Chemical Modification of Polynucleotides. Quantitative Studies of Polycytidylic Acid by Nuclear Magnetic Resonance Spectroscopy and Secondary-Ion Mass Spectrometry

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Chemical modification of polycytidylic acid with methyl methanesulfonate has been studied by ^{13}C NMR of the intact polymer, ^{1}H NMR of the polymer, and ^{1}H NMR, high-pressure liquid chromatography, and secondary-ion mass spectrometry (SIMS) of the mononucleosides following enzymatic degradation. The only site of modification is at the 3-position of cytidine. The extent of modification is quantitatively determined by ^{14}C incorporation, integration analysis of NMR signals, HPLC peaks, and SIMS, using [*methyl*-$^{2}H_3$]-3-methylcytidine as an internal standard. All analyses give consistent quantitative results.

As part of our continuing efforts to investigate the chemical modification of nucleic acids,[2-4] quantitative analysis of the reaction of polycytidylic acid with methyl methanesulfonate (MeMS) was undertaken. While many chemical methods of analysis,[5,6] are available for measuring the distribution of alkylated products, many if not all methods possess certain undesirable attributes which might lead to questionable conclusions as to the degree of reaction or distribution of products, usually as a result of chemical alterations of primary reaction products during analysis. ^{13}C NMR analysis[2-4] at the polynucleotide level, high-pressure liquid chromatography (HPLC)[7] which requires degradation to mononucleotides, mononucleosides, or bases,[8] and mass spectrometry[9,10] appear to be especially promising methods for qualitative and quantitative determinations.

The use of synthetic homopolynucleotides provides a useful model on which to obtain information on the chemical modification of nucleic acids.[11,12] Because the number of reactive sites is much smaller in these homopolymers, the identification and quantitation of the products by degradative and nondegradative methods are greatly facilitated. They allow us to critically evaluate the feasibility and reliability of different approaches for studying chemical modification of polynucleotides. In the case of polycytidylic acid, a number of sites are available for modification, including N_3, N_4, O_2, $O_{2'}$, and the phosphate oxygen. With methyl methanesulfonate (MeMS) as the alkylating agent, 3-methylcytidine (m^3C) has been shown to be the only methylated product,[13,14] thus providing a simple system for comparing the different methods of product analysis.

In this study we chose to evaluate the extent of the specific methylation induced in polycytidylic acid by comparing ^{14}C incorporation, ^{13}C NMR, and ^{1}H NMR of the polymer. After enzymatic degradation to mononucleosides the degree of modification was further analyzed by ^{1}H NMR, HPLC, and secondary-ion mass spectrometry (SIMS),[15] using the internal standard method of quantitative analysis.

Results and Discussion

Two sets of experiments were used to compare the several methods of analysis. In reaction 1, 0.269 mmol of poly C was allowed to react with 0.342 mmol of [^{12}C]MeMS at pH 7 for 24 h. The degree of methylation was then determined by ^{13}C NMR and ^{1}H NMR of the polymer and, after enzymatic degradation, HPLC and secondary-ion mass spectrometry of the mononucleosides produced from enzymatic degradation. In order to further compare with the traditional radioisotope labeling method, we performed reaction 2 in which 0.264 mmol of poly C was reacted with 0.618 mmol of [*methyl*-^{14}C]MeMS at pH 7 for 12 h. The percentage of methylation after this reaction was then determined by ^{14}C incorporation, ^{1}H NMR of the mononucleosides, and HPLC of the mononucleosides.

^{13}C NMR. Nuclear magnetic resonance has been a sucessful tool for probing the structure of biopolymers, including nucleic acids.[16-18] With the advent of the pulsed Fourier-transform technique, the sensitivity of carbon-13 magnetic resonance spectrometry has been greatly enhanced.[19,20] The great advantage of ^{13}C NMR over other methods is that the sites and the degree of modification can usually be directly determined without acid, base, or enzymatic degradation followed by isolation, separation, and purification of the resulting molecular fragments prior to analysis. This avoids any artifacts that might be generated during degradation such as deamination, glycosyl

(1) The following abbreviations are used: m^3C, 3-methylcytidine; C, cytidine; poly C, polycytidylic acid; MeMS, methyl methanesulfonate; NMR, nuclear magnetic resonance; HPLC, high-pressure (performance) liquid chromatography; SIMS, secondary-ion mass spectrometry.

