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Supporting Information

5-Oxyacetic Acid Modification Destabilizes Double Helical Stem Structures and Favors Anionic Watson–Crick like cmo^{5}U-G Base Pairs

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Electronic Supporting Information

The 5-oxyacetic acid modification destabilizes double helical stem structures and favors anionic Watson-Crick like cmo^{5}U-G base pairs

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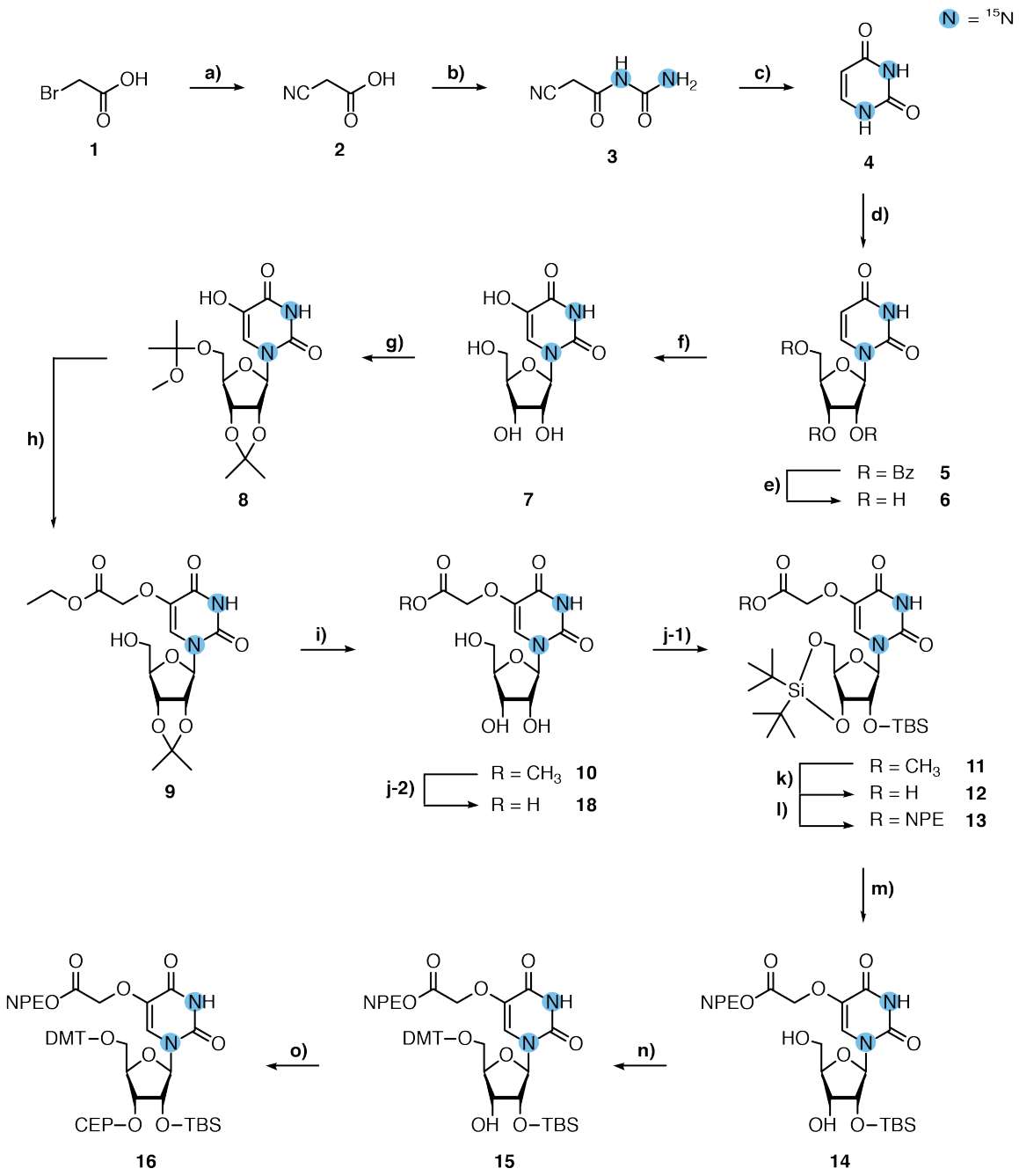
1 Synthesis of the 1,3-$^{15}N_2$-5-carboxymethoxy-2'-*O*-TBDMS-5'-*O*-DMT-uridine (cmo^{5}U) phosphoramidite (16).



