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GAS CHROMATOGRAPHY OF NUCLEOTIDES AND NUCLEOSIDES OF CYTOSINE

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SUMMARY

3,N^4-Etheno-O-persilyl derivatives of nucleotides and nucleosides of cytosine have been studied as derivatives for gas chromatography and mass spectrometry. Ethenylation blocks further derivatization of the base and therefore precludes chromatographic problems associated with trimethylsilylation at position N^4 of cytosine. The mixed derivatives exhibit satisfactory gas chromatographic properties and produce molecular ions of greater abundance than in the case of the corresponding trimethylsilyl derivatives. Relative retention times and mass spectral data from three nucleotides and three nucleosides are presented. Reaction with chloroacetaldehyde to form etheno derivatives is selective for cytosine and adenine bases, and so the reaction is potentially useful for characterization of nucleotides or nucleosides of unknown structure.

INTRODUCTION

The applications of gas chromatography (GC) and of GC–mass spectrometry (MS) to constituents of nucleic acids have been substantially hampered by the high polarity and chemical instability of nucleotides and nucleosides of cytosine. Although trimethylsilylation generally produces sufficiently volatile derivatives of nucleotides and nucleosides for GC[1,4], previous attempts to obtain a peak from cytidine 5′-monophosphate (as the derivative 1) and related nucleotides have failed[2,5–7]. The trimethylsilyl (TMS) derivative of the corresponding nucleoside cytidine produces a broad, low peak[3,5,8–10], and under some conditions two peaks[7,11]. The trifluoroacetyl-TMS derivative 2 is easily prepared on a micro scale and is suitable for MS but is insufficiently volatile for GC, while the derivative of cytidine is amenable to GC[12]. Likewise, the mixed methoxime–TMS derivative (3) which can be successfully applied to cytidine produced no GC peak from cytidine 2′-monophosphate[6]. Another generally

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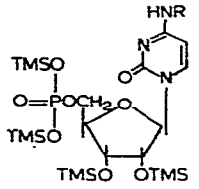
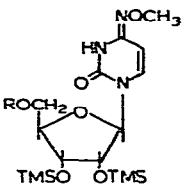
satisfactory alternative for GC of cytidine is N,O-permethylation[13], which is, however, not applicable to the corresponding nucleotide.

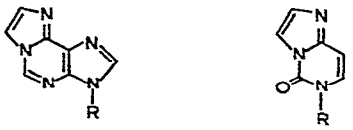


1, R = TMS
2, R = $COCF_3$

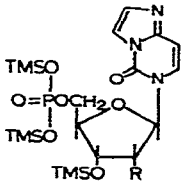


3, R = TMS or $PO(OTMS)_2$

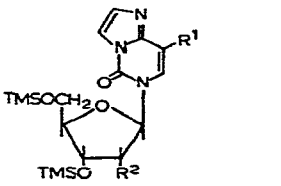
In view of the notable absence of a derivative suitable for the GC of nucleotides of cytosine, we sought a new derivative that can be prepared on a micro scale, exhibits good chromatographic and MS properties, and can be applied to both nucleotides and nucleosides of cytosine. Led by earlier work of Kochetkov *et al.*[14], and by Leonard and co-workers[15,16] on synthesis of etheno derivatives of the general types 4, we have developed a micro scale procedure for preparation of the etheno-TMS derivatives 5–10. These results provide the first successful gas chromatograms of nucleotides of cytosine derivatives (5, 6, 10), and show that etheno-TMS derivatives are useful alternatives for the GC and MS of cytosine-containing nucleosides.