(2) Chang, C.-j.; Lee, C. G. *Arch. Biochem. Biophys.* **1976**, *176*, 801.
(3) Chang, C.-j.; Lee, C. G. *Cancer Res.* **1978**, *38*, 3734.
(4) Chang, C.-j.; DaSilva Gomes, J.; Byrn, S. R. *J. Am. Chem. Soc.* **1981**, *103*, 2892.
(5) Lawley, P. D. Screening Tests in Chemical Carcinogenesis 1976, International Agency for Research on Cancer, Lyon, France, 1976.
(6) Ehrenpreis, S. *Drug Res.* **1970**, *14*, 59.
(7) Wehr, C. T. "Nucleic Acid Constituents by High Performance Liquid Chromatography"; Varian Associates, Inc.: Palo Alto, CA, 1980.
(8) Jensen, D. E.; Reed, D. J. *Biochemistry* **1978**, *17*, 5098.
(9) Schoen, A. E.; Cooks, R. G.; Wiebers, J. C. *Science* **1979**, *203*, 1249.
(10) Fenselau, C.; Kan, M. N.; Sack, G. H. *Adv. Mass Spectrom.* **1978**, *7*, 1572.
(11) Singer, B.; *Prog. Nucl. Acid Res. Mol. Biol.* **1975**, *15*, 219.
(12) Ludlum, D. B.; Kramer, B. S.; Wang, J.; Fenselau, C. *Biochemistry* **1975**, *14*, 5480.
(13) Ludlum, D. B.; Wilhelm, R. C. *J. Biol. Chem.* **1968**, *243*, 2750.
(14) Ludlum, D. B. *Mol. Pharmacol.* **1966**, *2*, 585.
(15) Day, R. J.; Unger, S. E.; Cooks, R. G. *Anal. Chem.* **1980**, *52*, 557A.
(16) Bovey, F. A. "High Resolution NMR of Macromolecules"; Academic Press: New York, 1972.
(17) Dwek, R. A. "Nuclear Magnetic Resonance in Biochemistry"; Clarendon Press: Oxford, 1973.
(18) Danchin, A. *Biochemie* **1975**, *57*, 445.
(19) Dorman, D. E.; Roberts, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 19.
(20) Chenon, M. T.; Pugmire, R. J.; Grant, D. M.; Panzica, R. P.; Townsend, L. B. *J. Am. Chem. Soc.* **1975**, *97*, 4627.

0022-3263/81/1946-4770$01.25/0

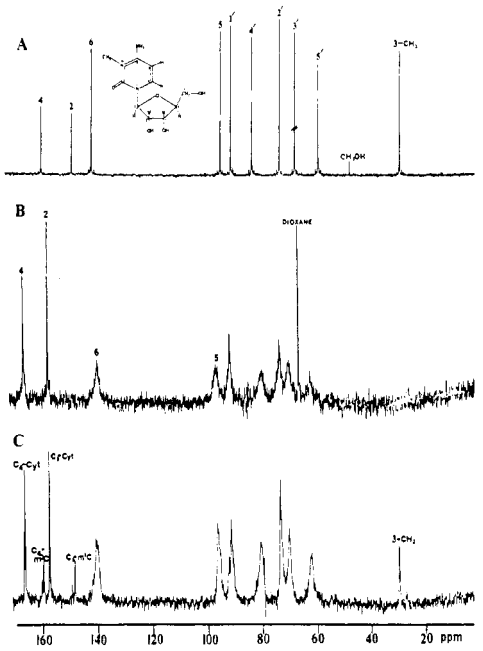

Figure 1. Natural-abundance proton-decoupled ^{13}C NMR spectra of (A) 3-methylcytidine, (B) polycytidylic acid, (C) polycytidylic acid after alkylation with methyl methanesulfonate.

bond cleavage, or loss of alkyl substituents in the case of oxygen alkylations. On the other hand, this method has been criticized as being very time consuming, requiring sometimes several days to obtain an adequate signal-to-noise ratio. This disadvantage can be minimized by the use of ^{13}C-enriched alkylating agents.

