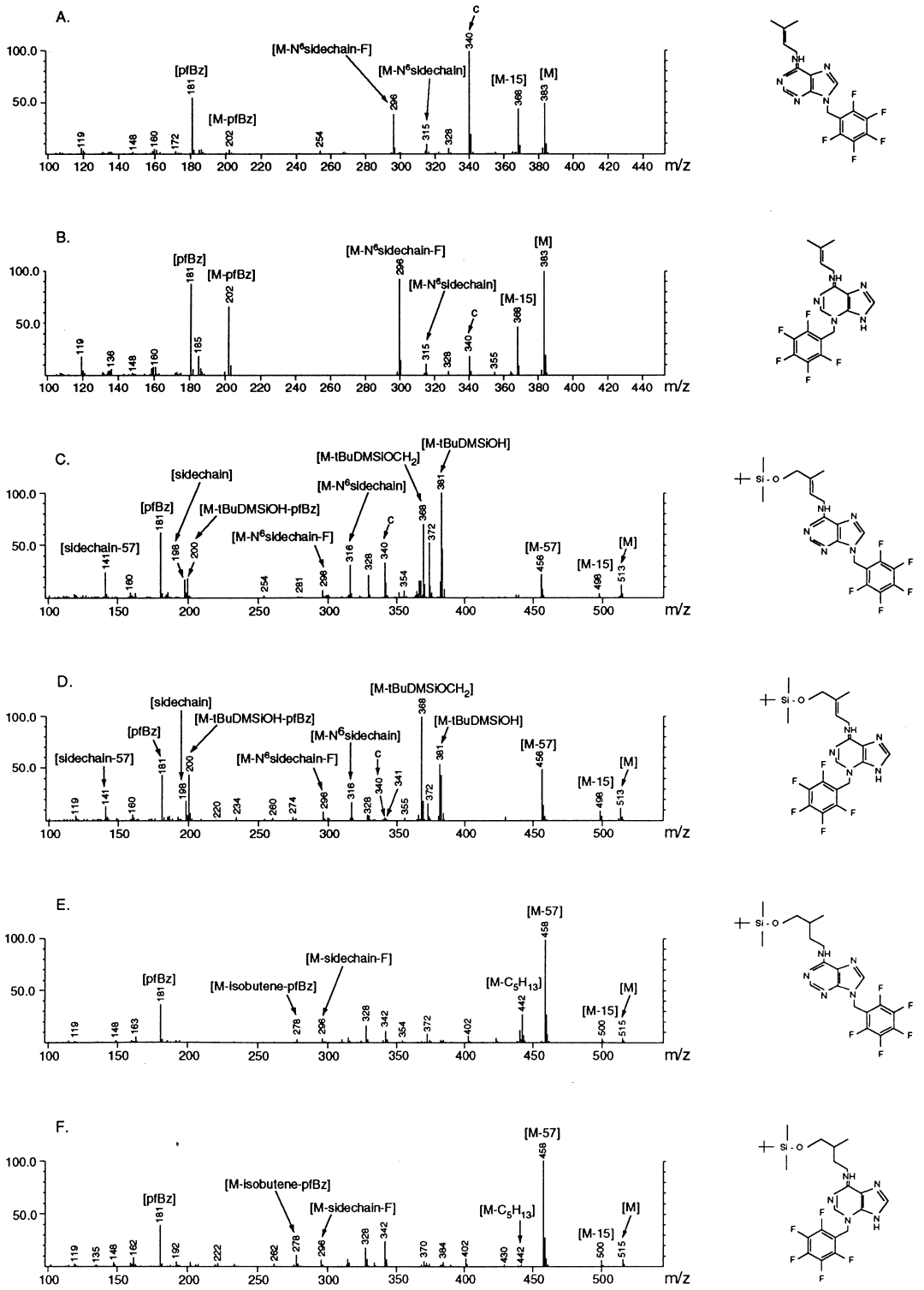

Fig. 1. EI mass spectra of (A) 9-pfBz-iP; (B) 3-pfBz-iP; (C) *O*-*t*BuDMSi-9-pfBz-Z; (D) *O*-*t*BuDMSi-3-pfBz-Z; (E) *O*-*t*BuDMSi-9-pfBz-DZ; (F) *O*-tBuDMSi-3-pfBz-DZ.

Table 1
UV absorbance maxima and minima (nm) of pfBz-iP, *t*BuDMSi/pfBz-Z and *t*BuDMSi/pfBz-DZ derivatives in acidic (0.1 *M* HCl in 95% aqueous EtOH), basic (0.1 *M* ammonia in 95% aqueous EtOH) and neutral solvent (95% aqueous EtOH)

Substitution pattern	Base		Neutral		Acid	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
N^6,9-iP[a]	268	233	267	233	265	234
N^6,3-iP[b]	297	252	298	252	290	244
N^6,9-Z[a]	268	233	268	233	265	234
N^6,3-Z[b]	297	252	298	252	289	244
N^6,9-DZ[a]	267	233	267	233	264	235
N^6,3-DZ[b]	297	252	296	252	289	244

[a] Major product.
[b] Minor product.

The positive and negative ion chemical ionisation mass spectra of the 3-pfBz and 9-pfBz isomers were essentially identical, being characterised by intense $[MH]^+$ and $[M-pfBz]^-$ ions, respectively. However, the EI mass spectra did permit the two series of isomers to be distinguished. It was apparent that substitution in the pyrimidine ring had a marked inhibitory effect on fragmentation processes that involve the *N*-1 position. Hence the cyclization ions (c, *m*/*z* 340, [3]) in the spectra of 3-pfBz-iP and *O*-*t*BuDMSi-3-pfBz-Z are much reduced in intensity by comparison with their 9-pfBz isomers. This in turn was complemented by enhanced losses of the 3-pfBz moiety as evidenced by a relatively increased intensity for *m*/*z* 202 ($[M-pfBz]^+$; 3-pfBz-iP), *m*/*z* 200 ($[M-t\text{BuDMSiOH}-pfBz]^+$; *O*-*t*BuDMSi-3-pfBz-Z), and *m*/*z* 278 ($[M-\text{isobutene}-pfBz]^+$; *O*-*t*BuDMSi-3-pfBz-DZ).

The ion *m*/*z* 372 in the EI mass spectra of *O*-*t*BuDMSi-9-pfBz-Z and -DZ is thought to derive from cleavage of the N^6 sidechain with silyl transfer to the *N*-1 position [1,3] and the relative decrease in intensity of this ion in the spectrum of the corresponding 3-pfBz isomers is consistent with this idea. The ions at *m*/*z* 381 ($[M-t\text{BuDMSiOH}]^+$) and *m*/*z* 382 ($[M-t\text{BuDMSiO}]^+$) in the EI mass spectra of *O*-*t*BuDMSi-9-pfBz-Z had reduced intensity in the spectrum of the 3-pfBz isomer and this may be indicative of a role for the *N*-1 position in this cleavage. A similar mechanism may be invoked to explain the reduced intensity of *m*/*z* 442 ($[M-C_5H_{13}]^+$, loss of *t*-butyl radicle and CH_4; [3]) in the spectrum of *O*-*t*BuDMSi-3-pfBz-DZ when compared with that of the 9-pfBz isomer.

When run by GC the 3-pfBz series of derivatives chromatographed with broad tailing peaks, later than the corresponding 9-pfBz isomers. Also interesting to note was the fact that the 3-pfBz moiety could be, at least partially, thermally rearranged to the 9-pfBz isomer when heated in a pyridine solution (approximately 30% at 90°C over 20 min).

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