Published in final edited form as:

Bioorg Med Chem. 2008 October 1; 16(19): 8795–8800. doi:10.1016/j.bmc.2008.09.016.

Synthesis and solution conformation studies of the modified nucleoside *N*⁴, 2'-*O*-dimethylcytidine (m⁴Cm) and its analogues

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Abstract

The dimethylated ribosomal nucleoside m⁴Cm and its monomethylated analogues Cm and m⁴C were synthesized. The conformations (*syn* versus *anti*) of the three modified nucleosides and cytidine were determined by CD and 1D NOE difference spectroscopy. The ribose sugar puckers were determined by the use of proton coupling constants. The position of modification (*e.g.*, *O* versus *N* methylation) was found to have an effect on the sugar pucker of cytidine.

1. Introduction

Nucleotide modification is a characteristic feature of most ribosomal and transfer RNAs. More than 100 different modified nucleotides are found in various RNAs.¹ The most frequent modifications are pseudouridylation, base methylation, and 2'-*O*-sugar methylation.² These alterations to the nucleoside bases or ribose moieties may enhance the diverse structural or functional properties of RNA.³ The ribosome is the molecular machinery for protein synthesis in all domains of life.⁴ Ribosomal RNAs (rRNAs) contain a wide range of modified nucleotides, which are present near or at the key functional sites of the ribosome.⁵ The modified cytidine m⁴Cm (*N*⁴, 2'-*O*-dimethylcytidine) is a rare nucleoside, in which both the base and sugar are methylated. To date, m⁴Cm has only been found in bacterial rRNA.^{1, 6–9} It is located in one of the most important functional sites of the small subunit 16S rRNA, the decoding region, which is involved in decoding the messenger RNA (mRNA) through interactions with mRNA, transfer RNAs (tRNAs), and the large subunit 23S rRNA.^{10–12} A number of known antibiotics also bind to the decoding region.¹³

There are three methylated nucleotides in the decoding region of bacterial 16S rRNA, namely m⁴Cm, 5-methylcytidine (m⁵C), and 3-methyluridine (m³U) at positions 1402, 1407, and 1498, respectively (*E. coli* numbering) (Figure 1, a-c).⁹ The nucleoside m⁴Cm was isolated from *E. coli* and characterized by Nichols and Lane in 1966.^{14, 15} The methyltransferase enzyme for *E. coli* m⁴Cm1402 in 16S rRNA is currently unknown.

Two of the decoding region modified nucleosides, m³U and m⁵C, are commercially available.¹⁶ In contrast, m⁴Cm is not available from commercial sources, and only two chemical syntheses of this modified nucleoside have been reported.^{17, 18} The Robins and Naik method requires only a few steps, but has relatively low overall yields (20% in four steps). The Nyilas and Chattopadhyaya method is facile (five steps) and can be carried out on a large scale with higher yields (~35% overall), but has one major drawback. This synthesis employs the

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relatively expensive TIPDSiCl₂ reagent for 3', 5'-*O*-ribose protection. For detailed structural studies of m⁴Cm, as well as its monomethylated analogues, N⁴-methylcytidine (m⁴C) and 2'-*O*-methylcytidine (Cm) (Figure 1, e and f), an inexpensive and versatile chemical method was desired in order to synthesize large quantities of the modified cytidine nucleosides and incorporate them into RNA. Cm is relatively expensive and m⁴C is not commercially available, although several synthetic methods have been reported.^{17–23} Thus, the other rationale for developing a new synthetic route for m⁴Cm was to generate the intermediates of interest, m⁴C and Cm. Herein, we report on the synthesis of m⁴Cm, m⁴C, and Cm using a convenient method for the generation of all three modified cytidine residues. One-dimensional ¹H NMR spectroscopy was used to determine the solution conformations of m⁴Cm, m⁴C, Cm, and cytidine. A comparison of the solution conformations of m⁴Cm and its analogues provides insight into the possible roles of the modifications at the RNA level.