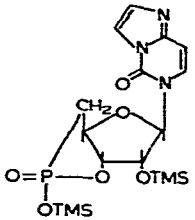
4, R = H or ribosyl



5, R = OTMS
6, R = H



7, R^1 = H, R^2 = OTMS
8, R^1 = R^2 = H
9, R^1 = CH_3, R^2 = H



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EXPERIMENTAL

Preparation of 3,N^4-etheno derivatives

Experiments were conducted using sample sizes of 20 μg and 1 mg. Chloracetaldehyde (45%, ICN Pharmaceuticals, Irvine, Calif., U.S.A.) was adjusted to pH 4 using sodium hydroxide; 100 μl was added to 1 mg (or 20 μg) of nucleotide or nucleoside and stirred for 12 h at room temperature. The water was removed *in vacuo* and remaining chloroacetaldehyde was removed by trituration with ether. Approximately 50 μg (or the entire product when starting with 20 μg) of the residue was reacted with 40 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (Regis, Chicago, Ill., U.S.A.) and 1% trimethylchlorosilane in 10 μl of dry pyridine at 100° for one hour in a PTFE-lined screw cap vial. Sample equivalent to 0.5–4 μg was submitted to GC or MS.

Gas chromatography

A Varian 2100 gas chromatograph with flame-ionization detector and 6 ft. × 0.25 in. silanized glass columns was used, with 1% OV-17 coated on 100–200 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). All runs were temperature programmed at 4°/min with carrier gas flow-rate of 20 ml/min using sample sizes in the range 0.5–4 μg. GC–MS measurements (see below) were made using a 3 ft. × 0.25 in., 1% OV-17 column.

Methylene unit (MU) values[17] were obtained as described earlier[18] using two *n*-alkanes as internal standards, one earlier and one later-eluting than the component of interest. Sample injections were made at column temperatures 50° lower than the elution temperature of the smaller alkane. MU values were derived by linear interpolation of distances on the recorded chromatogram.

Mass spectrometry

Mass spectra were acquired using an LKB 9000S instrument with ion source temperatures 270°, separator temperature 280°, and ionizing energy 70 eV. Compound 8 was introduced by direct probe after removal of reagents by the probe vacuum system; all others were introduced by gas chromatograph.

Exact mass measurements made on the derivative of cytidine 3',5'-cyclic monophosphate were done by peak matching at $M/\Delta M = 20{,}000$ using a Varian MAT 731 mass spectrometer, with sample introduction by direct probe.

RESULTS AND DISCUSSION

Gas chromatograms produced by etheno derivatives of the three nucleotides 5, 6, and 10 are shown in Fig. 1, and provide the first successful chromatograms of nucleotides of cytosine. Peak shapes were satisfactory over the range of sample size examined (0.5–4 μg). The greatest tailing observed was that exhibited in Fig. 1a. Low molecular weight artifacts related to the reagent were typically observed to elute shortly after the solvent front, as seen in Fig. 1.

The derivatives were examined by MS to confirm the expected structures as 3,N^4-etheno-O-persilyl derivatives and to examine the utility of MS for characterization of etheno derivatives of cytosine. Mass spectra of the etheno-TMS derivatives

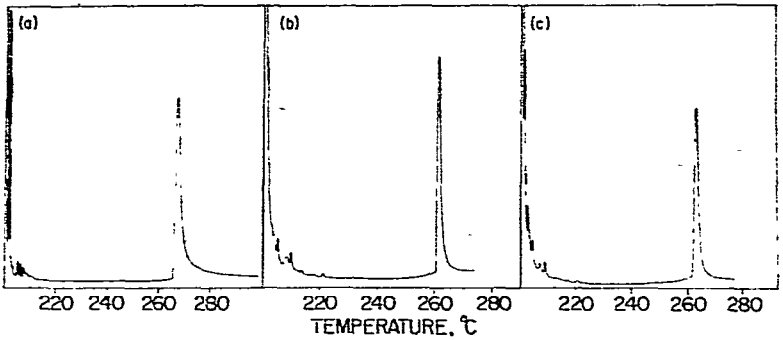


Fig. 1. Gas chromatograms of the 3,N⁴-etheno-TMS derivatives of (a) cytidine 5′-monophosphate, (b) cytidine 3′,5′-cyclic monophosphate, (c) 2′-deoxycytidine 5′-monophosphate.

5 and 10 are presented in Figs. 2 and 3, and principal ions from the spectra of the remaining derivatives are represented in Table I.

