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Detection of ribose-methylated nucleotides in enzymatic hydrolysates of RNA by thermospray liquid chromatography–mass spectrometry

NAOHITO TAKEDA, STEVEN C. POMERANTZ and JAMES A. McCLOSKEY*

Departments of Medicinal Chemistry and Biochemistry, University of Utah, Salt Lake City, UT 84112 (U.S.A.)

ABSTRACT

A procedure has been developed for the detection and characterization of ribose-methylated dinucleotides of the type NmpN′ in enzymatic digests of RNA. Differences in reaction products from hydrolysis using RNase T_2, which will not cleave NmpN′, and hydrolysis by nuclease P_1 are analyzed by thermospray liquid chromatography–mass spectrometry. The method is applicable to dinucleotides present in nanogram range quantities and is suited for the characterization of new or unexpected ribose-methylated nucleotides for which chromatographic mobilities are not known.

INTRODUCTION

Methylation of O-2′ of ribose in RNA occurs widely in nature [1], and is implicated in a variety of functional roles, such as enhancement of structural stability, codon–anticodon interactions, and resistance to ribonuclease attack [2–4]. An effective method for detection of ribose-methylated nucleosides (Nm) is as the so-called alkali-stable dinucleotides NmpN′, which are formed by alkali-catalyzed hydrolysis of RNA. This method is based on the requirement for a free 2′-hydroxyl for phosphodiester bond cleavage and has been extensively exploited in studies of structure and distribution of ribose-methylated nucleotides (*e.g* ref. 5). Detection of O-2′-methylated residues in this fashion has the added advantage that the residue on the 3′ side (usually A, U, G, or C) can be simultaneously established, although the use of alkali leaves open the possibility of inadvertent chemical degradation of some modified bases in either the 5′ or 3′ residues. If only unmodified bases are present, then the sixteen possible dinucleotides of the type NmpN′ can be detected by various standard chromatographic methods (*e.g.*, refs. 5 and 6). However, identification methods based on chromatographic mobility are poorly suited in the event that new or unexpected nucleoside residues are encountered, which may lead to structural misassignment. Even in the case of structurally known nucleosides, now known to number approximately 20 in rRNA and over 70 in tRNA, the possibility of incorrect assignment is significant

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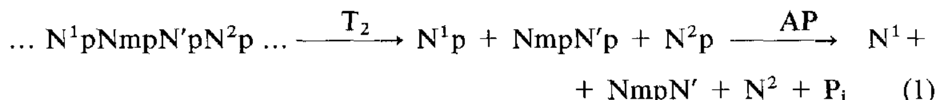
due simply to the potentially large number of combinations possible in dinucleotides.

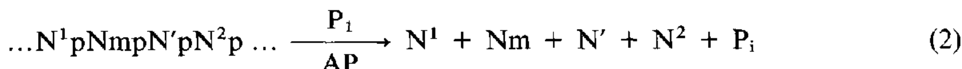
An efficient approach to the overall problem of detection and characterization of modified nucleosides in RNA is thermospray liquid chromatography–mass spectrometry (LC–MS) of enzymatic digests [7,8], which has been effective in the discovery of new nucleosides [9,10], including five which are modified both in the base and by ribose methylation [11,12].

We presently report an extension of the LC–MS method to dinucleotides of the type NmpN′, formed by hydrolysis of RNA by RNase T_2, which will not cleave 3′-phosphodiester bonds when O-2′ is methylated (reaction 1).



$$\ldots N^1pNmpN'pN^2p \ldots \xrightarrow{T_2} N^1p + NmpN'p + N^2p \xrightarrow{AP} N^1 + NmpN' + N^2 + P_i \quad (1)$$

where AP = alkaline phosphatase, T_2 = RNase T_2 and P_i = inorganic phosphate.



$$\ldots N^1pNmpN'pN^2p \ldots \xrightarrow[AP]{P_1} N^1 + Nm + N' + N^2 + P_i \quad (2)$$

where P_1 = nuclease P_1.

