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Synthesis and solution conformation studies of the modified nucleoside N^4,2′-*O*-dimethylcytidine (m^4Cm) and its analogues

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ABSTRACT

The dimethylated ribosomal nucleoside m^4Cm and its monomethylated analogues Cm and m^4C were synthesized. The conformations *(syn* vs *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 vs 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 m^4Cm (N^4,2′-*O*-dimethylcytidine) is a rare nucleoside, in which both the base and sugar are methylated. To date, m^4Cm 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 m^4Cm, 5-methylcytidine (m^5C), and 3-methyluridine (m^3U) at positions 1402, 1407, and 1498, respectively (*Escherichia coli* numbering) (Fig. 1a–c).[9] The nucleoside m^4Cm was isolated from *E. coli* and characterized by Nichols and Lane in 1966.[14,15] The methyltransferase enzyme for *E. coli* $m^4Cm1402$ in 16S rRNA is currently unknown.

Two of the decoding region modified nucleosides, m^3U and m^5C, are commercially available.[16] In contrast, m^4Cm 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 relatively expensive $TIPDSiCl_2$ reagent for 3′,5′-*O*-ribose protection. For detailed structural studies of m^4Cm, as well as its monomethylated analogues, N^4-methylcytidine (m^4C) and 2′-*O*-methylcytidine (Cm) (Fig. 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 m^4C is not commercially available, although several synthetic methods have been reported.[17–23] Thus, the other rationale for developing a new synthetic route for m^4Cm was to generate the intermediates of interest, m^4C and Cm. Herein, we report on the synthesis of m^4Cm, m^4C, 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 m^4Cm, m^4C, Cm, and cytidine. A comparison of the solution conformations of m^4Cm and its analogues provides insight into the possible roles of the modifications at the RNA level.

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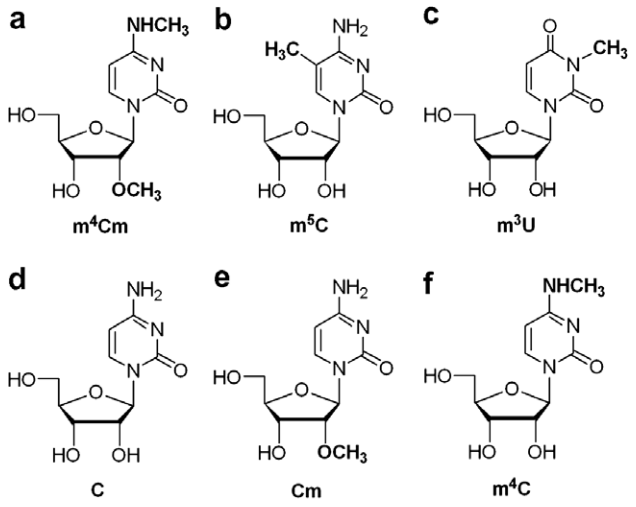