Scheme 1. Synthesis of $^{15}N_2$-labeled cmo^{5}U phosphoramidite **10**. **a)** Br_2, pyridine in H_2O, 0°C – rt, 14h, 45%; **b)** *p*-tolunesulfonic acid in dimethoxypropane, 50°C, 1h, 97%; **c)** ethyl-2-iodo acetate in EtOH (55%)/NaOH (1M) (5/1), rt, 16h, 65%; **d)** concentrated aqueous HCl in MeOH, 50°C, 1h, 92%; **e)** di-*tert.*-butylsilyl bistriflate, imidazole, then *tert.*-butyldimethylsilyl chloride in anhydrous DMF, 0 – 50°C, 3h, 68%; **f)** NaOH in pyridine/MeOH (1/1), rt, 10min, 87%; **g)** 4-nitrophenylethanol (NPE), DMAP, EDC in anhydrous THF, rt, 2h, quant.; **h)** HF, pyridine in anhydrous CH_2Cl_2, 0°C, 1h, then DMT-Cl in anhydrous pyridine, rt, 16h, 26%; **i)** BTT, TIPCEP in anhydrous acetonitrile, rt, 4h, 79%.

1.1 2-Cyano acetic acid (2)

2-Bromo acetic acid (**1**, 14.00 g, 100.8 mmol, 1.00 eq) was dissolved in 40 mL H_2O and stirred at room temperature. Thus, a solution of $NaHCO_3$ (2.71 g, 32.24 mmol, 0.32 eq) in 20 mL H_2O was added followed by a solution of KCN (6.36 g, 97.73 mmol, 0.97 eq) in 20 mL H_2O. The mixture was stirred at 80°C for 16 hours prior to being quenched with *conc*. HCl until pH 1. After evaporation of the solvent, 2-cyano acetic acid was extracted with ether. The salt was filtered off and the filtrate was concentrated and dried under high vacuum to yield 8.50 g 2-cyano acetic acid as a white solid (**2**, 99.9 mmol, quant.).

^{13}C-NMR (300 MHz, DMSO-d_6, 25°C): δ 165.81 (*C1*(O)OH); 115.66 (*C3*N); 24.74 (*C2*H_2) ppm.

1.2 $^{15}N_2$-*N*-Carbamoyl-2-cyanoacetamide (3)

A mixture of 2-cyano acetic acid (**2**, 8.80 g, 103 mmol, 1.00 eq) and $^{15}N_2$-urea (6.80 g, 110 mmol, 1.06 eq) was dissolved in acetic anhydride and stirred at 90°C for 30 minutes until white precipitation. Thus, the reaction mixture was cooled to 0°C, filtered, the white solid was washed with 500 mL ether and dried under high vacuum to yield 9.49 g $^{15}N_2$-*N*-carbamoyl-2-cyanoacetamide (**3**, 73.5 mmol, 71%), which was used without further purification and characterization.

1.3 1,3-$^{15}N_2$-Uracil (4)

Palladium on $BaSO_4$ (4.80 g, 5% Pd-basis) was dissolved in a mixture of 60 mL acetic acid and H_2O (1/1) and put under hydrogen atmosphere under vigorous stirring. Thus, $^{15}N_2$-*N*-carbamoyl-2-cyanoacetamide (**3**, 9.49 g, 73.6 mmol, 1.00 eq) was dissolved in a mixture of 240 mL acetic acid and H_2O (1/1) at 80°C and was added to the stirring Palladium-acetic acid-water suspension. The reaction mixture was stirred under hydrogen atmosphere at room temperature for 20 hours, followed by 1.5 hours at 70°C. After the suspension was filtered over celite and washed with hot water, the mother lye was concentrated at 45°C until precipitation started. The product crystallized at 0°C, was filtrated, washed with cold water and dried under high vacuum to give 3.33 g 1,3-$^{15}N_2$-uracil (**4**, 28.9 mmol, 40%) as a white powder.

^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.14 - 10.60 (m, 2H, 2x ^{15}N*H*); 7.38 (m, 1H, C6*H*); 5.44 (m, 1H, C5*H*) ppm.