Figure 1A shows the ^{13}C natural abundance spectrum of m^3C, indicating the 3-methyl resonance at 30.7 ppm. Figures 1B and 1C are the natural abundance spectra of poly C (97 mg, 0.269 mmol) before and after reaction with 0.342 mmol of MeMS for 24 h (reaction 1). The signal at 30.4 ppm can be assigned to the methyl resonance of m^3C based on model studies with the N_4, O_2, $O_{2'}$, and phosphate methyl resonances occurring at 27.5, 57.5, 58.4, and 53.3 ppm, respectively. The percentage of m^3C present in the polymer was determined by dividing the integral for the C_2 resonance of m^3C (147.4 ppm) by the total integral for the C_2 resonances of m^3C and cytidine (156.4 ppm). This showed that 22.2% of the nucleotides in the polymer were m^3C.

^1H NMR. While ^1H NMR has been used for analysis of high molecular weight polymers, the spectra often suffer from poor resolution of the individual resonances primarily due to the chemical shift anisotopic effect caused by the secondary and tertiary structures of polymers.[16] The line-broadening phenomenon of polymers is mainly attributed to the short spin–spin relaxation time.[16] This problem can be partially eliminated by using high-field spectrometers (in our case 470 MHz). Figure 2A is the ^1H NMR spectrum of the polymer (5 mg) after reaction 1. The H_6 of m^3C appears as an unresolved doublet at 8.1 ppm. H_6 of cytidine occurs as a resolved doublet at 7.9 ppm. Integration of these areas showed the m^3C to be 22.7%. The H_5 of m^3C occurs at 6.3 ppm and H_5 of cytidine at 5.9 ppm. Analysis of this region is less desirable due to the partial overlap with the $H_{1'}$ signals.

Figure 2B is the 470-MHz ^1H spectrum of the same sample measured following enzymatic degradation in Tris buffer solution (pH 7.3) with ribonuclease A, snake venom phosphodiesterase, and alkaline phosphatase for 12 h. Removal of the enzymes was not necessary due to their low concentration (see Experimental Section) and only the

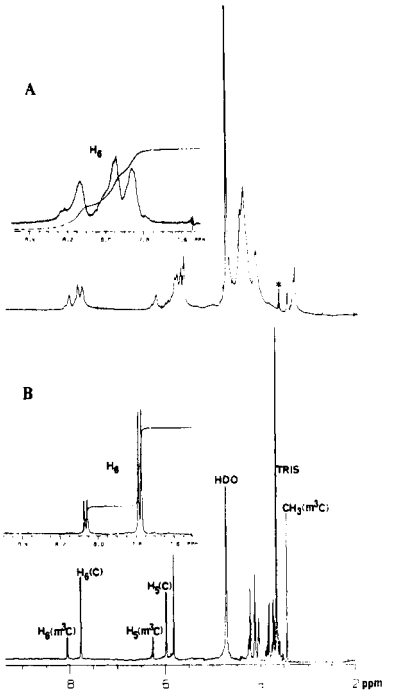

Figure 2. ^1H NMR spectra of (A) polymer of polycytidylic acid after reaction with methyl methanesulfonate, (B) after enzymatic degradation. Inserts of region of H_6 signal with integration. (*) Unknown impurity.

Tris signal appears in the spectrum at 3.65 ppm. This spectrum shows obvious improvement in resolution over Figure 2A. All aromatic protons are clearly resolved with H_6 of m^3C at 8.04 ppm, H_6 of cytidine at 7.75 ppm, H_5 of m^3C at 6.25 ppm, and H_5 of cytidine at 5.97 ppm. Furthermore $H_{1'}$ of m^3C and cytidine coincide as a doublet at 5.81 ppm. The 3-methyl peak of m^3C appears as a singlet at 3.42 ppm. Integral analysis of the proton ratios of H_5, H_6, $H_{1'}$, and CH_3 gave the percentage of m^3C present. These values gave an average value of 22.3% of m^3C present. We were also interested at this stage whether enzymatic degradation at pH 8.2 resulted in any deamination of m^3C to m^3U. Proton NMR, thin-layer chromatography, and SIMS, using authentic 3-methyluridine as a reference, showed that such deamination is insignificant.