Dinucleotides are detected by comparison of high-performance liquid chromatography (HPLC) or LC–MS data from products of reaction 1 with those from reaction 2 in which RNA is completely hydrolyzed to the nucleoside level. Under the conditions employed, dinucleotides dissociate in the heated spray of the thermospray interface and are detected as the constituent nucleosides, with sequence defined by the mass spectrum of the 5′ member, Nm. For additional confirmation of results, the procedure can be further extended by collection of the putative NmpN′ fraction from reaction 1, followed by complete hydrolysis of the fraction as in reaction 2 and LC–MS. Nm and N′ are then observed in stoichiometric amounts, which aids in their distinction from other nucleosides which partially coelute with NmpN′.

The thermospray method is disadvantageous in that molecular ions or sequence-determining fragment ions from NmpN′ are not observed, but is advantageous in providing nanogram range sensitivity and applicability to complex mixtures, with retention of very high chromatographic fidelity in the thermospray interface. Further, the method is complementary to conventional reversed-phase HPLC, for which relative elution times of a large number of natural nucleosides have been catalogued [8,13,14].

EXPERIMENTAL

Materials

The following were obtained from Sigma (St. Louis, MO, U.S.A.); transfer RNA^{Phe} from brewer's yeast (*Saccharomyces cerevisiae*); nuclease P_1; phosphodiesterase I (type VII, *Crotalus atrox* venom); RNase T_2 (grade V, *Aspergillus oryzae*); alkaline phosphatase (type VII-S, bovine intestinal mucosa). Unfractionated tRNA was isolated from *Pyrodictium occultum* cells provided by K. O. Stetter, Regensburg University, by an earlier described procedure [15].

Enzymatic hydrolysis of tRNA

Vacuum-dried $tRNA^{Phe}$ (50 μg) was dissolved in 48 μl of 10 m*M* potassium acetate buffer, pH 4.5. After addition of 2 U of RNase T_2 (2 U in 2 μl of potassium acetate buffer), the mixture was digested at 37°C for 16 h and then divided in half (parts A and B). Part A was dried under reduced pressure, redissolved in 29 μl of 20 m*M* triethylammonium bicarbonate (TEAB) buffer, pH 8.0, and then incubated with alkaline phosphatase (1 U in 1 μl of TEAB buffer) at 37°C for 3.6 h. A 12-μl aliuot (10 μg) was injected directly into the LC–MS instrument for acquisition of the data shown in Figs. 1a and 2.

To part B were added 10 μl of 50 m*M* ammonium acetate buffer, pH 5.3, containing 1.0 U of nuclease P_1. The mixture was incubated at 45°C for 7 h, then dried under reduced pressure and redissolved in 29 μl of 20 m*M* TEAB buffer, pH 8.0. Alkaline phosphatase (1 U) was added and the mixture incubated at 37°C for 3.5 h. A 12-μl aliquot (10 μg) was injected directly into the LC–MS instrument for acquisition of the data shown in Fig. 1b.

Note: for purposes of illustration for Fig. 1, the $tRNA^{Phe}$ digests were slightly underreacted for hydrolysis and dephosphorylation. For discussion of procedures suitable for cleavage of the dinucleotide (A,yW) and complete dephosphorylation, see ref. 16.

P. occultum tRNA (15 μg) was hydrolyzed by a procedure similar to that used for part A (preceding section). The entire hydrolysate was submitted to LC–MS analysis, resulting in data shown in Fig. 3. A second 15-μg sample was hydrolyzed according to the procedure for part B above in order to effect complete hydrolysis (resulting data not shown). Unlike the procedure for $tRNA^{Phe}$, it was necessary to further incubate the nuclease P_1 digest using 0.002 U phosphodiesterase (20 m*M* TEAB buffer, pH 7.4), 37°C for 2 h. This additional step is due to the unusual stability of *P. occultum* tRNA and its constituent ribose-methylated nucleotides, toward hydrolysis.

