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GAS CHROMATOGRAPHY–MASS SPECTROMETRY CHARACTERISTICS OF METHYLATED PERFLUOROACYL DERIVATIVES OF CYTOSINE AND 5-METHYLCYTOSINE

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SUMMARY

Pentafluorobenzoyl and heptafluorobutyryl derivatives of cytosine and pentafluorobenzoyl-5-methylcytosine have been examined by mass spectrometry following permethylation. Electron impact mass spectra were used to confirm the structures of the derivatives. The permethylated pentafluorobenzoyl derivatives of cystosine and 5-methylcytosine are sensitive for quantitative analysis by capillary gas chromatography–mass spectrometry. Under negative ion chemical ionization conditions 1 femtogram of each is detected.

INTRODUCTION

Mass spectrometry (MS) has contributed significantly to structural characterization in nucleic acid chemistry. The contributions include sequencing[1,2], determination of the structures of minor bases[3,4], and characterization of carcinogen–DNA

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adducts[5]. Recent developments and prospects have been briefly reviewed[6].

We are interested in advancing MS in the characterization and quantitation of carcinogen–DNA and mutagen–DNA adducts. Such measurements are needed to advance our understanding as well as control over such modifications, particularly of human DNA. This requires a special emphasis on sensitivity since routine human physiological samples contain little DNA.

Along these lines, we have begun to label nucleic acid monomers with electrophores, substances that readily attach low energy electrons in the gas phase. The resulting electrophore–monomer conjugates then can be detected with high sensitivity by both gas chromatography with electron-capture detection (GC–ECD) and GC with negative ion chemical ionization MS. Initially the pyrimidine base cytosine was labeled preferably with a pentafluorobenzoyl (PFB) electrophore[7]. Also, the other common pyridine bases, thymine and uracil, were correspondingly labelled with a pentafluorophenylsulfonyl electrophore. After subsequent permethylation, the products as standards were detected down to the low femtogram level by GC–ECD.

In this work several cytosine and 5-methylcytosine derivatives involving either the pentafluorobenzoyl or heptafluorobutyryl electrophores were characterized by mass spectrometry. These derivatives included the permethylated products analyzed before by GC–ECD[7]. Electron impact, positive chemical ionization, and negative ion chemical ionization (NICI) modes were employed. Electron impact MS provided necessary information to confirm the structural assignments of the derivatives. On the other hand, negative ion chemical ionnization was shown to be ideally suited for trace level analysis of the products. The latter was shown to reach the low femtogram level of detection for the PFB, permethylated derivatives of cystosine and 5-methylcytosine.

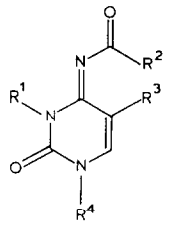
EXPERIMENTAL

Electron impact mass spectra were obtained with a Nuclide 12-90-G magnetic mass spectrometer. GC–MS analyses were conducted with a Finnigan 4021B quadrupole mass spectrometer operated in the pulsed positive–negative ion chemical ionization mode. Samples were introduced via the GC inlet using an on-column injector. The GC column was a 20 m × 0.025 mm I.D. fused-silica capillary column coated with SE 54 (J. & W. Scientific). The mass spectrometer was scanned in the range from 40 to 500 a.m.u. at a rate of 1 sec/scan for full mass spectral acquisition. For the quantitative calibration curve, the mass spectrometer was operated in the selective ion monitoring mode. Standards of the compounds were dissolved in toluene and dilutions were made in this solvent using silanized syringes. Appropriate solvent blank injections were made into the GC–MS instrument when low fg amounts of these compounds were analyzed.

RESULTS AND DISCUSSION

The compounds examined in this study consisted of cytosine and several of its methylated analogues. Two different types of N-acyl derivatives were investigated: N-heptafluorobutyryl and N-pentafluorobenzoyl. The structures of the compounds are summarized in 1–6. Mass spectra were obtained in the electron impact (EI) ion-

ization mode in order to establish the fragmentation characteristics of the derivatives. Chemical ionization (CI) spectras were recorded, both in the positive and negative ion mode, for the pentafluorobenzoyl derivatives 5 and 6.