^1H NMR analysis of the mononucleosides of reaction 2, in which ^{14}C incorporation (reaction 2) showed the percentage of m^3C to be 11.5, showed the ratio of H_6 (m^3C)/H_6 (m^3C + C) to be 11.7% and the ratio of H_6 (m^3C)/$H_{1'}$ (m^3C + C) to be 11.8%.

HPLC. In recent years, high-pressure liquid chromatography has found widespread use in the separation of complex reaction products[21] and in the determination of product distributions from small amounts of reaction mixtures. While this method still requires degradation of polynucleotides prior to analysis, and the availability of reference compounds, once a suitable stationary and mobile phase have been chosen, complete analysis of the products can often be achieved in less than 10 min.

Prior to HPLC analysis 5 mg of the methylated polynucleotide was enzymatically degraded (as described earlier) and 9.29×10^{-6} mmol of the mononucleoside mixture injected. A clean separation of m^3C (t_R 0.82 min) and cytidine (t_R 1.9 min) is observed. Integration of the peak areas was performed with a Waters data module and each component quantitatively determined by using cal-

(21) Kuo, K. C.; McCune, R. A.; Gehrke, C. W. *Nucleic Acids Res.* **1980**, *8*, 20, 4763.

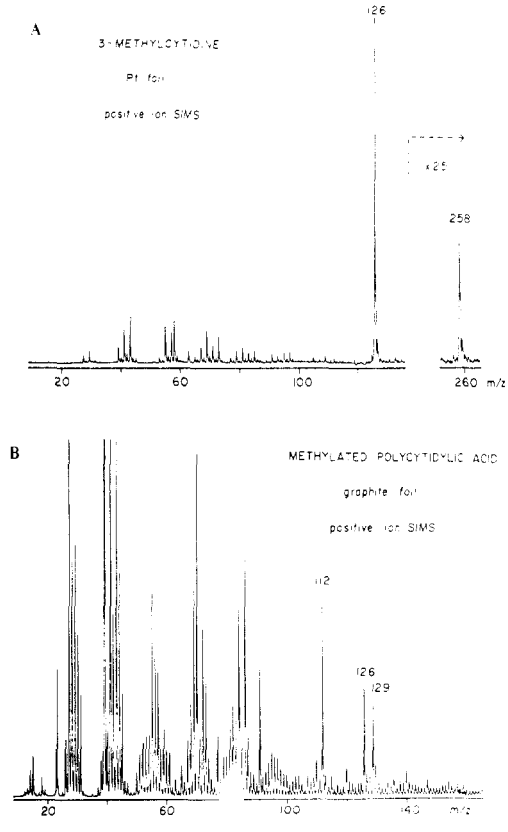

Figure 3. Secondary-ion mass spectra (SIMS) of (A) 3-methylcytidine and (B) methylated polycytidylic acid after enzymatic degradation in the presence of the internal reference [*methyl*-$^2H_3$]m^{3}C.

ibration curves for m^{3}C (correlation coefficient 0.999) and cytidine (0.998) before analysis and then subjecting the peak areas for the methylation reaction m^{3}C and cytidine to least-squares analysis. Five injections were made, and analysis showed a mean value of 21.4% m^{3}C with a standard deviation of ±0.7. The enzymatic degradation products of reaction 2 were also separated by HPLC. Least-squares analysis showed the percentage of m^{3}C to be 12.2 with a standard deviation after three injections of ±0.5.