2. Results and Discussion

2.1 Synthesis of N⁴, 2'-*O*-dimethylcytidine and its analogues

Uridine was converted to the intermediate 2,2'-anhydrouridine, **1**, and then reacted with magnesium methoxide to generate 2'-*O*-methyluridine, **2**, using a method developed by Roy and Tang (79% yield for two steps; Scheme 1).^{24, 25} Compound **2** was acetylated using catalytic amounts of DMAP in pyridine with acetic anhydride to afford 3', 5'-diacetyl-2'-*O*-methyluridine, **3**, in 95% yield.²⁶ The C-4 position of methyluridine was then activated with tetrazole using the method developed by Reese and Ubasawa to give intermediate **4** in 95% yield.^{27, 28}

The tetrazoyl derivative **4** can be substituted with a variety of nucleophiles to generate a range of cytidine analogues. Methylamine was employed as the nucleophile to give 3', 5'-diacetyl-N⁴, 2'-*O*-dimethylcytidine, **5**, in 95% yield. Removal of the acetyl protecting groups by treatment with 2.0 M NH₃ in methanol yielded N⁴, 2'-*O*-dimethylcytidine, **6**, in 99% yield. The overall yield of m⁴Cm by this method (Scheme 1) was 67% in six steps from uridine. The reagents used in this procedure are relatively inexpensive, and the synthesis can be carried out on a multigram scale. This method is versatile in that it produces intermediates for other modified nucleotide syntheses.

Ammonium hydrochloride was used as the nucleophile (Scheme 1, vii) in place of methylamine hydrochloride (step v) to generate 3', 5'-diacetyl-2'-*O*-methylcytidine, **7**, followed by removal of the acetyl groups with 2.0 M NH₃ in methanol to give 2'-*O*-methylcytidine, **8**, in 99% yield.

To generate m⁴C (Scheme 1), uridine was acetylated using catalytic amounts of DMAP in pyridine with acetic anhydride to afford 2', 3', 5'-tri-O-acetyl uridine, **9**, in 94% yield. Compound **9** was reacted with tetrazole (step x) to give intermediate **10** in 95% yield. Methylamine was employed as the nucleophile (step xi) to produce 2', 3', 5'-tri-*O*-acetyl, N⁴-methylcytidine, **11**, in 95% yield. Deprotection of the acetyl groups was carried out by treatment with 2.0 M NH₃ in methanol to yield N⁴-methylcytidine, **12**, in 99% yield. The overall yield was 84% in four steps from uridine.

2.2. Circular dichroism studies

The nucleoside base can exist in two main conformations relative to the ribose sugar due to rotation about the glycosidic C1'-N1 bond, referred to as the *anti* and *syn* conformers (Figure 2).^{29, 30} Circular dichroism (CD) spectroscopy can be used to determine the solution conformations of nucleosides.³¹ The m⁴Cm and its analogues all have peak maxima at 271 nm. This feature indicates that they all prefer the *anti* conformation in solution (Figure 3). There are subtle effects on the solution conformation of cytidine nucleoside due to methylation,

which is evident by slight differences in the CD spectra of cytidine and the three analogues. The CD spectra of cytidine is consistent with prior work by Miles *et al.*, even though the solvents in that study were different.^{31, 32}

2.3. One-dimensional ¹H NMR studies

Nuclear magnetic resonance (NMR) spectroscopy can be used to determine the solution conformations of nucleosides.^{33–35} In particular, 1D ¹H NOE experiments are valuable for examining relative proton positions and deducing the *anti* or *syn* conformations of the nucleoside.^{33, 34} The distance between two protons is inversely proportional to the magnitude of the NOE signal; therefore, relative proton-proton distances in the nucleoside can be determined (Figure 2). In an *anti* conformation, the base H6 (pyrimidine) or H8 (purine) protons are closest to the sugar H2' and H3' protons; therefore, irradiation of pyrimidine H6 gives a strong NOE to H2' and H3'. In contrast, if the nucleoside has a *syn* conformation, base H6 or H8 protons are closest to the sugar H1' proton, and irradiation of H6 gives a strong NOE to H1'.³⁴ Hence, the magnitude of the sum of H2' and H3' NOEs compared to the H1' NOE is the basis for determining the *anti* to *syn* ratio of the nucleoside.³⁴