1.4 1,3-$^{15}N_2$-2',3',5'-*O*-Benzoyl uridine (5)

A mixture of 1,3-$^{15}N_2$-uracil (**4**, 3.30 g, 28.9 mmol, 1.00 eq) and 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose (ATBR, 14.01 g, 28.9 mmol, 1.00 eq) was dissolved in 40 mL anhydr. acetonitrile and was stirred at room temperature under Argon atmosphere. Bis(trimethylsilyl)acetamide (BSA, 21.2 mL, 86.8 mmol, 3.00 eq) was added and the solution was refluxed at 60°C for 30 minutes. Thus, trimethylsilyltriflate (18.3 mL, 101 mmol, 3.50 eq) was added slowly and the reaction mixture was stirred at 60°C for another 30 minutes, before being allowed to cool to room temperature. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2, washed twice with sat. $NaHCO_3$, dried over Na_2SO_4, filtered and concentrated to yield 16.20 g 1,3-$^{15}N_2$-2',3',5'-*O*-benzoyl uridine (**5**, 29.0 mmol, quant.).

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.85
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.17 - 7.29 (m, 16H, 15x arom. *H*, C6*H*); 6.32 (d, 1H, C1'*H*, $^3J_{HH}$ = 5.4 Hz); 5.89 (t, 1H, C3'*H*, $^3J_{HH}$ = 5.4 Hz); 5.76 (t, 1H, C2'*H*, $^3J_{HH}$ = 5.4 Hz); 5.61 (m, 1H, C5*H*); 4.84 (m, 1H, C5'*H*); 4.69 (m, 2H, C4'*H*, C5'*H*) ppm.

1.5 1,3-$^{15}N_2$-Uridine (6)

1,3-$^{15}N_2$-2',3',5'-*O*-Benzoyluridine (**5**, 16.05 g, 28.74 mmol, 1.00 eq) was dissolved in a solution of methylamine in EtOH (103 mL, 767.3 mmol, 26.70 eq) and was stirred at room temperature for 14 hours under Argon atmosphere. Thus, the solvent was evaporated and the residue was dissolved in H_2O. The

aq. phase was washed 6 times with CH_2Cl_2, concentrated and dried under high vacuum to give 7.07 g 1,3-$^{15}N_2$-uridine (**6**, 28.72 mmol, quant.) as an off-white foam which was not further purified.

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.05
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 7.87 (m, 1H, C5*H*); 5.77 (s, 1H, C1'*H*); 5.63 (m, 1H, C6*H*); 4.01 (s, 1H, C2'*H*); 3.95 (s, 1H, C3'*H*); 3.83 (s, 1H, C4'*H*); 3.57 (m, 2H, C5'H_2) ppm.

1.6 1,3-$^{15}N_2$-5-Hydroxy uridine (7)

1,3-$^{15}N_2$-Uridine (**6**, 7.11 g, 28.9 mmol, 1.00 eq) was dissolved in H_2O and bromine (14.1 mL, 274 mmol, 9.50 eq) was added slowly under stirring. Excess of bromine was extracted with ethyl acetate. Thus, pyridine (20.9 mL, 260 mmol, 9.00 eq) was added dropwise at 0°C and the solution was stirred at room temperature for 14 hours. After evaporation of the solvent and co-evaporation with toluene, the crude was dissolved in H_2O and washed with ethyl acetate. The org. phase was extracted with H_2O and the combined aq. phases were concentrated and dried under high vacuum. Recrystallization from ethanol yielded 3.40 g 1,3-$^{15}N_2$-5-hydroxy uridine (**7**, 28.9 mmol, 45%).

TLC: ethyl acetate, R_f = 0.20
^{1}H-NMR (400 MHz, DMSO-d_6, 25°C): δ 11.42 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 7.36 (s, 1H, C6*H*); 5.79 (d, 1H, C1'*H*, $^3J_{HH}$ = 5.8 Hz); 4.00 (t, 1H, C2'*H*, $^3J_{HH}$ = 5.8 Hz); 3.94 (m, 1H, C3'*H*); 3.81 (m, 1H, C4'*H*); 3.56 (m, 2H, C5'H_2) ppm.
^{13}C-NMR (400 MHz, DMSO-d_6, 25°C): δ 160.52 (*C4*); 149.49 (*C2*); 132.46 (*C5*); 119.81 (*C6*); 87.26 (*C1'*); 84.85 (*C4'*); 73.11 (*C2'*); 70.24 (*C3'*); 61.17 (*C5'*) ppm.