SIMS. Secondary-ion mass spectrometry has been used to obtain mass spectra of many biologically important compounds, including nucleosides,[22] sugars, and amino acids.[23] Results presented here demonstrate the characterization of methylated nucleosides and the quantitation of alkylated polynucleotides by SIMS. The choice of SIMS over other ionization methods is based on the capabilities established in the accompanying paper, viz., low detection limits, ease of sample preparation, and applicability to involatile compounds. The spectra obtained by SIMS closely resemble those obtained by other methods which ionize directly from the solid (such as laser desorption and plasma desorption). Figure 3A demonstrates the ability of SIMS to provide an excellent mass spectrum of a methylated nucleoside (ca. 1 μg). Ion bombardment (Ar^+, 5 keV, 1×10^{-9} A/cm^2) of 3-methylcytidine on a Pt support generates both the molecular ion of the intact nucleoside at *m/z* 258 and the 3-methylcytosine ion at *m/z* 126 by cleavage of the ribose sugar. Essentially no other fragmentation is observed; however, spectra recorded at higher primary ion fluxes show NH_3 loss at *m/z* 109.

(22) Liu, L. K.; Busch, K. L.; Cooks, R. G. *Anal. Chem.* **1981**, *53*, 109.
(23) Benninghoven, A.; Sichtermann, W. K. *Anal. Chem.* **1978**, *50*, 1180.

Table I. Summary of 3-Methylcytidine Percentages for Various Analytical Methods

reaction	method	% m^{3}C
1	^{13}C NMR (polymer)	22.2
1	^{1}H NMR (polymer)	22.7
1	^{1}H NMR (monomers)	22.3
1	HPLC (monomers)	21.4
1	SIMS (monomers)	22.0
2	^{14}C incorporation (polymer)	11.5
2	^{1}H NMR (monomers)	11.8
2	HPLC (monomers)	12.2

Ion bombardment of ca. 1 μg of a mixture (10.55 mg) of enzymatically degraded methylated polycytidylic acid (reaction 1) in the presence of [*methyl*-$^2H_3$]m^{3}C generated molecular secondary ions at *m/z* 112, 126, and 129 (Figure 3B). Protonated cytosine is observed at *m/z* 112; m^{3}C and [*methyl*-$^2H_3$]m^{3}C are seen at *m/z* 126 and 129, respectively. Since the spectra are not corrected for background, many lower mass ions are observed which correlate with common background species. On the basis of the values of ^{13}C and ^{1}H NMR and HPLC analysis, the predicted ratio of the peaks due to the bases, viz., m^{3}C/[*methyl*-$^2H_3$]m^{3}C, would be 1.23 to yield a percentage of m^{3}C in the methylated polymer of 22.3. Repetitive scans of the 120–140-amu region for each of three identically prepared samples resulted in a $126^+/129^+$, viz., m^{3}C/[*methyl*-$^2H_3$]m^{3}C, ratio of 1.22 ± 0.17. This value then yielded 22% of m^{3}C in the methylated polycytidylic acid. Quantitation by SIMS may be performed on as little as 50 ng of the modified base provided an equal or larger amount of internal standard is added.

Conclusion

Table I summarizes the values obtained for the percentage of 3-methylcytidine present in each methylated polynucleotide as determined by the methods of analysis employed here. The average values for reaction 1 was found to be 22.1%. The ^{14}C incorporation data included in the analysis of reaction 2 showed the percentage of m^{3}C to be 11.8. All methods employed here appear to give consistent results, indicating their reliability for quantitative analysis of chemical modification of polynucleotides. NMR methods can often afford direct determination of the sites of reaction and the product distribution without going through degradation and separation. These are probably among the best approaches for in vitro studies. However they require relatively large amounts of sample which is not always available, especially in the in vivo reactions. Other methods are more sensitive but require degradation prior to analysis. In order to avoid any decomposition and rearrangement of primary reaction products, it is essential to fully understand the exact reaction mechanisms and chemical properties of these reaction products.

The use of mass spectrometry for quantitative analysis is especially attractive due to its extreme sensitivity. Coupled with mass spectrometry/mass spectrometry,[9] SIMS may be particularly useful in characterizing and quantitating nanogram levels of more complex alkylated polynucleotides. The combination of MS/MS with SIMS ionization would not only eliminate much of the chemical noise observed in SIMS spectra but substantially decrease analysis time. A similar approach involves the coupling of a liquid chromatographic system to a SIMS instrument[24] for separation of components prior to the ionization ev-

(24) Benninghoven, A.; Eicke, A.; Junack, M.; Sichtermann, W.; Crizek, J.; Peters, H. *Org. Mass. Spectrom.* **1980**, *15*, 459.

ent.[25] The use of deuterated internal reference has allowed accurate quantitation of the extent of reaction and served as a carrier to minimize the loss during transferring a minute amount of sample. Furthermore, it is also eliminates the possible errors resulting from partial decomposition due to chemical or enzymatic degradation.