When H6 of cytidine was irradiated in D₂O at 25 °C, combined NOEs of 9.5% for H2' and H3' were determined, compared to 4.9% for the H1' NOE (summarized in Table 1). This data confirms that cytidine prefers the *anti* conformation. The reverse experiment in which H2' and H3' were irradiated gave a 9.0% combined NOE at H6; whereas, irradiation of H1' gave a 3.1% NOE at H6. This data is consistent with prior work of Rosemeyer and coworkers (8.5% for H2' and H3'; 3.6% for H1' in (CD₃)₂SO₄).³⁴ In the case of m⁴Cm, the H1' and H5 proton signals overlapped in D₂O; therefore, the NOE to H1' could not be measured accurately. Reverse studies (*i.e.*, irradiation of H2', H3', and H1') showed a preference for the *anti* conformation (Table 1). To further confirm these results, NOE studies of m⁴Cm were carried out in CD₃OD. Each proton signal was irradiated to determine the NOEs (Table 1). Similar results were obtained, in which irradiation of H6 gave an 8.7% NOE at H2' and H3', but only a 3.4% NOE at H1'. The two monomethylated cytidine analogues, m⁴C and Cm, also prefer the *anti* conformation (at 25 °C, the combined NOEs at H2' and H3' with H6 irradiation were 12.2% and 8.4% NOE for m⁴C and Cm, respectively; Table 1). One-dimensional ¹H NOE experiments were also done at 4 and 37 °C. Only minor variations in the conformation populations were observed with varying temperature (Table 1).

The ribose moieties of nucleosides most often adopt either a C_{3'}-*endo* (north, N) or a C_{2'}-*endo* (south, S) orientation (Figure 4).³⁶ These two preferred sugar puckering modes refer to the movement of the carbon atoms out of the plane (*i.e.*, C3' is above the plane for C_{3'}-*endo* and C2' is above the plane for C_{2'}-*endo*). Altona and Sundaralingam characterized the C_{3'}-*endo* and C_{2'}-*endo* sugar pucker using coupling constants of the sugar protons, J_{H1'}-J_{H2'} and J_{H3'}-J_{H4'}.³⁷ The levels of C_{2'}-*endo* and C_{3'}-*endo* sugar pucker can be calculated from the following equations: [%C_{2'}-*endo* (S)] = 100 × J_{1'2'}/(J_{1'2'} + J_{3'4'}); [%C_{3'}-*endo* (N)] = 100 – [%C_{2'}-*endo*].³⁷ Coupling constants for m⁴Cm and the monomethylated analogues are given in Table 2. The data reveal that cytidine and the three analogues all prefer the C_{3'}-*endo* conformation. The N/S ratio (*K_{eq}*) is similar for cytidine and m⁴Cm (1.6–1.7), but slightly lower for m⁴C (1.3) and slightly higher for Cm (1.9–2.0) (Table 3). Thus, 2'-*O*-methylation more strongly favors the C_{3'}-*endo* (N) sugar pucker in contrast to N⁴-methylation, in which C_{3'}-*endo* is slightly less favored. The differences appear to be balanced such that dimethylated m⁴Cm is similar to unmodified cytidine in the preference for the C_{3'}-*endo* sugar conformation.

3. Conclusions

The modified nucleosides m⁴Cm, Cm, and m⁴C were synthesized in good overall yields (67%, 67%, and 84%, respectively) on multigram scales. Circular dichroism and one-dimensional

NOE difference spectroscopy were employed for determination of the *syn* and *anti* preferences of the cytidine analogues. All of the cytidine nucleosides examined here (m^4Cm , Cm , m^4C , and cytidine) prefer the *anti* conformation. The sugar puckers were determined by using sugar proton coupling constants from the 1D 1H NMR spectra. The 2'-*O* and N^4 methylations have opposing effects in the ribose sugar pucker. Sugar methylation leads to a slight enhanced preference for the C_3' -*endo* (north) sugar pucker; whereas, base methylation leads to a slight decreased preference for the C_3' -*endo* conformation. These results show that the effect of modifications on nucleoside conformation are subtle, which is consistent with previous studies on methylated or alkylated uridine and pseudouridine monomers.^{38, 39} Nonetheless, the methylations may have significant effects on RNA conformation when present in certain sequences or structural motifs due to long-range tertiary contacts within the RNA. Hence, we are currently examining the effects of m^4Cm and its analogues at the oligonucleotide level (in RNA model systems) and full-length rRNAs. Multiple modifications may have additive effects on RNA conformation and play a role in fine-tuning the RNA structure and function.