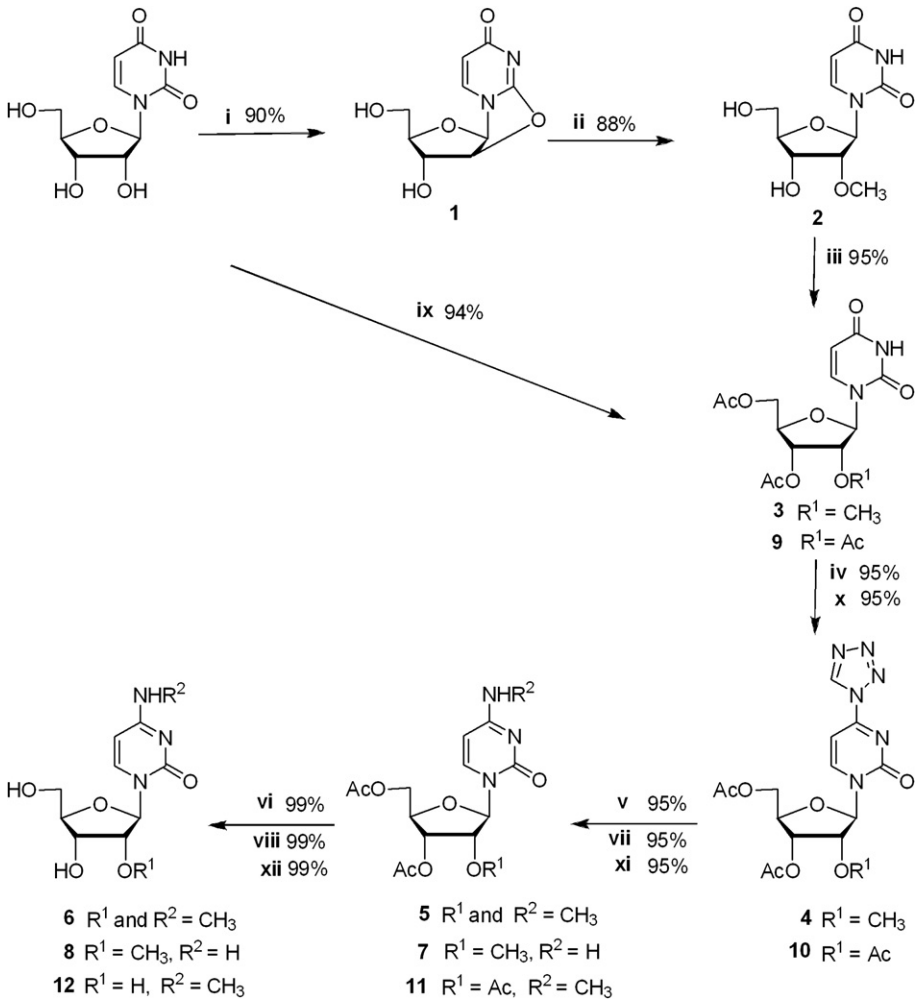
Figure 1. The structures of the modified nucleosides. (a) N^4,2′-*O*-dimethylcytidine (m^4Cm), (b) 5-methylcytidine (m^5C), (c) 3-methyluridine (m^3U), (d) cytidine (C), (e) 2′-*O*-methylcytidine (Cm), and (f) N^4-methylcytidine (m^4C) are shown.

2. Results and discussion

2.1. Synthesis of N^4,2′-*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-N^4,2′-*O*-dimethylcytidine, **5**, in 95% yield. Removal of the acetyl protecting groups by treatment with 2.0 M NH_3 in methanol yielded N^4,2′-*O*-dimethylcytidine, **6**, in 99% yield. The overall yield of m^4Cm 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.



Scheme 1. Synthesis of N^4,2′-*O*-dimethylcytidine and its analogues. Reagents and conditions: (i) $(PhO)_2CO$, $NaHCO_3$, DMF, 80 °C, 3 h; (ii) $Mg(OCH_3)_2$, CH_3OH, reflux, 5 h; (iii) $(CH_3CO)_2O$, DMAP, pyridine, rt, overnight; (iv) tetrazole, TsCl, diphenyl phosphate, pyridine, rt, 36 h; (v) $CH_3NH_3^+Cl^-$, KOH, $(C_2H_5)_3N$, CH_3CN–H_2O, rt, 24 h; (vi) NH_3 in CH_3OH, rt, overnight; (vii) NH_4Cl, KOH, $(C_2H_5)_3N$, CH_3CN–H_2O, rt, 24 h; (viii) NH_3 in CH_3OH, rt, overnight; (ix) $(CH_3CO)_2O$, DMAP, pyridine, rt, overnight; (x) tetrazole, TsCl, diphenyl phosphate, pyridine, RT, 36 h; (xi) $CH_3NH_3^+Cl^-$, KOH, $(C_2H_5)_3N$, CH_3CN–H_2O, rt, 24 h; (xii) NH_3 in CH_3OH, rt, overnight.

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 NH_3 in methanol to give 2′-*O*-methylcytidine, **8**, in 99% yield.

To generate m^4C (Scheme 1), uridine was acetylated using catalytic amounts of DMAP in pyridine with acetic anhydride to afford 2′,3′, 5′-triacetyl 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, N^4-methycytidine, **11**, in 95% yield. Deprotection of the acetyl groups was carried out by treatment with 2.0 M NH_3 in methanol to yield N^4-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 (Fig. 2).[29,30] Circular dichroism (CD) spectroscopy can be used to determine the solution conformations of nucleosides.[31] The m^4Cm and its analogues all have peak maxima at 271 nm. This feature indicates that they all prefer the *anti* conformation in solution (Fig. 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 are consistent with prior work by Miles et al., even though the solvents in that study were different.[31,32]