Assignments of the major ions largely follow those of trimethylsilylated nucleotides[19] and nucleosides[20]. In addition, Chheda *et al.*[21] have published the mass spectrum of 1,N⁶-ethenoadenosine and of the TMS derivative of a related compound. In every case the molecular ion (M) and M—CH_3 values are consistent with the presence of an etheno function in the base, and fully silylated ribose or ribose phosphate. Ions of the base series are of relatively low abundance with the exception of compound 9 (see Table I), while ions of the sugar series predominate in the spectra of ribonucleotides (*m/e* 169), the deoxyribonucleotide 6 (*m/e* 81), and nucleosides (*m/e* 103 from C-5′)[19,20]. The fragment ion at base + 41 a.m.u. that is characteristic

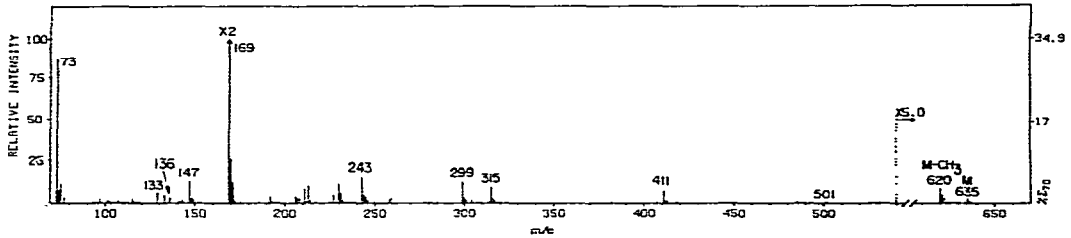


Fig. 2. Mass spectrum of the trimethylsilyl derivative of 3,N⁴-ethenocytidine 5′-monophosphate.

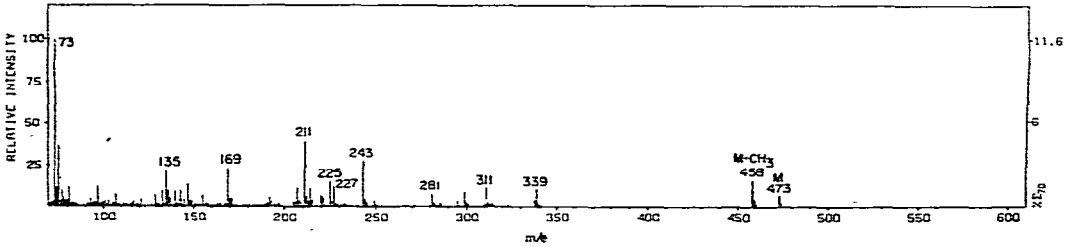


Fig. 3. Mass spectrum of the tris(trimethylsilyl) derivative of 3,N⁴-ethenocytidine 3′,5′-cyclic monophosphate.

TABLE I

SELECTED IONS FROM THE MASS SPECTRA OF ETHENO-TMS DERIVATIVES OF NUCLEOTIDES AND NUCLEOSIDES

Parent compound	*No. TMS groups*	*m/e (relative intensity)*						
		M	*M−CH₃*	*Base + H*	*Base + 2H*	*Other ions*		
Cytidine	3	483 (4.9)	468 (2.6)	135 (3.1)	136 (1.1)	349 (5.0	103 (34)	73 (100)
2′-Deoxycytidine	2	395 (3.5)	380 (0.5)	135 (11.5)	136 (8.8)	261 (4.7)	103 (100)	73 (70)
5-Methyl-2′-deoxy-cytidine	2	409 (16)	394 (1.5)	149 (37)	136 (5.7)	261 (1.9)	103 (100)	73 (75)
2′-Deoxycytidine 5′-monophosphate	3	547 (0.6)	532 (0.3)	135 (1.6)	136 (2.2)	413 (1.6)	299 (3.0)	81 (100)
Cytidine*	0	267 (3.9)		135 (100)	136 (20)	178 (1.6)	148 (2.6)	133 (3.2)
Adenosine	3	507 (6.7)	492 (2.5)	159 (4.4)	160 (6.9)	259 (21)	103 (29)	73 (100)

* Compound 4; R = ribosyl.

of cytidine analogs is absent from all mass spectra, as required by its mechanism of formation and the presence of an imino, rather than amino, group at C-4 (ref. 22). Molecular ion abundances of all etheno-TMS derivatives are markedly greater than in the corresponding TMS derivatives, suggesting their use for selected ion monitoring or quantitative applications of MS. For example the spectrum of cytidine 5′-monophosphate-$(TMS)_5$ exhibits no molecular ion[19] (*vs.* 0.3% in Fig. 1), while that of cytidine-$(TMS)_4$ is 1.3%[22] (*vs.* 6% in Table I).