Enzymatic hydrolysis of collected dinucleotide fractions

Two chromatographic separations equivalent to 15 μg each of *P. occultum* tRNA were made for collection of individual peaks corresponding to Fig. 3 over the range 28–32 min. The two sets of fractions were combined and hydrolyzed by

nuclease P_1, phosphodiesterase, and alkaline phosphatase as described above. In the case of the eluate at 28.6 min (Fig. 3), the quantity collected from 30 μg of tRNA was used to produce data shown in Fig. 4, which was estimated from peak areas shown in Fig. 4a to correspond to 59 ng of dinucleotide, or 25 ng (G) and 27 ng (m^2Gm) of the constituent nucleosides.

High-performance liquid chromatography

Reversed-phase HPLC carried out in direct combination with MS utilized a Beckman Instruments (Fullerton, CA, U.S.A.) Model 342 system as described in ref. 8, with a 250 mm × 4.6 mm I.D. LC-18S octadecyldimethylsilyl, reversed-phase analytical column, 5 μm particle diameter (Supelco, Bellefonte, PA, U.S.A.) preceded by a 30 mm × 4.6 mm I.D. RP-18 guard column (Applied Biosystems, Foster City, CA, U.S.A.).

Chromatography for collection of dinucleotide fractions for further hydrolysis was performed using the same column and pre-column described above. The gradient profile used [8] was based on the system of Buck *et al.* [13] for nucleoside separations.

Liquid chromatography–mass spectrometry

Thermospray LC–MS measurements of enzymatic hydrolysates were made using a non-commercial quadrupole mass analyzer and a Vestec (Houston, TX, U.S.A.) interface and vaporizer controller, with an intermediate Waters (Milford, MA, U.S.A.) 440 UV detector, as described in ref. 8. The vaporizer exit temperature was maintained at 245–270°C, with the ion source temperature set at 350°C to provide a vapor temperature at the point of ion sampling of 290–300°C. Further details concerning the acquisition of LC–MS data and interpretation of mass spectra are described in refs. 7 and 8.

RESULTS AND DISCUSSION

The examples presented in the following sections were chosen to represent the characteristics and problems which can arise in the analysis of nucleoside and nucleotide mixtures by thermospray LC–MS. The model discussed, $tRNA^{Phe}$, is a small nucleic acid (76 nucleotides) containing the four major nucleotides and eleven different modified residues. These include two sugar-methylated nucleosides (2′-O-methylcytidine-32 and 2′-O-methylguanosine-34) and six other residues, all of which occur once per tRNA molecule, in addition to dihydrouridine, pseudouridine, and 5-methylcytidine which each occur twice in the sequence [17]. If the method is applied to larger ribosomal RNAs the resulting chromatogram will be somewhat simpler due to presence of few modified residues, which will in general be present in lower concentrations. In the case of mixtures of RNAs, such as unfractionated tRNA, the resulting chromatogram will be somewhat more complex, with a potentially larger number of dinucleotides produced by reaction 1.

Detection of dinucleotides in enzymatic digests

The hydrolysis products of tRNA^{Phe} from reaction 1 are shown in Fig. 1a, and from reaction 2 in Fig. 1b. The identification of nucleosides (*e.g.* peaks 1–13) is made by their thermospray mass spectra and by comparison of HPLC retention times with those of authentic standards [13,14]. Thermospray spectra of nucleosides [7,18] consist primarily of the protonated molecule (MH^+) and the protonated base ($BH_2{}^+$ from base fragment B), with the differences in mass between these ions reflecting the loss of ribose (132 u), methylribose (146 u) or deoxyribose (116 u) in the case of DNA contaminants. By chromatographic comparison of the