1.7 1,3-$^{15}N_2$-5-Hydroxy-2',3'-*O*-isopropylidene-5'-*O*-(2''-methoxypropyl) uridine (8)

A mixture of 1,3-$^{15}N_2$-5-hydroxy uridine (**7**, 1.90 g, 7.25 mmol, 1.00 eq) and *para*- toluene sulfonic acid (125 mg, 725 µmol, 0.10 eq) was suspended in dimethoxypropane and stirred at room temperature for 16 hours. Thus, the solution was neutralized with 100 µl TEA, concentrated, co-evaporated with CH_2Cl_2 and dried under high vacuum to give 2.62 g as a mixture of 1,3-$^{15}N_2$-5-hydroxy-2',3'-*O*-isopropylidene-5'-*O*-(2''-methoxypropyl) uridine (**8**) and 1,3-$^{15}N_2$-5-hydroxy-2',3'-*O*-isopropylidene uridine (**17**, approx. 7.00 mmol in total, 97%).

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.66
^{1}H-NMR (400 MHz, DMSO-d_6, 25°C): δ 11.53 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 8.69 (s, 1H, C5O*H*); 7.29 (s, 1H, C6*H*); 5.87 (s, 1H, C1'*H*); 4.87 (m, 1H, C2'*H*); 4.75 (m, 1H, C3'*H*); 4.15 (m, 1H, C4'*H*); 3.52 (m, 2H, C5'H_2); 3.08 (s, 3H, OCH_3); 1.48 (s, 3H, CH_3); 1.29 (s, 9H, 3x CH_3) ppm.
^{13}C-NMR (400 MHz, DMSO-d_6, 25°C): δ 160.59 (*C4*); 148.94 (*C2*); 132.9 (*C5*); 120.65 (*C6*); 113.08 (*C*(CH_3)$_2$); 99.84 (*C*(CH_3)$_2$); 90.40 (*C1'*); 84.43 (*C4'*); 83.54 (*C2'*); 80.49 (*C3'*); 60.75 (*C5'*); 47.95 (O*C*H_3); 27.07 (*C*H_3); 25.22 (*C*H_3); 24.05 (*C*H_3); 23.98 (*C*H_3) ppm.

1.8 1,3-$^{15}N_2$-5-Ethylcarboxymethoxy-2',3'-*O*-isopropylidene uridine (9)

1,3-$^{15}N_2$-5-Hydroxy-2',3'-*O*-isopropylidene-5'-*O*-(2''-methoxypropyl) uridine (**8**, 2.62 g, 7.00 mmol, 1.00 eq) was dissolved in a mixture of EtOH (55%) and NaOH (1M) (5/1) and stirred at room temperature. Thus, ethyl-2-iodo acetate (3.31 mL, 28.0 mmol, 4.00 eq) was added in portions over 2 hours and the solution was stirred for additional 30 minutes. After neutralization with acetic acid, the solvent was evaporated and the dry residue was dissolved in $CHCl_3$. The org. phase was washed with cold H_2O, dried over Na_2SO_4, concentrated and dried under high vacuum. 1.76 g 1,3-$^{15}N_2$-5-ethylcarboxymethoxy-2',3'-*O*-isopropylidene uridine (**9**, 4.53 mmol, 65%) were obtained and used without further purification.

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.60
^{1}H-NMR (400 MHz, DMSO-d_6, 25°C): δ 11.60 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 7.60 (s, 1H, C6*H*); 5.84 (s, 1H, C1'*H*); 5.13 (t, 1H, C5'O*H*); 4.88 (m, 1H, C2'*H*); 4.75 (m, 1H, C3'*H*); 4.56 (s, 2H, C(O)CH_2O); 4.16

(q, 2H, OC*H*$_2$CH$_3$, $^3J_{HH}$ = 7.1 Hz); 4.08 (m, 1H, C4'*H*); 3.58 (m, 2H, C5'*H*$_2$); 1.48 (s, 3H, C*H*$_3$); 1.29 (s, 3H, C*H*$_3$); 1.22 (t, 3H, CH$_2$C*H*$_3$, $^3J_{HH}$ = 7.1 Hz) ppm.
^{13}C-NMR (400 MHz, DMSO-d$_6$, 25°C): δ 168.16 (O*C*(O)CH$_2$); 159.15 (*C4*); 149.01 (*C2*); 133.58 (*C5*); 125.44 (*C6*); 112.91 (*C*(CH$_3$)$_2$); 90.97 (*C1'*); 86.23 (*C4'*); 83.44 (*C2'*); 80.47 (*C3'*); 66.67 (C(O)*C*H$_2$O); 61.28 (*C5'*H$_2$); 60.58 (O*C*H$_2$CH$_3$); 27.03 (*C*H$_3$); 25.17 (*C*H$_3$); 14.00 (CH$_2$*C*H$_3$) ppm.