The mass spectrum of the TMS derivative of 3,N^4-ethenocytidine 3′,5′-cyclic-monophosphate (10) shown in Fig. 2 shares numerous sugar-derived peaks in common with the TMS derivative of adenosine 3′,5′-cyclic monophosphate[19] (*m/e* 169, 211, 225, 227, 243), but exhibits several notable differences. The sugar fragment *m/e* 339 (*s*) predominates over the *s*-H species (*m/e* 338), while the prominent peak at *m/e* 311 in Fig. 3 (*vs. m/e* 310 in the adenosine analog) was shown by measurement of exact mass to result from expulsion of CO from *m/e* 339 (found: 311.0902; calculated: 311.0899 for $C_{10}H_{24}O_5Si_2P$). The significant peak at *m/e* 281, which is somewhat smaller in the case of the adenosine analog, corresponds in exact mass (281.0792) to further loss of CH_2O from the *m/e* 311 ion, presumably from C-5′ (calculated: 281.0795 for $C_9H_{22}O_4Si_2P$). The base + H ion (*m/e* 135) is the only notable member of the base series, in contrast to the adenosine analog in which base-containing ions play a more prominent role.

MU values of the compounds studied are given in Table II, and for comparison some literature values for other derivatives of cytidine and 2′-deoxycytidine are included. The latter data show that the entheno-TMS derivatives are substantially more volatile than the corresponding TMS derivatives and slightly more polar than trifluoroacetyl-TMS derivatives. Success in obtaining a product which is sufficiently stable for GC lies in the inherent stability of the imidazo[1,2-*c*]pyrimidine system and the absence of an active hydrogen or functional group at N^4. By contrast, the N^4-trimethylsilyl function in derivatives of cytidine or cytidylic acid is easily displaced or hydrolyzed, leaving an unprotected and highly polar amino group at C-4 that is detrimental to good chromatographic behavior. In cases when sample quantity is limited and the structure of the component under examination is not known, im-

perfect silylation conditions can often lead to a mixture of derivatives (*e.g.*, ref. 23) in which the base is the site of under-silylation[20]. This problem is reduced or eliminated in the case of etheno derivatives because no labile hydrogen remains on the exocyclic amino group and silylation cannot occur.

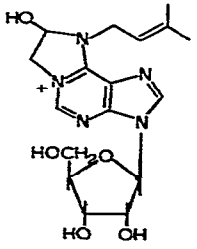
TABLE II

MU VALUES OF DERIVATIVES OF NUCLEOTIDES AND NUCLEOSIDES OF CYTOSINE

Parent compound	*Etheno-TMS*	*TMS**	*Methoxime-TMS***	*Trifluroacetyl-TMS****
Cytidine	27.26	31.73	24.91	26.68
2′-Deoxycytidine	26.97		23.79	26.60
5-Methyl-2′-deoxy-cytidine	27.42			
Cytidine 5′-mono-phosphate	32.57			
Cytidine 3′,5′-cyclic monophosphate	31.35			
2′-Deoxycytidine 5′-monophosphate	32.42			

* 1% OV-17, 4°/min (ref. 18).
** 5% SE-30, program rate not given (ref. 6).
*** 1% OV-17, 4°/min (ref. 12).