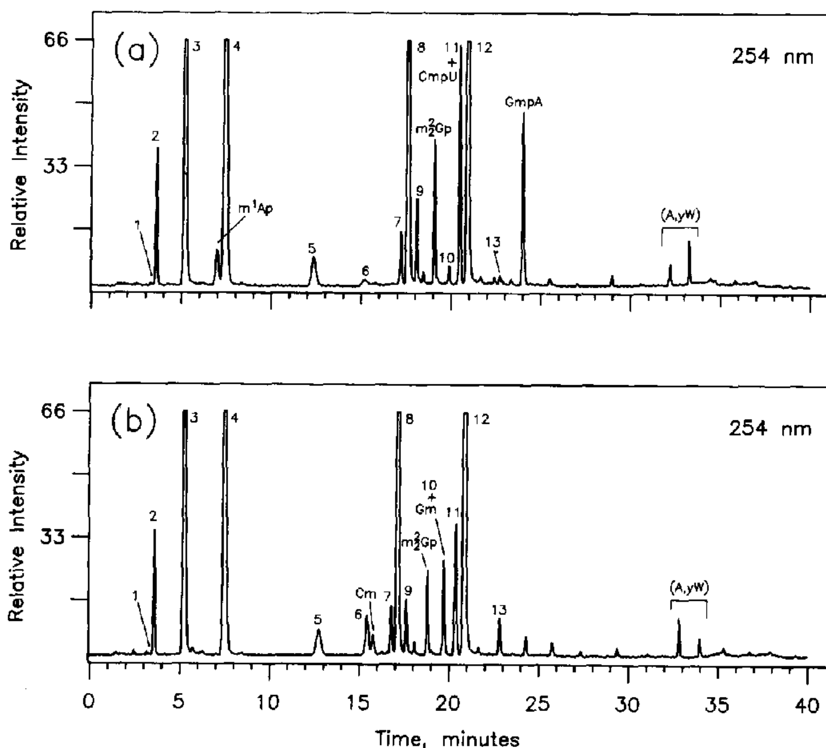


Fig. 1. (a) Chromatography of *S. cerevisiae* tRNA^{Phe} following digestion with RNase T_2 and alkaline phosphatase (reaction 1). UV detection at 254 nm. Numbers refer to nucleosides expected from the previously determined tRNA sequence [17]. Peaks: 1 = dihydrouridine; 2 = pseudouridine; 3 = cytidine; 4 = uridine; 5 = 5-methylcytidine; 6 = 1-methyladenosine; 7 = thymine riboside; 8 = guanosine; 9 = 7-methylguanosine; 10 = 1-methylguanosine; 11 = N^2-methylguanosine; 12 = adenosine; 13 = N^2,N^2-dimethylguanosine. Other components: m^{1}Ap = 1-methyladenosine 3′-phosphate; m$^2_2$Gp = N^2,N^2-dimethylguanosine 3′-phosphate; CmpU = 2′-O-methylcytidylyl-3′,5′-uridine; GmpA = 2′-O-methylguanosinyl-3′,5′-adenosine; (A,yW) = dinucleotide (sequence unspecified) composed of adenosine and wybutosine, yW. Unlabeled peaks are due to non-nucleoside UV-absorbing impurities or to traces of nucleosides at very low stoichiometric amounts arising from other isoaccepting tRNAs in the tRNA^{Phe} sample. (b) Chromatography of *S. cerevisiae* tRNA^{Phe} following digestion with nuclease P_1 and alkaline phosphatase (reaction 2). Cm = 2′-O-methylcytidine; Gm = 2′-O-methylguanosine. Other notations as in (a).

products of reactions 1 and 2, it is expected to observe dinucleotides of the type NmpN′ in Fig. 1a, but not in Fig. 1b. Conversely, sugar-methylated nucleosides (Cm and Gm in the present example) should not appear as products of reaction 1 (Fig. 1a), but are released by total hydrolysis represented in Fig. 1b. Comparison of the two chromatograms readily provides candidate peaks for testing by LC–