1; $R^1 = R^3 = R^4 = H$, $R^2 = C_6F_5$

2; $R^1 = R^3 = R^4 = H$, $R^2 = CF_2CF_2CF_3$

3; $R^1 = R^3 = H$, $R^2 = C_6F_5$, $R^4 = COC_6F_5$

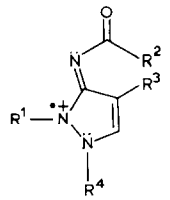
4; $R^1 = R^4 = H$, $R^2 = C_6F_5$, $R^3 = CH_3$

5; $R^1 = R^4 = CH_3$, $R^2 = C_6F_5$, $R^3 = H$

6; $R^1 = R^3 = R^4 = CH_3$, $R^2 = C_6F_5$

Electron impact mass spectra

The EI mass spectra of 1–6 are tabulated in Table I. The mass spectra of the compounds are characterized by well defined molecular ion peaks and a fragmentation behavior common to all of them is indicated in the table. Typical of fluorinated compounds is the presence of, for the most part, weak ion peaks corresponding to the elimination of F· and HF from the molecular ion[8]. This is followed or preceded by the loss of a CO molecule. Presumably the latter fragmentation originates from the ring system to give the five-membered ring conjugated ion a. This fragmentation is consistent with the general tendency of alicyclic and/or heterocyclic ring compounds which contain a carbonyl group to eliminate CO[9].



a

As expected, the bulk of the fragmentation is associated with cleavage of the N-acyl bond. The ion $[R^2CO]^+$ is prominent in the spectra of 1 and 3–6, but virtually absent in the spectrum of the heptafluorobutynyl derivative, 2, where the charge cannot be stabilized by an aromatic system. For the same reason the $[R^2]^+$ ion is also of low relative intensity in the spectrum of 2 while it is prominent in the spectra of the pentafluorobenzoyl derivatives. Instead, the spectrum of 2 is dominated by the $[M-R^2]^+$ ion, *m/z* 138. Finally an ion containing the heterocyclic ring structure is present in all spectra as a moiety comprising the groups R^1 and R^2 and the elements N, C and O. Probably this reflects a loss of the fragments NR^1CO and R^2. The identity of this ion is confirmed by its shift from *m/z* 95 in the spectra of 1 and 2 to

TABLE I

ELECTRON IMPACT MASS SPECTRA OF 1-6

Type of ion	*1*	*2*	*3*	*4*	*5*	*6*
M^+.	305*(63)**	307 (52)	499 (52)	319 (34)	333 (35)	347 (74)
$[M-F]^+$	286 (29)	288 (2)	480 (3)	300 (6)	314 (7)	328 (22)
$[M-HF]^+$.	285 (18)	287 (2)	479 (2)	299 (5)	313 (2)	327 (10)
$[M-CO]^+$	277 (21)	279 (2)	471 (8)	291 (6)	305 (66)	319 (4)
$[M-CO-F]^+$	258 (35)	260 (2)	452 (2)	272 (26)	286 (46)	300 (28)
$[R^2CO]^+$	195 (100)	198 (2)	195 (100)	195 (100)	195 (44)	195 (64)
$[R^2]^+$	167 (83)	169 (10)	167 (36)	167 (55)	167 (44)	167 (42)
$[M-R^2]^+$	138 (65)	138 (100)	332 (2)	152 (52)	166 (100)	180 (100)
$[M-COR^2]^+$	110 (2)	109 (18)	304 (3)	124 (28)	138 (9)	152 (10)
$[M-NR^1COR^2]^+$	95 (21)	95 (62)	289 (2)	109 (26)	109 (36)	123 (18)
				81 (48)		95 (40)

* Refers to *m/z* value of indicated ion.