In addition to preparation of a derivative which is sufficiently volatile for GC, chemical derivatization, if selective, provides a degree of information in the case of compounds of unknown structure. For example, silylation of 7-methylpurine nucleosides or nucleotides uniquely leads to the 7-methyl-8-oxo derivative[24], and methoxime formation[6] or trifluoroacetylation at N^4 is characteristic of cytidine derivatives[12]. In the present case derivatization is only partly selective in that chloroacetaldehyde reacts to form derivatives of cytidine and adenosine (or their nucleotides), as well as closely related analogs[25,26] but not of the other common nucleosides such as uridine,



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guanosine, thymidine or inosine[15]. The mass spectrum of the TMS derivative of 1,N^6-ethenoadenosine has been included in Table I, and as expected follows the general behavior of compound 7 and of adenosine-$(TMS)_5$ (ref. 27). In related compounds the ethenylation reaction is blocked by substitution at N-1 of adenine or N-3 of cyosine moieties[15]. A product assigned structure 11 was obtained from N^6-isopentenyl-adenosine[15], but its polarity presumably precludes GC or MS. Similarly, analogs of

N^4-substituted cytidine presumably do not form derivatives that, after silylation, are detectable by GC or MS. Use of derivatives with specific structural requirements regarding substitution in the base is also potentially useful in establishing the site of substitution when only microgram quantities of material are available.

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REFERENCES

1 R. L. Hancock and D. L. Coleman, *Anal. Biochem.*, 10 (1965) 365.
2 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 15 (1966) 199.
3 Y. Sasaki and T. Hashizume, *Anal. Biochem.*, 16 (1966) 1.
4 K. H. Schram and J. A. McCloskey, in K. Tsuiji and W. Morowich (Editors), *GLC and HPLC Determination of Therapeutic Agents*, Vol. III, Marcel Dekker, New York, in press.
5 R. L. Hancock, *J. Gas Chromatogr.*, 6 (1968) 431.
7 A. B. Patel and C. W. Gehrke, *J. Chromatogr.*, 130 (1977) 115.
8 V. Miller, V. Pacakova and E. Smolkova, *J. Chromatogr.*, 119 (1976) 355.
9 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 123 (1976) 335.
10 J. P. Seiler, *Anal. Biochem.*, 75 (1976) 45.
11 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 130 (1977) 103.
12 U. I. Krahmer, J. G. Liehr, K. J. Lyman, E. A. Orr, R. N. Stillwell and J. A. McCloskey, *Anal. Biochem.*, 82 (1977) 217.
13 D. L. von Minden and J. A. McCloskey, *J. Amer. Chem. Soc.*, 95 (1973) 7480.
14 N. K. Kochetkov, V. W. Shibaev and A. A. Kost, *Tetrahedron Lett.*, (1971) 1993.
15 J. R. Barrio, J. A. Secrist, III, and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 46 (1972) 597.
16 J. A. Secrist, III, J. R. Barrio, N. J. Leonard and G. Weber, *Biochemistry*, 11 (1972) 3499.
17 C. E. Dalgliesh, E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 10 (1966) 792.
18 S. E. Hattox and J. A. McCloskey, *Anal. Chem.*, 46 (1974) 1378.
19 A. M. Lawson, R. N. Stillwell, M. M. Tacker, K. Tsuboyama and J. A. McCloskey, *J. Amer. Chem. Soc.*, 93 (1971) 1014.
20 J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger and R. N. Stillwell, *J. Amer. Chem. Soc.*, 90 (1968) 4182.
21 G. B. Chheda, S. P. Dutta, A. Mittelman and L. Baczynskyj, *Tetrahedron Lett.*, (1974) 433.
22 J. G. Liehr, D. L. von Minden, S. E. Hattox and J. A. McCloskey, *Biomed. Mass Spectrom.*, 1 (1974) 281.
23 H. Kasai, K. Murao, S. Nishimura, J. G. Liehr, P. F. Crain and J. A. McCloskey *Eur. J. Biochem.*, 69 (1976) 435.
24 D. L. von Minden, R. N. Stillwell, W. A. Koenig, K. J. Lyman and J. A. McCloskey, *Anal. Biochem.*, 50 (1972) 110.
25 K. H. Schram and L. B. Townsend, *Tetrahedron Lett.*, (1974) 1345.
26 K. F. Yip and K. C. Tsou, *Tetrahedron Lett.*, (1973) 3087.
27 J. A. McCloskey, in P. O. P. Ts'o (Editor), *Basic Principles in Nucleic Acid Chemistry*, Vol. I, Academic Press, New York, 1974, Ch. 3.