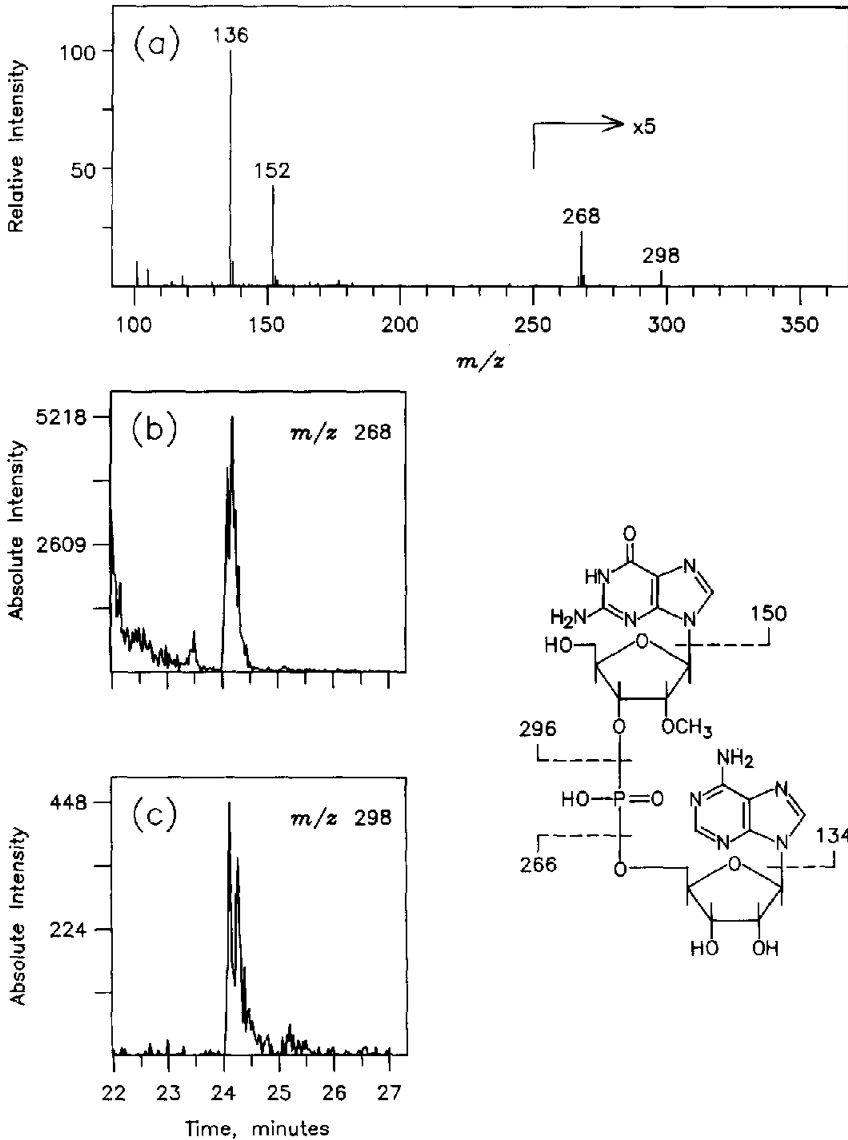


Fig. 2. (a) Thermospray mass spectrum of the dinucleotide GmpA eluting at 24.0 min in Fig. 1a. Cleavages shown are accompanied by net addition of two hydrogens to form the observed ions. (b,c) Selected ion profiles from scanned mass spectra over the range 22–27 min in Fig. 1a.

MS in the same experiment. These effects are observed in Figs. 1a and 1b as discussed below, but are accompanied by other abundance changes which could be misinterpreted in the absence of mass spectral data.

Increased amounts of the components eluting at 20.5 and 24.0 min in Fig. 1a compared with Fig. 1b reflect the failure to cleave the dinucleotides CmpU and GmpA, respectively. These assignments result from mass spectra of these peaks, which exhibit MH^+ and BH_2^+ ions of the constituent nucleosides, as shown for the dinucleotide GmpA in Fig. 2a: Gm (*m/z* 152, 298) and A (*m/z* 136, 268). In general, three or all four of the possible nucleoside peaks resulting from dissociation in the spray are usually observed for dinucleotides. Although MH^+ and sequence-related ions from thermospray have been reported for dinucleotides [19,20], the conditions required include microgram level quantities which are impractical for applications of the type reported here.