4. Experimental

4.1. Synthesis of modified nucleosides

4.1.1. General—Tetrazole was purchased from Glen Research. All NMR solvents were from Cambridge Isotope Laboratories, Inc. Dry methanol and dimethylformamide were purchased from Acros. Methylene chloride (CH_2Cl_2) was purchased from Fisher, distilled over CaH_2 . All other chemicals were purchased from Sigma-Aldrich or Fisher and used without further purifications. Moisture-sensitive reactions were performed under dry argon. Flame-dried equipment (syringes, round-bottom flasks, etc.) was used in those reactions. The compounds were azeotroped using benzene or toluene.

ESI mass spectra and high resolution mass spectra (HRMS) were obtained on Waters Micromass Zq and Micromass GCT spectrometers, respectively. Flash column chromatography was carried out on silica gel 60 (240-400 mesh). 1H and ^{13}C NMR spectra were recorded on a Varian Unity 500 spectrometer.

4.1.2. 3',5'-Diacetyl-2'-*O*-methyluridine (3)—Compounds **1** (2, 2'-anhydrouridine) and **2** (2'-*O*-methyluridine) were prepared according to literature methods.²⁵ Compound **2** (1.80 g, 7.0 mmol) was placed in 24 mL of pyridine and stirred for 15 min. DMAP (214 mg, 1.75 mmol) was added, followed by 2.3 mL (24.5 mmol) of acetic anhydride. The reaction mixture was stirred overnight at room temperature. The reaction was quenched with saturated $NaHCO_3$. The compound was purified by column chromatography (hexane:ethyl acetate, 8:2) to give a yellow oil (2.27 g, 95%). 1H NMR (500 MHz, CD_3Cl) δ (ppm) 2.13 (s, 3H), 2.15 (s, 3H), 3.47 (s, 3H), 4.04 (m, 1H), 4.36 (m, 3H), 4.98(m, 1H), 5.75 (d, 1H), 5.91 (dd, 1H), 7.52 (d, 1H), 9.10 (s, 1H), ^{13}C NMR (500 MHz, CD_3Cl) δ (ppm) 20.86, 59.30, 62.65, 70.08, 79.40, 81.77, 88.81, 102.94, 127.23, 139.43, 145.69, 150.18, 163.06, 170.37, HRMS calculated for $C_{14}H_{18}N_2O_8$ 342.1063, found 342.1067.

4.1.3. 4-(Tetrazol-1-yl)-1-(3', 5'-di-*O*-acetyl-2'-*O*-methyl- β -D-ribofuranosyl)pyrimidine-2-(1H)-one (4)—In a round-bottom flask containing 15 mL of pyridine, compound **3** (2.05 g, 6 mmol), tetrazole (0.84 g, 12 mmol), tosyl chloride (2.29 g, 12 mmol), and diphenyl phosphate (2.25 g, 9 mmol) were added. The mixture was stirred for 36 h at room temperature, and then 3.75 mL of water was added. The solution was poured over saturated $NaHCO_3$ solution. The desired product was extracted with methylene chloride. The solvent was dried over Na_2SO_4 and concentrated on a rotary evaporator. The product was purified by column chromatography ($CH_2Cl_2:CH_3OH$, 95:5) to give a yellow foam (2.25 g, 95%). 1H NMR (500 MHz, CD_3Cl) δ (ppm) 2.11(s, 3H), 2.14(s, 3H), 3.6(s,3H), 4.17(d, 1H), 4.42(m, 2H), 4.54(m, 1H), 4.77(m, 1H), 5.97(s, 1H), 7.21(d, 1H), 8.56(d, 1H), 9.59(s, 1H) ^{13}C NMR

(500 MHz, CD₃Cl) δ (ppm) 20.75, 21.07, 59.43, 61.8, 69.24, 79.50, 81.75, 90.89, 95.16, 140.95, 147.5, 153.79, 157.69, 170.21, 170.317 ESI-MS (ES⁺) calculated for C₁₅H₁₈N₆O₇ 394.1, found 395.1 (MH⁺).