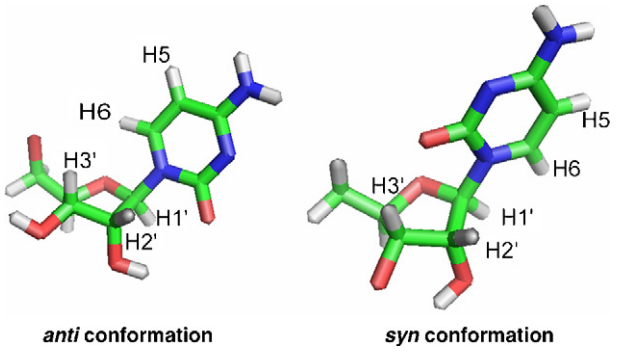


Figure 2. The *anti* and *syn* conformations of the cytidine nucleoside are represented by stick models.

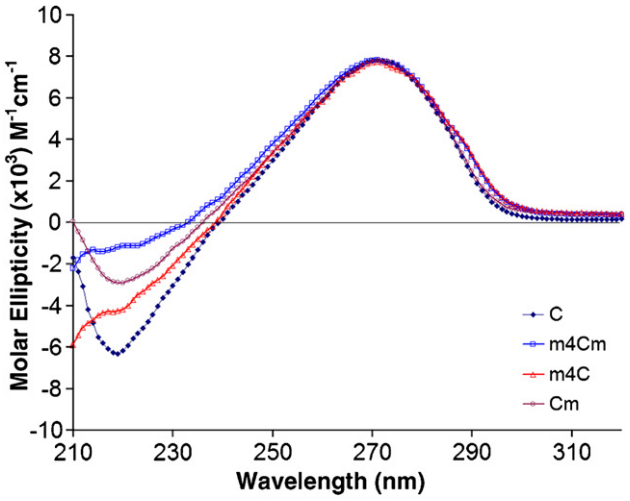


Figure 3. The circular dichroism (CD) spectra of cytidine and its methylated analogues at room temperature are shown. Each curve represents the average of five scans.

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 (Fig. 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 D_2O 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 $(CD_3)_2SO_4$).[34] In the case of m^4Cm, the H1′ and H5 proton signals overlapped in D_2O; therefore,

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

Compound	Irradiated proton	Enhanced proton	NOE%		
			4 °C	25 °C	37 °C
C (D_2O)	H6	H1′	3.3	4.9	4.3
		H2′ + H3′	8.6	9.5	8.1
		H5	7.3	11.4	10.1
	H1′	H6	1.8	3.1	3.1
	H2′	H6	5.6	6.0	5.2
	H3′	H6	2.8	3.0	2.6
Cm (D_2O)	H6	H1′	3.5	3.7	3.6
		H2′ + H3′	6.7	8.4	7.6
		H5	9.2	10.8	9.4
	H1′	H6	2.2	4.1	2.8
	H2′	H6		5.5	4.9
	H3′	H6	2.9	3.7	2.6
m^4C (D_2O)	H6	H1′	4.7	8.5	4.8
		H2′ + H3′	10.5	12.2	10.7
		H5	8.2	11.6	9.2
	H1′	H6	1.7	2.3	1.9
	H2′	H6	6.2	5.8	4.5
	H3′	H6	2.2	1.9	1.9
m^4Cm (D_2O)	H6	H1′ + H5	5.4	17.6	16.1
		H2′ + H3′	7.2	11.9	10.9
	H1′	H6	1.5	4.8	4.2
	H2′	H6	4.3	6.0	5.2
	H3′	H6	1.9	2.8	2.4
m^4Cm (CD_3OD)	H6	H1′	3.4	3.4	3.3
		H2′ + H3′	11.0	8.7	8.5
		H5	11.2	9.3	8.9
	H1′	H6	2.7	2.1	2.3
	H2′	H6	3.9	3.4	3.3
	H3′	H6	5.0	3.5	3.1

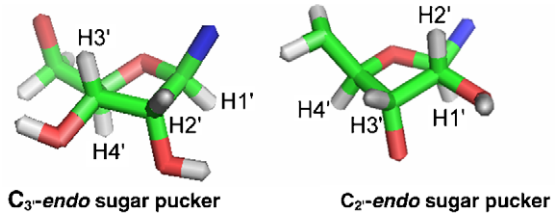


Figure 4. Two types of sugar puckers, $C_{3'}$-*endo* (north) and $C_{2'}$-*endo* (south), are represented by stick models.