The required correspondence in elution times for ions in Fig. 2a can be tested by plotting of ion profiles, as indicated in Fig. 2b and c for *m/z* 268 and 298. Because the methyl group must reside in the 5′ residue, the sequence is thus defined as 5′-GmpA-3′.

Similarly, the mass spectrum of the 20.5-min eluate in Fig. 1a leads to the assignment CmpU (data not shown), which coelutes with N^2-methylguanosine, m^2G. Recognition of coeluates such as m^2G can be made principally from the fact that m^2G elutes at the expected retention time [8], while signals for Cm and U do not correspond to the times expected for the free nucleosides; the same observation holds for GmpA. Coeluates can also often be distinguished by slight differences in retention time when observed as selected ion profiles of the type shown in Fig. 2b and c. Additionally, as shown by the case of CmpU in Fig. 1a, the dinucleotide component is hydrolyzed by nuclease P_1, and so is absent in Fig. 1b, leaving only the peak (and corresponding mass spectrum) of m^2G (peak 11).

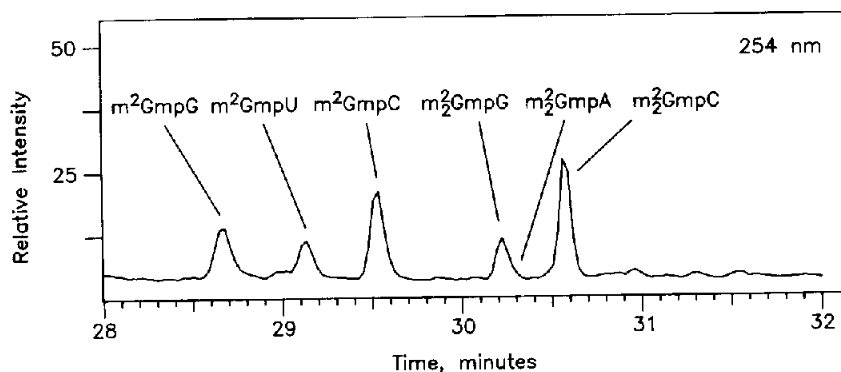
If the dinucleotides of the type NpN′ are formed, either inadvertently or by intentional partial hydrolysis, the component can usually be identified as a dinucleotide, but sequence context is lost. In the present example, this is shown by the pair of peaks eluting in the region 32–34 min in Figs. 1a and b. The mass spectra of these components show them to be dinucleotides composed of adenosine and the fluorescent yW (wye) nucleoside from position 37, but the sequences ApyW and yWpA cannot be distinguished by their mass spectra. Because the tRNA sequence is 5′-ApyWpA-3′ both nucleotides are therefore possible products; the yW nucleotide has been demonstrated to exhibit unusual resistance to nuclease digestion [21].

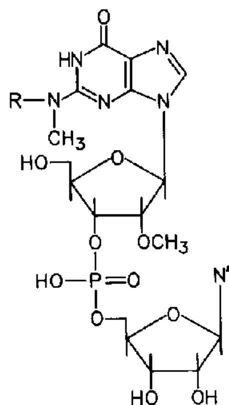
Several other features of Figs. 1a and 1b illustrate the formation of unexpected products. Incomplete dephosphorylation of the mononucleotides of 1-methyladenosine (m^1A) and N^2,N^2-dimethylguanosine (m_2^2G) in Fig. 1a leads to nucleotide peaks at 7.0 and 19.0 min, respectively. They are recognized by the presence of ions due only to one nucleoside in each peak (m^1A and m_2^2G), at retention times earlier than required for chromatography of the free nucleosides [9]. A

small amount of 1-methylguanosine is observed (peak 10, Fig. 1a and b), although it is absent in the published sequence. Although it may be derived from other isoaccepting tRNAs as impurities in the commercial sample of $tRNA^{Phe}$, it is more likely to be due to undermodification of wybutosine-37.

Dihydrouridine is a special case because it is essentially transparent at 254 nm due to absence of a significant chromophore in the base. It is readily recognized by its mass spectrum, which shows its elution on the leading edge of pseudouridine [7] (see peak 1, Fig. 1a and b).