4.1.4. 3',5'-Diacetyl-N⁴, 2'-O-dimethylcytidine (5)—Potassium hydroxide (89%, 0.32 g, 5 mmol) and CH₃NH₃Cl (0.34 g, 5 mmol) were added to a 50 mL round bottom flask. The flask was sealed with a septum. Water (10 mL), acetonitrile (10 mL), triethylamine (770 μ L), and a solution of compound **4** (1.97 g, 5 mmol) in acetonitrile (20 mL) were added sequentially by syringe. The mixture was stirred vigorously for 24 h. The solvent was removed with a rotary evaporator. The crude product was purified by column chromatography (CH₂Cl₂:CH₃OH, 9:1) to give a yellow oil (1.69 g, 95%). ¹H NMR (500 MHz, CD₃Cl) δ (ppm) 2.11(s, 3H), 2.13 (s, 3H), 3.06 (s, 3H), 3.55 (s, 3H), 4.10(m, 1H), 4.40 (m, 3H), 4.88 (m, 1H), 5.75 (d, 1H), 6.00 (d, 1H), 7.50 (d, 1H), ¹³C NMR (500 MHz, CD₃Cl) δ (ppm) 20.85, 20.99, 28.07, 59.15, 62.85, 70.43, 78.43, 78.66, 81.93, 89.93, 96.20, 138.63, 164.52, 170.35, 170.52 HRMS calculated for C₁₅H₂₁N₃O₇ 355.1380, found 355.1383.

4.1.5. N⁴,2'-O-Dimethylcytidine (6)—Compound **5** (1.60 g, 4.5 mmol) was placed in a dry 50 mL round-bottom flask. The flask was fitted with a septum. Next, 25 mL of 2 M NH₃ in methanol was added, and the mixture was stirred overnight at room temperature. The mixture was dried on a rotary evaporator. The residue was coevaporated twice with CH₃OH and once with CH₃OH–CH₂Cl₂ (1:1) under a vacuum. The residue was heated to 100 °C under vacuum for 2 h to give **6** as a white powder (1.20 g, 99%). ¹H NMR (500 MHz, D₂O) δ (ppm) 2.73 (s, 3H), 3.34 (s, 3H), 3.63 (dd, 1H), 3.75 (dd, 1H), 3.82 (m, 1H), 3.91 (m, 1H), 4.13 (m, 1H), 5.78 (m, 2H), 7.52 (d, 1H), ¹³C NMR (500 MHz, D₂O) δ (ppm) 27.27, 58.29, 60.54, 68.42, 82.90, 83.13, 88.31, 97.48, 139.45, 157.78, 164.59, HRMS calculated for C₁₁H₁₇N₃O₅ 271.1168, found 271.1176.

4.1.6. 3',5'-Diacetyl-2'-O-methycytidine (7)—Potassium hydroxide (89%, 0.32 g, 5 mmol) and NH₄Cl (0.27 g, 5 mmol) were added to a round-bottom flask. The flask was sealed with a septum. Water (10 mL), acetonitrile (10 mL), triethylamine (770 μ L), and solution of compound **4** (1.97 g, 5 mmol) in acetonitrile (20 mL) were added sequentially with a syringe. The mixture was stirred vigorously for 24 h. The solvent was removed with a rotary evaporator. The crude product was purified by column chromatography (CH₂Cl₂:CH₃OH, 9:1) to give a yellow oil (1.62 g, 95%). ¹H NMR (500 MHz, CD₃Cl) δ (ppm) 2.09 (s, 3H), 2.11 (s, 3H), 3.47 (s, 3H), 4.01 (d, 1H), 4.32 (m, 1H), 4.34 (m, 1H), 4.35 (m, 1H), 4.95 (m, 1H), 5.88 (m, 1H), 6.01 (d, 1H), 7.55 (d, 1H) ¹³C NMR (500 MHz, CD₃Cl) δ (ppm) 20.89, 21.08, 58.18, 62.71, 78.84, 81.84, 90.31, 95.59, 125.96, 140.82, 155.48, 165.84, 170.41, 170.58 ESI-MS (ES⁺) calculated for C₁₄H₁₉N₃O₇ 341.3, found 342.3 (MH⁺).