1.9 1,3-$^{15}N_2$-5-Methylcarboxyethoxy uridine (10)

1,3-$^{15}N_2$-5-Ethylcarboxymethoxy-2',3'-*O*-isopropylidene uridine (**9**, 1.76 g, 4.53 mmol, 1.00 eq) was dissolved in methanol and conc. HCl was added until pH = 1. The solution was stirred at 50°C for 1 hour. Thus, the solvent was evaporated, co-evaporated with methanol and dried under high vacuum to yield 1.40 g 1,3-$^{15}N_2$-5-methylcarboxymethoxy uridine (**10**, 4.53 mmol, 92%), which was not further purified or characterized.

1.10 1,3-$^{15}N_2$-5-Methylcarboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (11)

1,3-$^{15}N_2$-5-Methylcarboxymethoxy uridine (**10**, 1.40 g, 4.19 mmol, 1.00 eq) was dissolved in anhydr. DMF and stirred at 0°C under Argon. Thus, di-*t*-butylsilyl bis(trifluoromethanesulfonate) (3.55 mL, 10.9 mmol, 2.60 eq) was added and the solution was stirred at 0°C for 30 minutes. Imidazole (1.43 g, 20.9 mmol, 5.00 eq) was added and the reaction mixture was allowed to warm to room temperature and stir for 20 minutes. After the addition of *t*-butyl dimethylsilyl chloride (1.70 g, 11.3 mmol, 2.70 eq) the solution was stirred at 50°C for 3 hours. The solvent was evaporated and the residue was dissolved in CH_2Cl_2. The org. phase was washed with half-sat. NaCl, dried over Na_2SO_4, concentrated and dried under high vacuum. The crude was purified via column chromatography (SiO_2, ethyl acetate/hexane 1/9 - 2/8 - 3/7 - 4/6) to give 1.67 g 1,3-$^{15}N_2$-5-methylcarboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**11**, 2.84 mmol, 68%) as a white foam.

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.85
^{1}H-NMR (400 MHz, $CDCl_3$, 25°C): δ 9.06 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 7.44 (s, 1H, C6*H*); 5.66 (s, 1H, C1'*H*); 4.59 (m, 2H, C(O)C*H*$_2$O); 4.51 (m, 1H, C5'*H*$_2$); 4.26 (m, 1H, C2'*H*); 4.18 (m, 1H, C3'*H*); 4.08 (m, 1H, C5'*H*$_2$); 3.89 (1, 1H, C4'*H*); 3.74 (s, 3H, OC*H*$_3$); 1.04 (d, 18H, 6x C*H*$_3$); 0.93 (s, 9H, 3x C*H*$_3$); 0.18 (s, 3H, C*H*$_3$); 0.14 (s, 3H, C*H*$_3$) ppm.
^{13}C-NMR (400 MHz, $CDCl_3$, 25°C): δ 169.68 (O*C*(O)CH$_2$); 159.68 (*C*4); 148.68 (*C*2); 133.28 (*C*5); 129.98 (*C*6); 93.62 (*C*1'); 76.10 (*C*4'); 75.62 (*C*2'); 74.86 (*C*3'); 68.25 (C(O)*C*H$_2$O); 67.63 (*C*5'H$_2$); 52.13 (C(O)O*C*H$_3$); 27.65 (3x *C*H$_3$); 27.13 (3x *C*H$_3$); 26.00 (3x *C*H$_3$); 22.80 (*C*(CH$_3$)$_3$); 20.50 (*C*(CH$_3$)$_3$); 18.38 (*C*(CH$_3$)$_3$); -4.18 (*C*H$_3$); -4.91 (*C*H$_3$) ppm.