** Values in parenthesis refer to % relative intensity.

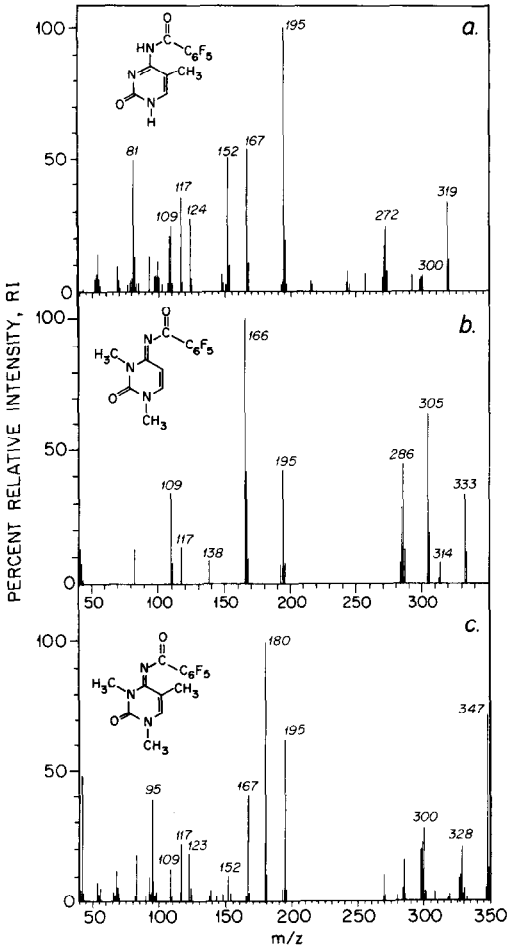


Fig. 1. Electron impact masss spectra of N^4-pentafluorobenzoyl derivatives of (a) 5-methylcytosine, 4; (b) 1,3-dimethylcytosine, 5; (c) 1,3-dimethylcytosine, 6.

m/z 289 in the spectrum of 3, to *m/z* 109 in those of 4 and 5, and to *m/z* 123 in that of 6. Significantly, further loss of CO from the $[M-NR^1COR^2]^+$ ion also occurs. This supports our earlier suggestion that the loss of CO from the molecular ion originates from the ring system as opposed to the N-acyl group.

These fragmentation processes provide necessary criteria for the structural characterization of the derivatives. This is more fully illustrated in the mass spectra of the homologs 4, 5 and 6 (Fig. 1). The important contribution of the N-methyl substituent at R^1 towards stabilization of the positive charge in the ion $[M-R^2]^+$ is exemplified by the inxcreased relative intensity of the related ion. Note, for example, the shift of *m/z* 152 in the top spectrum (4) to *m/z* 166 and *m/z* 180 in the spectra of 5 and 6, respectively. The latter two ions constitute the base peaks in these spectra. Interestingly, the tendency for subsequent loss of CO from $[M-R^1]^+$ to yield $[M-R^1CO]^+$ (ions of *m/z* 138 and *m/z* 152) is reduced significantly in the spectra of 5 and 6 compared to that of 4. This again reflects the influence of the N-methyl on the amide nitrogen towards stabilization of the $[M-R^2]^+$ ion and inhibition of its further fragmentation.

Chemical ionization mass spectra

Positive and negative ion chemical ionization mass spectra using methane as

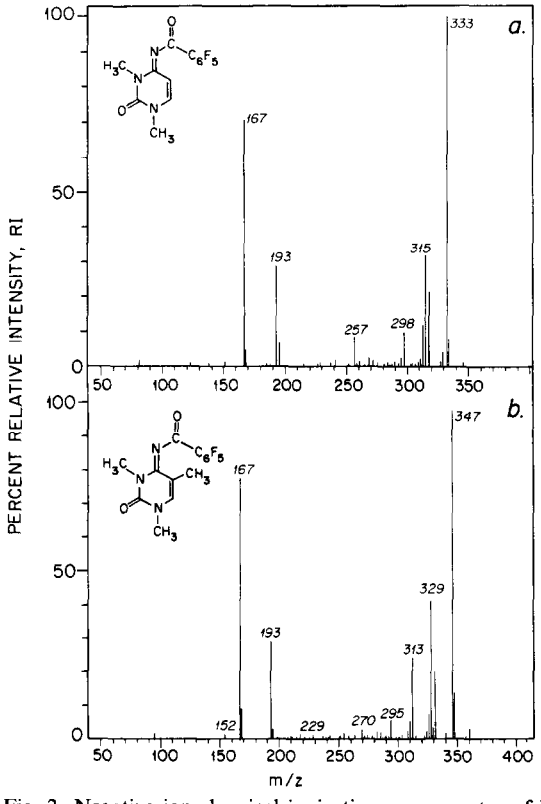


Fig. 2. Negative ion chemical ionization mass spectra of N^4-pentafluorobenzoyl derivatives of (a) 1,3-dimethylcytosine, 5; (b) 1,3-dimethyl-5-methylcytosine, 6.

a reagent gas were obtained for the methylated derivatives 5 and 6. In both cases the spectra are dominated by the molecular ion peaks at $[M+H]^+$ in the positive ion mode and $M^{-\cdot}$ in the negative ion mode. The NICI spectra of 5 and 6 are shown in Fig. 2a and b. In addition to the $M^{-\cdot}$ ion, in both spectra the pentafluorophenyl anion (*m/z* 167) contributes substantially to the total ion current of the NICI spectrum. Notable is the observed loss of 18 mass units from the negative molecular ion to yield the peaks at *m/z* 315 and *m/z* 329 in the NICI spectra of 5 and 6, respectively. Perhaps these ions occur as a result of a complex structural rearrangement leading to elimination of H_2O.