Experimental Section

Synthesis of 3-Methylcytidine (m^3C) and [*methyl*-$^2H_3$]-3-Methylcytidine. Cytidine (200 mg, 0.82 mmol) was dissolved in 3 mL of *N,N*-dimethylacetamide (DMA). To this solution was added 3.21 mmol of methyl iodide (either CH_3I or CD_3I) in 2 mL of cold DMA. The reaction was run at room temperature and monitored by TLC on silica (2-propanol/acetic acid/H_2O, 12:1:4, R_f cytidine 0.54, m^3C 0.43). After 2.4 h the reaction mixture was transferred to a 50-mL flask and the DMA was removed under vacuum (10 μm) at room temperature. The remaining syrup was dissolved in 2 mL of absolute methanol and chloroform added to induce turbidity. After 24 h at −20 °C the crystalline material was obtained (99% yield): ^{13}C NMR (D_2O) δ 159.4 (C_4), 148.5 (C_2), 141.5 (C_6), 95.2 (C_5), 91.5 ($C_{1'}$), 84.0 ($C_{4'}$) 74.2 ($C_{2'}$), 68.7 ($C_{3'}$), 60.2 ($C_{5'}$), 30.7 (3-methyl), 49.0 (CH_3OH reference).

^1H NMR (D_2O) δ 8.00 (d, 8.0 Hz, H_6), 6.18 (d, 8.0 Hz, H_5), 5.75 (d, 3.2 Hz, $H_{1'}$, 4.20 (dd, 4.8, 3.2 Hz, $H_{2'}$), 4.05 (dd, 6.7, 4.8 Hz, $H_{4'}$), 4.03 (ddd, 6.7, 3.9, 2.5 Hz, $H_{4'}$), 3.82 (dd, 12.9, 2.5 Hz, $H_{5'}$), 3.68 (m, 12.9, 3.9 Hz, $H_{5'}$), 3.37 (s, CH_3), 2.04 (acetone-d_6 reference); mp 182–185 °C (iodide salt). Anal. Calcd for [*methyl*-$^2H_3$]-3-methylcytidine: C, 30.94; N, 10.83; $^1H + {}^2H$, 4.94; I, 32.69. Found: C, 30.93; N, 11.00; $^1H + {}^2H$, 4.89; I, 32.60.

Alkylation Procedure. Polycytidylic acid (P.L. Biochemicals) was purified prior to reaction by dialysis (Spectrapor 4 membranes, molecular weight cutoff 12 000–14 000) against double distilled water, pH 7, at 4 °C until no UV-absorbing material (λ_{max} 268 at pH 7) was present in the dialysis water and then lyophilized. Both alkylations were performed in a Radiometer ETS822 pH stat at pH 7, maintaining the pH value by addition of 0.125 N NaOH.

Reaction 1. Poly C (0.269 mmol, 97.0 mg) was weighed and added to the reaction vessel. Three milliliters of double distilled water (pH 7) was then added and 26.4 μL of MeMS (0.342 mmol) added. Reaction was allowed to continue for 24 h at which time the contents of the reaction vessel was transferred to a dialysis bag and dialyzed for 12 h at 4 °C, changing the 100 mL of double distilled water (pH 7) each hour. The contents of the dialysis bag was then lyophilized. All dialysis solutions were concentrated and the total loss of poly C, as determined by UV_{268}, was found to be 0.877 mg (0.9%).

Reaction 2. Poly C (0.264 mmol, 95.4 mg) was weighed and added to the reaction vessel. Five milliliters of double distilled water (pH 7) was then added and 0.618 mmol of [$^{12}C/^{14}C$]MeMS (sp act. 2.59 μCi/mM) was added. Reaction continued for 12 h at which time the contents of the reaction vessel was dialyzed until no radioactivity was detected in the dialysis water (12 h). The ^{14}C incorporation was determined by analysis of 3–20-μL samples of the final 10-mL volume on a Beckman LS 7000 liquid scintillation counter and the remainder was lyophilized.