1.11 1,3-$^{15}N_2$-5-Carboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (12)

1,3-$^{15}N_2$-5-Methylcarboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**11**, 1.67 g, 2.84 mmol, 1.00 eq) was dissolved in a mixture of pyridine and methanol (1/1) and was stirred at room temperature. Thus, NaOH in methanol (8.51 mL, 17.0 mmol, 2 M, 6.00 eq) was added and the solution was stirred for 10 minutes. After evaporation of the solvent mixture, the residue was suspended in CH_2Cl_2, neutralized with conc. HCl and washed with 0.5 M HCl. The org. phase was dried over Na_2SO_4 and concentrated to yield 1.41 g 1,3-$^{15}N_2$-5-carboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**12**, 2.45 mmol, 87%) as a white foam. The product was used for the next step without further purification or characterization.

1.12 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (13)

A mixture of 1,3-$^{15}N_2$-5-carboxymethoxy-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**12**, 1.41 g, 2.45 mmol, 1.00 eq), nitrophenyl ethanol (902 mg, 5.40 mmol, 2.20 eq) and *N*-dimethyl amino pyridine (30.0 mg, 245 µmol, 0.10 eq) was dissolved in anhydr. THF and stirred at room temperature under Argon. Thus, *N*-Ethyl-*N'*-(3-dimethyl aminopropyl) carbodiimide (EDC, 1.13 ml, 6.38 mmol, 2.60 eq) was added and the solution was stirred at room temperature for 2 hours. After dilution with CH_2Cl_2, the org. phase was washed with half-sat. $NaHCO_3$, dried over Na_2SO_4 and concentrated to yield 1.77 g of 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**13**, 2.44 mmol, quant.) as a yellowish oil and was used without further purification.

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.85
^{1}H-NMR (400 MHz, $CDCl_3$, 25°C): δ 9.26 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 8.15 (d, 2H, *arom. H*, $^3J_{HH}$ = 8.5 Hz); 8.06 (s, 1H, C6*H*); 7.38 (d, 2H, *arom. H*, $^3J_{HH}$ = 8.5 Hz); 5.59 (s, 1H, C1'*H*); 4.55 (s, 2H, C(O)*C*H_2O); 4.40 (d, 2H, C(O)OC*H*$_2$, $^3J_{HH}$ = 6.7 Hz); 4.36 (1H, m, C3'*H*); 4.22 (m, 1H, C2'*H*); 4.15 (m, 1H, C4'*H*); 3.99 (m, 2H, C5'*H*$_2$); 3.07 (t, 2H, OCH$_2$C*H*$_2$, $^3J_{HH}$ = 6.7 Hz); 1.01 (d, 18H, 6x C*H*$_3$); 0.92 (s, 9H, 3x C*H*$_3$); 0.19 (s, 3H, C*H*$_3$); 0.11 (s, 3H, C*H*$_3$) ppm.
^{13}C-NMR (400 MHz, $CDCl_3$, 25°C): δ 169.37 (O*C*(O)CH$_2$); 159.61 (*C4*); 148.93 (*C2*); 147.14 (*arom. qC*); 145.17 (*arom. qC*); 133.50 (*C5*); 129.93 (2x *arom. C*); 127.20 (*C6*); 123.93 (2x *arom. C*); 90.77 (*C1'*); 83.02 (*C4'*); 76.36 (*C2'*); 69.51 (*C3'*); 67.54 (C(O)*C*H$_2$O); 64.82 (O*C*H$_2$CH$_2$); 60.22 (*C5'*); 34.80 (OCH$_2$*C*H$_2$); 27.66 (3x *C*H$_3$); 27.38 (3x *C*H$_3$); 25.89 (3x *C*H$_3$); 21.11 (*C*(CH$_3$)$_3$); 20.24 (*C*(CH$_3$)$_3$); 18.16 (*C*(CH$_3$)$_3$); -4.39 (*C*H$_3$); -4.74 (*C*H$_3$) ppm.

1.13 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl) uridine (14)

1,3-$^{15}N_2$ -5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-3',5'-*O*-(di-*t*-butylsilyl) uridine (**13**, 1.77 g, 2.44 mmol, 1.00 eq) was dissolved in anhydr. CH_2Cl_2 and stirred at 0°C under Argon. Thus, hydrogen fluoride in pyridine (240 µL, 9.41 mmol, 3.85 eq) was diluted with excess pyridine (1.52 mL, 18.8 mmol, 7.70 eq) and was added to the cooled solution and stirred for 1 hour. After complete conversion, the org. phase was washed with sat. $NaHCO_3$ and 5% citric acid, dried over Na_2SO_4 and concentrated. 1.42 g 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl) uridine (**14**, 2.43 mmol, quant.) were established and used without further purification.