The ion at *m/z* 193 in both spectra is interestihng. It is likely that this ion is associated with the pentafluorobenzoyl moiety. While the expected *m/z* value of the pentafluorobenzoyl anion is 195, it is conceivable that the 2 a.m.u. decrease in its mass value is due to exchange of a fluorine atom with an oxygen from impurities in the CH_4 reagent gas to form a tetrafluorohydroxybenzoyl system. Aromatic halogen exchange reactions with oxygen in the vapor phase under NICI-MS conditions have been reported in similar systems[10–12]. Surprisingly, no analogous peak is observed at *m/z* 165 for the corresponding fragment accompanying the pentafluorophenyl anion, or at *m/z* 331 for the molecular ion. Conceivably this may be due to different sta-

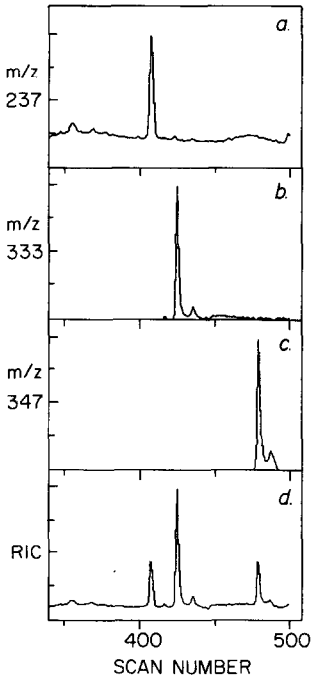


Fig. 3. GC–NICI-MS profiles of a standard mixture of 1 fg of derivatives 5 and 6 with heptachlor as the internal standard. Bottom trace (d) represents the reconstructed total ion current chromatogram. Single ion profiles of the internal standard, and compounds 5 and 6 are traces a, b and c, respectively.

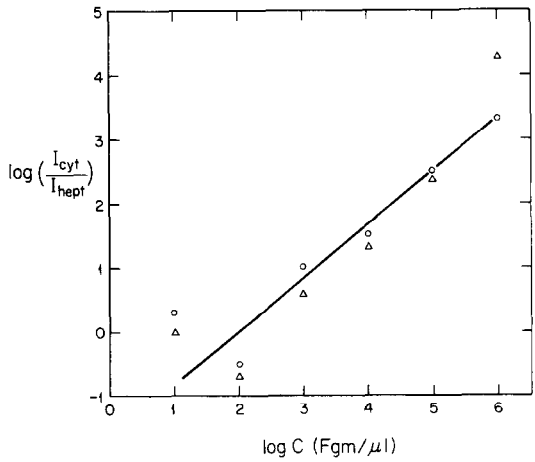


Fig. 4. Calibration curve of N^4-pentafluorobenzoyl-1,3-dimethylcytosine in the range from 1 fg to 1 ng using heptachlor as the internal standard. The points shown are the averages from duplicate injections. ○, Cytosine; △, methylcytosine.

bilization requirements or mechanistic pathways leading to the formation of these species. Further work to confirm these hypotheses is contemplated.

CONCLUSIONS

Despite the presence of some unexpected fragment ions, the utility of the NICI-MS spectra is affirmed by the high intensity of the molecular ion peaks. These ion peaks provide the necessary selectivity for analysis of these type of compounds by GC–MS using NICI-MS. To demonstrate the usefulness of this feature for trace level analysis, a series of standard mixtures of 5 and 6 was prepared at varying dilutions in the range from 1 μg/μl to 1 fg/μl. Fig. 3 shows a GC–NICI-MS chromatogram of a mixture of 1 fg each of 5 and 6 using heptachlor as an internal standard. A total ion chromatogram and selective ion chromatograms of the three compounds in the mixture are shown. Notable is the excellent signal-to-nose ratio obtained for the cytosine derivatives even at the 1 fg level. This suggests that even lower detection limits are feasible. The potential analytical applicability of this approach is further illustrated in Fig. 4 which shows a standard calibration plot for 5 and 6 in the range from 1 fg to 1.0 ng. Except at the highest concentration, the close pairing of the data points, and the linearity in the mid range even with heptachlor as a reference compound, encourages the use of these derivatives for trace level analysis.

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