4.1.7. 2'-O-Methycytidine (8)—Compound **7** (1.54 g, 4.5 mmol) was placed in a dry round-bottom flask fitted with a septum. Next, 25 mL of 2 M NH₃ in methanol was added, and the mixture was stirred overnight at room temperature. The mixture was dried on a rotary evaporator. The residue was coevaporated twice with CH₃OH and once with CH₃OH–CH₂Cl₂ under vacuum. The residue was heated to 100 °C under a vacuum for 2 h to give **8** as a white powder (1.15 g, 99%). The NMR data matched that in the literature.²⁵ ESI-MS (ES⁺) calculated for C₁₀H₁₅N₃O₅ 257.1, found 258.1 (MH⁺).

4.1.8. 4-(Tetrazol-1-yl)-1-(2', 3', 5'-Tri-O-acetyl- β -D-ribofuranosyl)pyrimidine-2-(1H)-one (10)—Compound **9** (2',3',5'-triacetyluridine) was prepared according to literature methods.²⁶ Compound **9** (2.22 g, 6 mmol), tetrazole (0.84 g, 12 mmol), tosyl chloride (2.29 g, 12 mmol), and diphenyl phosphate (2.25 g, 9 mmol) were added to a round bottom flask containing 15 mL of pyridine. The mixture was stirred for 36 h at room temperature, and then

3.75 mL of water was added. The solution was poured over a saturated sodium carbonate solution. The desired product was extracted with methylene chloride. The solvent was dried over Na_2SO_4 and concentrated on a rotary evaporator. The product was purified by column chromatography ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$, 95:5) to give a yellow foam (2.40 g, 95%). ^1H NMR (500 MHz, CD_3Cl) δ (ppm) 2.07 (s, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 4.40 (m, 3H), 5.27 (m, 1H), 5.48 (m, 1H), 6.09 (d, 1H), 7.24 (d, 1H), 8.38 (d, 1H), 9.60 (s, 1H), ^{13}C NMR (500 MHz, CD_3Cl) δ (ppm) 20.66, 20.68, 21.05, 62.66, 69.65, 73.99, 80.51, 90.31, 95.72, 141.04, 147.69, 153.98, 157.84, 169.76, 169.76, 170.33, ESI-MS (ES^+) calculated for $\text{C}_{16}\text{H}_{18}\text{N}_6\text{O}_8$ 422.1, found 423.1 (MH^+).

4.1.9. 2',3',5'-Tri-O-acetyl- N^4 -methylcytidine (11)—Potassium hydroxide (89%, 0.32 g, 5 mmol) and $\text{CH}_3\text{NH}_3\text{Cl}$ (0.34 g, 5 mmol) were added in a 50 mL round-bottom flask. The flask was sealed with a septum. Water (10 mL), acetonitrile (10 mL), triethylamine (770 μL), and a solution of the compound **10** (2.11 g, 5 mmol) in acetonitrile (20 mL) were added sequentially by syringe. The mixture was stirred vigorously for 24 h. The solvent was removed with a rotary evaporator. The crude product was purified by column chromatography ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$, 9:1) to give a yellow oil (1.82 g, 95%). ^1H NMR (500 MHz, CD_3Cl) δ (ppm), 2.09 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 4.34 (m, 2H), 5.40 (dd, 1H), 5.90 (d, 1H), 5.99 (d, 1H), 6.71 (d, 1H), 7.39 (d, 1H), (s, 3H) 8.27 (d, 1H), ^{13}C NMR (500 MHz, CD_3Cl) δ (ppm), 20.48, 20.53, 20.68, 63.0, 70.04, 73.41, 79.12, 89.96, 96.11, 141.03, 155.59, 166.10, 169.58, 169.71, 170.38, ESI-MS (ES^+) calculated for $\text{C}_{16}\text{H}_{21}\text{N}_3\text{O}_8$ 383.1, found 384.1 (MH^+).