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.40
^{1}H-NMR (400 MHz, DMSO-d_6, 25°C): δ 11.56 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 8.16 (d, 2H, arom. *H*, $^3J_{HH}$ = 8.5 Hz); 7.75 (s, 1H, C6*H*); 7.55 (d, 2H, arom. *H*, $^3J_{HH}$ = 8.5 Hz); 5.77 (d, 1H, C1'*H*, $^3J_{HH}$ = 4.6 Hz); 5.19 (t, 1H, C5'O*H*, $^3J_{HH}$ = 4.6 Hz); 4.95 (d, 1H, C3'O*H*, $^3J_{HH}$ = 5.4 Hz); 4.54 (s, 2H, C(O)OC*H*$_2$); 4.38 (t, 2H, OC*H*$_2$ CH$_2$, $^3J_{HH}$ = 6.4 Hz); 4.13 (d, 1H, C2'*H*, $^3J_{HH}$ = 4.6 Hz); 3.95 (m, 1H, C3'*H*); 3.88 (m, 1H, C4'*H*); 3.62 (m, 2H, C5'*H*$_2$); 3.07 (t, 2H, C*H*$_2$, $^3J_{HH}$ = 6.4 Hz); 0.83 (s, 9H, 3x C*H*$_3$); 0.02 (s, 3H, C*H*$_3$); 0.00 (s, 3H, C*H*$_3$) ppm.
^{13}C-NMR (400 MHz, DMSO-d_6, 25°C): δ 167.96 (O*C*(O)CH$_2$); 158.94 (*C4*); 149.15 (*C2*); 146.10 (2x *arom. qC*); 133.62 (*C5*); 130.23 (2x *arom. C*); 124.20 (*C6*); 123.38 (2x *arom. C*); 87.93 (*C1'*); 84.25 (*C4'*); 75.38 (*C2'*); 69.59 (*C3'*); 66.57 (C(O)*C*H$_2$O); 63.99 (O*C*H$_2$CH$_2$); 60.25 (*C5'*); 33.90 (OCH$_2$*C*H$_2$); 25.47 (3x *C*H$_3$); 17.83 (*C*(CH$_3$)$_3$); -4.86 (2x *C*H$_3$) ppm.

1.14 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy) -2'-*O*-(*t*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl) uridine (15)

1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl) uridine (**14**, 1.43 g, 2.46 mmol, 1.00 eq) was co-evaporated with anhydr. pyridine and dissolved in anhydr. pyridine. 4,4'-Dimethoxytrityl chloride (1.66 g, 4.92 mmol, 2.00 eq) was added in three portions and the solution was stirred at room temperature for 16 hours under Argon. Thus, the solvent was evaporated, the residue was dissolved in CH_2Cl_2 and washed with sat. $NaHCO_3$ and 5% citric acid. The org. fractions were combined, dried over Na_2SO_4 and concentrated. The crude was purified via column chromatography (SiO_2, ethyl acetate/hexane 1/9 - 7/3) to give 570 mg pure 1,3-$^{15}N_2$-5-(4-

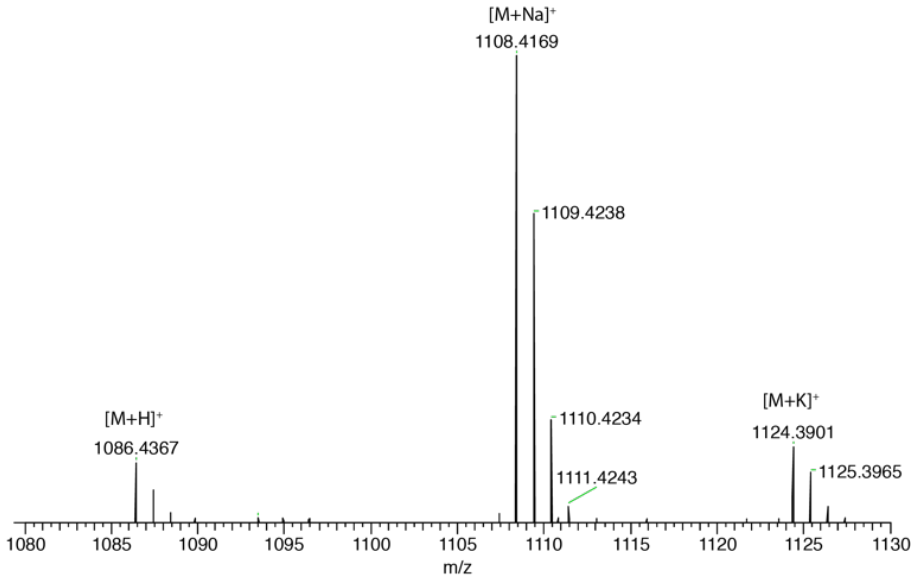
nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl) uridine (**15**, 2.45 mmol, 26% over 3 steps).