Application of the presently described method to unfractionated tRNA leads to a significantly increased number of possible combinations of dinucleotides of the type NmpN′, so that less useful sequence information will generally result. However, for certain ribose-methylated nucleotides at highly conserved sequence locations, RNase T_2 hydrolysis will in principle produce a complete picture of the distribution of the corresponding 3′-nucleotide partners in all of the tRNAs pre-





Peak	R	N′
m^2GmpG	H	G
m^2GmpU	H	U
m^2GmpC	H	C
m^2_2GmpG	CH_3	G
m^2_2GmpA	CH_3	A
m^2_2GmpC	CH_3	C

Fig. 3. Chromatographic separation of dinucleotides formed from hydrolysis of unfractionated *P. occultum* tRNA by RNase T_2 and alkaline phosphatase (reaction 1). Structure: dinucleotides indicated in the above chromatogram, as deduced from their mass spectra and further hydrolysis (see text).

sent. An example is given by the occurrence of N^2,2′-O-dimethylguanosine (m^2Gm) and N^2,N^2,2′-O-trimethylguanosine (m^2_2Gm) which are likely to occur mainly at sequence position 26. Hydrolysis by reaction 1 of tRNA from the archaebacterium *P. occultum* yields the partial dinucleotide pattern shown in Fig. 3. The assignments shown were made from mass spectra of the constituent nucleosides as discussed above and verified in each case by isolation and further hydrolysis as described in the following section. It is noteworthy that the qualitative amounts of 3′ partners observed in Fig. 3 ($C > G > U > A$) is approximately the same as in the 27 published archaebacterial sequences in which a methylated guanosine occurs at position 26 [17].

Collection and hydrolysis of dinucleotides

When desired, additional evidence on dinucleotide structure can be gained by collection of the putative dinucleotides produced by reaction 1, followed by complete hydrolysis to nucleosides (reaction 2) and analysis by LC–MS. This procedure can be carried out on nanogram range quantities of dinucleotides, with the following advantages. (a) Chromatography of the product will yield retention times for the constituent nucleosides which can be compared with standard values [8,13], which is not possible using the approach represented in Fig. 2. When the quality of the chromatogram permits, characteristic 254/280 nm UV absorbance ratios can also be measured, although for nucleosides of known structure, retention time data and mass spectra are usually definitive. (b) Using standard molar response factors for UV absorbance (*e.g.* ref. 14), the 1:1 stoichiometry of the nucleosides released by reaction 2 can be verified. Although in our experience such measurements when carried out in the nanogram range are not highly accurate, they may be useful to clarify data when more than one component elutes with the dinucleotide and the identity of the 3′ residue is uncertain. (c) The resulting mass spectra of constituent nucleosides will generally be of higher quality than those produced as composites from gas phase dissociation of the dinucleotide.

An example of this approach is shown in Fig. 4, which results from collection and rehydrolysis of approximately 59 ng of the earliest eluting component shown in Fig. 3. The resulting hydrolysis products are clearly shown by retention times [8] (Fig. 4a) and mass spectra (*e.g.* Fig. 4b) to be guanosine and N^2,2′-O-dimethylguanosine, thus requiring the parent structure to be 5′-m^2GmpG-3′. The signal-to-noise ratios shown in Fig. 4b–d are typical for such quantities, estimated here as 25 ng of G and 27 ng of m^2Gm. The UV absorbance ratio observed in Fig. 4a for m^2Gm/G (as peak areas) is 1.03, compared with a standard response ratio of 1.09, taken from a similar chromatographic system [15] and using the absorbance value for m^2G rather than m^2Gm, since the chromophores are identical.