4.1.10. N^4 -Methylcytidine (12)—Compound **11** (1.53 g, 4 mmol) was added to a round-bottom flask fitted with a septum. Next, 25 mL of 2 M NH_3 in methanol was added, and the mixture was stirred overnight at room temperature. The mixture was dried on a rotary evaporator. The residue was coevaporated twice with CH_3OH and once with $\text{CH}_3\text{OH}-\text{CH}_2\text{Cl}_2$ (1:1) under vacuum. The residue was heated at 100 $^\circ\text{C}$ under vacuum for 2 h to give **12** as a white powder (1.01 g, 99%). ^1H NMR (500 MHz, D_2O) δ (ppm) 2.75 (s, 3H), 3.63 (dd, 1H), 3.75 (dd, 1H), 3.98 (m, 1H), 4.05 (m, 1H), 4.17 (m, 1H), 5.77 (d, 1H), 5.83 (d, 1H), 7.54 (d, 1H), ^{13}C NMR (500 MHz, D_2O) δ (ppm) 27.30, 60.99, 69.60, 73.89, 83.94, 90.13, 97.51, 139.62, 158.17, 164.71 ESI-MS (ES^+) calculated for $\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_5$ 257.1, found 258.2 (MH^+).

4.2. Circular dichroism and NMR studies of modified nucleosides

4.2.1. Sample preparation—The nucleosides were dissolved in a 20 mM sodium cacodylate buffer, pH 7.0. The concentrations were calculated by using the Beer-Lambert equation $A = \epsilon \cdot C \cdot \lambda$, in which ϵ , C and λ are extinction coefficient, concentration, and pathlength, respectively. The extinction coefficients (ϵ) are 11,000 $\text{cm}^{-1}\text{M}^{-1}$, 11,700 $\text{cm}^{-1}\text{M}^{-1}$, and 9,700 $\text{cm}^{-1}\text{M}^{-1}$ for m^4Cm , m^4C , and Cm , respectively.^{17, 20, 40}

4.2.2. Circular dichroism and NMR spectroscopy—CD spectra were acquired on a Chirascan circular dichroism spectrometer equipped with a water bath to control the temperature. The molar ellipticities were normalized from a concentration of 1.83 mM, 1.45 mM, 1.01 mM, and 1.24 mM for cytidine, m^4Cm , m^4C , and Cm , respectively. ^1H NMR spectra were recorded on a Varian Unity 500 MHz spectrometer. NMR experiments were performed in 99.99% D_2O . ^1H NMR and NOE experiments were done at 4, 25, and 37 $^\circ\text{C}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are thankful to Dr. B. Ksebati and Dr. J.-P. Desaulniers for their technical assistance and helpful discussions. This work was supported by the National Institutes of Health (AI061192 and AI055496).

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Figure 1.

The structures of the modified nucleosides a) *N*⁴, 2'-*O*-dimethylcytidine (m⁴Cm), b) 5-methylcytidine (m⁵C), c) 3-methyluridine (m³U), d) cytidine (C), e) 2'-*O*-methylcytidine (Cm), and f) *N*⁴, methylcytidine (m⁴C) are shown.

Figure 2.
The *anti* and *syn* conformations of the cytidine nucleoside are represented by stick models.

Figure 3. The circular dichroism (CD) spectra of cytidine and its methylated analogues at room temperature are shown. Each curve represents the average of five scans.

Figure 4. Two types of sugar pucker, C_{3'}-endo (north) and C_{2'}-endo (south), are represented by stick models.