Table 2
^1H–^1H coupling constants of m^4Cm and its analogues at 4, 25, and 37 °C

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

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 m^4Cm were carried out in CD_3OD. 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, m^4C 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 m^4C 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 (Fig. 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 puckers 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: $[\%C_{2'}\text{-}endo\ (S)] = 100 \times J_{1'2'}/(J_{1'2'} + J_{3'4'})$; $[\%C_{3'}\text{-}endo\ (N)] = 100 - [\%\ C_{2'}\text{-}endo]$.[37] Coupling constants for m^4Cm 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 m^4Cm (1.6–1.7), but slightly lower for m^4C (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 N^4-methylation, in which $C_{3'}$-*endo* is slightly less favored. The differences appear to be balanced such that dimethylated m^4Cm is similar to unmodified cytidine in the preference for the $C_{3'}$-*endo* sugar conformation.

3. Conclusions

The modified nucleosides m^4Cm, Cm, and m^4C 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 effects 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.

Table 3
Type of sugar pucker of m^4Cm 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^4C	4	56	44	1.3
	25	56	44	1.3
	37	56	56	1.3
m^4Cm	4	63	37	1.7
	25	61	39	1.6
	37	61	39	1.6

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%). ^{1}H 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%). ^{1}H 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, CD_3Cl) 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 $C_{15}H_{18}N_6O_7$ 394.1, found 395.1 (MH^+).

4.1.4. 3′,5′-Diacetyl-N^4,2′-*O*-dimethylcytidine (5)

Potassium hydroxide (89%, 0.32 g, 5 mmol) and CH_3NH_3Cl (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 μL), 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 (CH_2Cl_2/CH_3OH, 9:1) to give a yellow oil (1.69 g, 95%). ^{1}H NMR (500 MHz, CD_3Cl) 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), ^{13}C NMR (500 MHz, CD_3Cl) 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 $C_{15}H_{21}N_3O_7$ 355.1380, found 355.1383.

4.1.5. N^4,2′-*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 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 CH_3OH/CH_2Cl_2 (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%). ^{1}H NMR (500 MHz, D_20) 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),^{13}C NMR (500 MHz, D_2O) 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 $C_{11}H_{17}N_3O_5$ 271.1168, found 271.1176.

4.1.6. 3′,5′-Diacetyl-2′-*O*-methycytidine (7)

Potassium hydroxide (89%, 0.32 g, 5 mmol) and NH_4Cl (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 μL), 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 (CH_2Cl_2/CH_3OH, 9:1) to give a yellow oil (1.62 g, 95%). ^{1}H NMR (500 MHz, CD_3Cl) 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) ^{13}C NMR (500 MHz, CD_3Cl) 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 $C_{14}H_{19}N_3O_7$ 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 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 $CH_3OH–CH_2Cl_2$ 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 $C_{10}H_{15}N_3O_5$ 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 (CH_2Cl_2/CH_3OH, 95:5) to give a yellow foam (2.40 g, 95%). ^{1}H 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 $C_{16}H_{18}N_6O_8$ 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 CH_3NH_3Cl (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 (CH_2Cl_2/CH_3OH, 9:1) to give a yellow oil (1.82 g, 95%). ^{1}H 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 $C_{16}H_{21}N_3O_8$ 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 CH_3OH/CH_2Cl_2 (1:1) under vacuum. The residue was heated at 100 °C under vacuum for 2 h to give **12** as a white powder (1.01 g, 99%). ^{1}H NMR (500 MHz, D_20) 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 $C_{10}H_{15}N_3O_5$ 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 $A = \varepsilon \cdot C \cdot \lambda$, in which ε, C, and λ are extinction coefficient, concentration, and pathlength, respectively. The extinction coefficients (ε) are 11,000 cm^{-1} M^{-1}, 11,700 cm^{-1} M^{-1}, and 9700 cm^{-1} 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. ^{1}H NMR spectra were recorded on a Varian Unity 500 MHz spectrometer. NMR experiments were performed in 99.99% D_2O. ^{1}H NMR and NOE experiments were done at 4, 25, and 37 °C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.016.

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