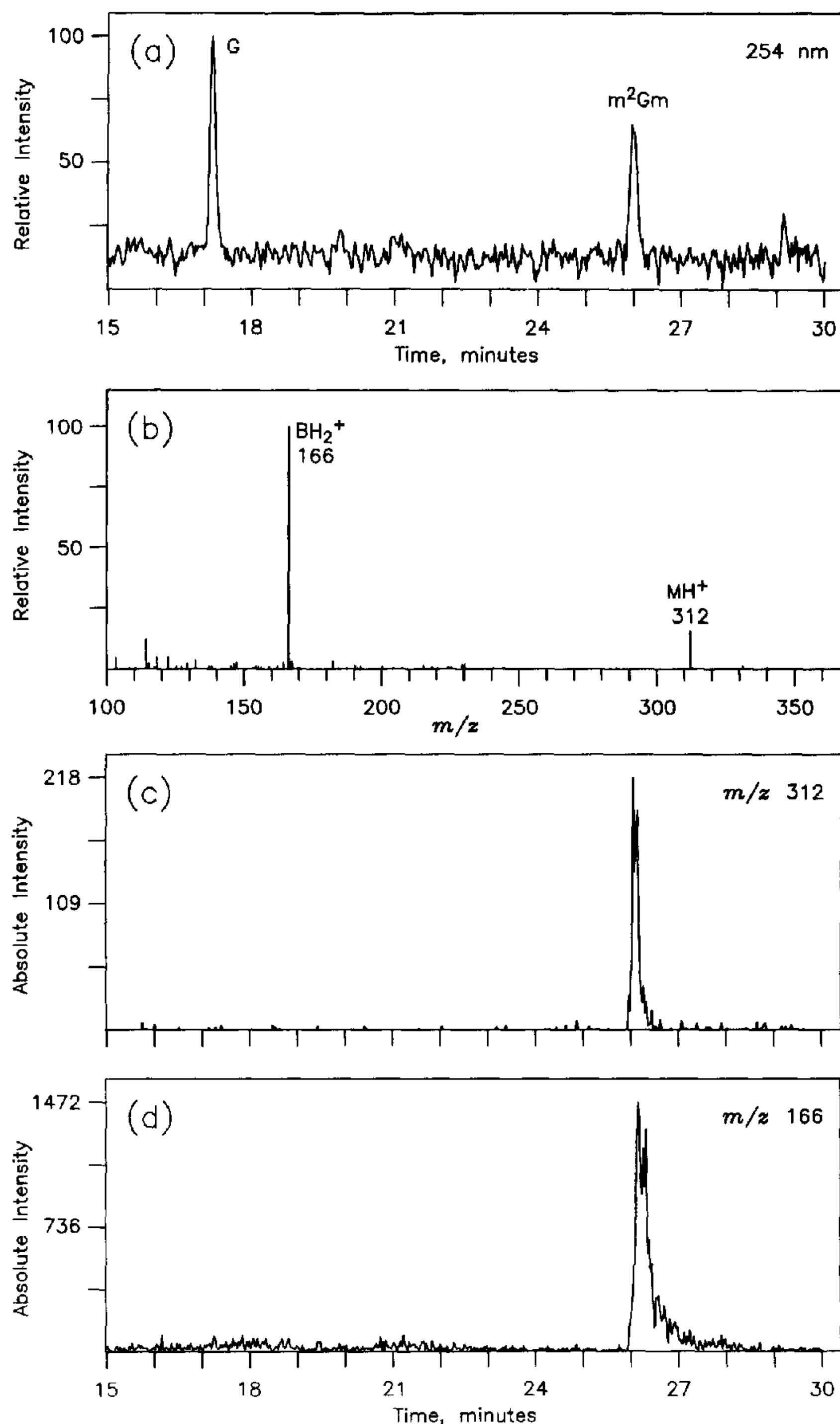


Fig. 4. LC–MS data of component eluting at 28.6 min in Fig. 3, following collection and hydrolysis by nuclease P_1 and phosphodiesterase. (a) Chromatogram, UV detection at 254 nm. (b) Thermospray mass spectrum of the component eluting at 26.0 min in panel (a). (c,d) Selected ion profiles for ions shown in panel (b) taken from scanned mass spectra over the range 15–30 min.

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