Scheme 1. Synthesis of N^4 , 2'-O-dimethylcytidine and its analogues

Reagents and conditions: i) $(\text{PhO})_2\text{CO}$, NaHCO_3 , DMF, 80°C , 3 h; ii) $\text{Mg}(\text{OCH}_3)_2$, CH_3OH , reflux, 5 h; iii) $(\text{CH}_3\text{CO})_2\text{O}$, DMAP, pyridine, rt, overnight; iv) tetrazole, TsCl , diphenyl phosphate, pyridine, rt, 36 h; v) $\text{CH}_3\text{NH}_3^+\text{Cl}^-$, KOH , $(\text{C}_2\text{H}_5)_3\text{N}$, $\text{CH}_3\text{CN-H}_2\text{O}$, rt, 24 h; vi) NH_3 in CH_3OH , rt, overnight; vii) NH_4Cl , KOH , $(\text{C}_2\text{H}_5)_3\text{N}$, $\text{CH}_3\text{CN-H}_2\text{O}$, rt, 24 h; viii) NH_3 in CH_3OH , rt, overnight; ix) $(\text{CH}_3\text{CO})_2\text{O}$, DMAP, pyridine, rt, overnight; x) tetrazole, TsCl , diphenyl phosphate, pyridine, RT, 36 h; xi) $\text{CH}_3\text{NH}_3^+\text{Cl}^-$, KOH , $(\text{C}_2\text{H}_5)_3\text{N}$, $\text{CH}_3\text{CN-H}_2\text{O}$, rt, 24 h; xii) NH_3 in CH_3OH , rt, overnight.

Table 1
Irradiation and NOE data for cytidine and its methylated analogues at 4, 25, and 37 °C.

Compound	Irradiated proton	Enhanced proton	4 °C	NOE % 25 °C	37 °C
C (D ₂ O)	H6	H1'	3.3	4.9	4.3
		H2' + H3'	8.6	9.5	8.1
	H1' H2' H3'	H5	7.3	11.4	10.1
		H6	1.8	3.1	3.1
		H6	5.6	6.0	5.2
Cm (D ₂ O)	H6	H1'	3.5	3.7	3.6
		H2' + H3'	6.7	8.4	7.6
	H1' H2' H3'	H5	9.2	10.8	9.4
		H6	2.2	4.1	2.8
		H6	2.9	5.5	4.9
m ⁴ C (D ₂ O)	H6	H1'	4.7	8.5	4.8
		H2' + H3'	10.5	12.2	10.7
	H1' H2' H3'	H5	8.2	11.6	9.2
		H6	1.7	2.3	1.9
		H6	6.2	5.8	4.5
m ⁴ Cm (D ₂ O)	H6	H1' + H5	5.4	17.6	16.1
		H2' + H3'	7.2	11.9	10.9
	H1' H2' H3'	H6	1.5	4.8	4.2
		H6	4.3	6.0	5.2
		H6	1.9	2.8	2.4
m ⁴ Cm (CD ₃ OD)	H6	H1'	3.4	3.4	3.3
		H2' + H3'	11.0	8.7	8.5
	H1' H2' H3'	H5	11.2	9.3	8.9
		H6	2.7	2.1	2.3
		H6	3.9	3.4	3.3
		H6	5.0	3.5	3.1

Table 2
¹H-¹H coupling constants of m⁴Cm and its analogues at 4, 25, and 37 °C.

Compound	Temperature	$J_{1,2'}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5'}$	$J_{4,5''}$
C	4° C	3.8	5.1	6.3	2.8	4.3
	25° C	3.9	5.3	6.1	2.8	4.4
	37° C	4.0	5.4	6.1	2.9	4.5
Cm	4° C	3.3	5.2	6.8	2.6	4.2
	25° C	3.5	5.3	6.7	2.8	4.4
	37° C	3.5	5.4	6.5	2.8	4.4
m ⁴ C	4° C	4.3	5.4	5.5	2.8	4.4
	25° C	4.4	5.4	5.6	2.9	4.6
	37° C	4.4	5.4	5.6	2.9	4.6
m ⁴ Cm	4° C	3.8	5.3	6.6	2.8	4.3
	25° C	3.9	5.4	6.2	2.8	4.3
	37° C	3.9	5.4	6.2	2.9	4.5

Table 3

Type of sugar puckered of m⁴Cm and its analogues at 4, 25, and 37 °C.

Compound	Temperature (° C)	% C ₃ -endo (N)	% C ₂ -endo (S)	K _{eq} (N/S)
C	4	62	38	1.6
	25	61	39	1.6
	37	60	40	1.5
Cm	4	67	33	2.0
	25	66	34	1.9
	37	65	35	1.9
m ⁴ C	4	56	44	1.3
	25	56	44	1.3
	37	56	56	1.3
m ⁴ Cm	4	63	37	1.7
	25	61	39	1.6
	37	61	39	1.6