Enzymatic Degradation. Methylated poly C (5 mg) was dissolved in 0.5 mL of 0.1 M Tris buffer, pH 7.3. Ribonuclease A (60 units, Sigma Chemical Co., R-4875) was added in 0.2 mL of H_2O and allowed to react 1 h at 37 °C in a shaker bath. Snake venom phosphodiesterase (0.04 unit; Sigma Chem. Co., P-6761) and 3.06 units of alkaline phosphatase (Sigma Chem. Co., P-4252) in 0.05 mL of Tris buffer were then added. Degradation was complete after 12 h as determined by TLC (2-propanol/acetic acid/water, 12:1:4) on silica (R_f m^3C 0.43, C 0.54, poly C 0.0).

^{13}C NMR. The natural abundance ^{13}C spectra reaction 1 (97 mg) was obtained on a JEOL PFT-100 spectrometer operating at 25.0 MHz and interfaced with a JEOL EC-100 Fourier-transform computer with a 20K memory. The spectra were recorded at ambient temperature with a deuterium lock; the chemical shifts were measured for a 5-KHz sweep width. The pulse width was 22.2 μs (90° pulse) and the repetition time was 2 s. All proton resonances were decoupled by a broad-band (2.5 kHz) irradiation from an 99.99-MHz source.

^1H NMR. All proton spectra were run in D_2O on an NTC-470 instrument operating at 470 MHz. Five-microsecond pulse angles and 5-s pulse delay were used with a total spectral width of 5 kHz. Each sample contained an average of 5 mg either as the polymer or mononucleosides, which were subjected to three deuterium exchanges prior to the measurements. Chemical shifts were referenced to acetone (2.04 ppm), which was added after acquiring the initial spectra.

HPLC. Analysis was performed on a Waters Model 6000 A solvent delivery system with a Model 440 absorbance detector interfaced with a Waters data module. Samples were analyzed directly following enzymatic degradation, injecting 25.0 μL each time onto a Waters 10 μ RC-C_{18} column. Mobile phase was 5% methanol in 0.004 M $NH_4H_2PO_4$ and 0.0009 M tetrabutylammonium chloride (flow rate, 3 mL/min; pressure, 1500 psi; chart speed, 1.5 cm/min).

SIMS. The internal reference [*methyl*-$^2H_3$]m^3C was synthesized as stated above and the exact molecular weight of the compound, 388.177, existing as the iodide salt was verified by elemental analysis.

Analysis of reaction 1 was performed by weighing 10.55 mg (.03 mmol) of the methylated polymer. The average molecular weight of the partially methylated mononucleotides, 341.44, was determined by ^1H NMR, taking into account that at pH 7, the pH at which the alkylation was performed, 90.2% of the phosphates is ionized (pK_a = 6.04) and exist as the sodium salt (NaOH used to maintain pH 7 during alkylation and dialysis). Addition of 5.35×10^{-3} mmol of [*methyl*-$^2H_3$]m^3C followed by enzymatic degradation gave a mixture of the mononucleosides of cytidine, m^3C, and [*methyl*-$^2H_3$]m^3C. The degraded sample was then adsorbed from degradation solution onto a graphite or Pt support and introduced into the secondary-ion mass spectrometer. A commercial Riber system, which has been described previously,[14] was used to obtain SIMS spectra. An incident primary ion beam (Ar^+, 5 keV, 1×10^{-9} A/cm^2) generates molecular secondary ions of the degraded, alkylated polycytidylic acid. These ions were subjected to mass analysis, using a Riber SQ 156 quadrupole mass filter, and detected with a Galileo Channeltron 4830 electron multiplier, and the resulting signal was processed by a Princeton Applied Research 1121 amplifier/discriminator. Spectra are not corrected for background.

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(25) Unger, S. E.; Vincze, A.; Cooks, R. G.; Chrisman, R.; Rothman, L. D. *Anal. Chem.*, in press.