TLC: CH_2Cl_2/MeOH 9/1, R_f = 0.85
^{1}H-NMR (400 MHz, DMSO-d_6, 25°C): δ 11.67 (d, 1H, ^{15}N*H*, $^1J_{NH}$ = 90.0 Hz); 8.12 (d, 2H, arom. *H*, $^3J_{HH}$ = 8.5 Hz); 7.45 (d, 2H, arom. *H*, $^3J_{HH}$ = 8.5 Hz); 7.43 (s, 1H, C6*H*); 7.40 (d, 2H, arom. *H*); 7.32 - 7.15 (m, 7H, arom. *H*); 6.87 (d, 4H, arom. *H*); 5.78 (d, 1H, C1'*H*); 5.04 (s, 1H, C3'O*H*); 4.41 (d, 2H, C(O)OC*H*$_2$); 4.32 – 4.19 (m, 3H, C2'*H*, OC*H*$_2$CH$_2$) ; 3.98 (m, 1H, C4'*H*); 3.92 (m, 1H, C3'*H*); 3.72 (s, 6H, 2x OC*H*$_3$); 3.23 (m, 2H, C5'*H*$_2$); 2.99 (t, 2H, OCH$_2$C*H*$_2$); 0.83 (s, 9H, 3x C*H*$_3$); 0.04 (s, 3H, C*H*$_3$); 0.01 (s, 3H, C*H*$_3$) ppm.
^{13}C-NMR (400 MHz, DMSO-d_6, 25°C): δ 167.94 (O*C*(O)CH$_2$); 159.28 (*C4*); 158.08 (2x *arom. qC*); 149.25 (*C2*); 146.26 (*arom. qC*); 146.16 (*arom. qC*); 144.75 (*arom. qC*); 135.32 (*arom.* qC); 135.22 (*arom. qC*); 133.31 (*C5*); 130.11 – 123.36 (*C6*, 13x *arom. C*); 113.20 (4x *arom. C*); 88.27 (*C1'*); 85.82 (O*C*(CR)$_3$ 83.19 (*C4'*); 74.82 (*C2'*); 69.78 (*C3'*); 66.99 (C(O)*C*H$_2$O); 64.00 (O*C*H$_2$CH$_2$); 63.50 (*C5'*); 54.98 (2x O*C*H$_3$); 33.86 (OCH$_2$*C*H$_2$); 25.58 (3x *C*H$_3$); 17.87 (*C*(CH$_3$)$_3$); -5.23 (2x *C*H$_3$) ppm.

1.15 1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl) uridine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (16)

1,3-$^{15}N_2$-5-(4-nitrophenylethylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl) uridine (**15**, 570 mg, 645 µmol, 1.00 eq) was dissolved in anhydr. acetonitrile and stirred at room temperature under Argon. Thus, 5-(benzylthio)-1*H*-tetrazole (136 mg, 709 µmol, 1.10 eq) was added and the solution was stirred for 1 hour. After addition of *N,N*-(tetraisopropyl)-cyanoethyl phosphane (307 µl, 967 µmol, 1.50 eq) the solution was stirred at room temperature for another 4 hours. The reaction mixture was diluted with CH_2Cl_2, washed with half-sat. $NaHCO_3$, dried over Na_2SO_4 and concentrated. The crude was purified via column chromatography (SiO_2, ethyl acetate/hexane 8/2 - 1/0 + TEA 1%) to yield 550 mg 1,3-$^{15}N_2$-5-(4-nitrophenylcarboxymethoxy)-2'-*O*-(*t*-butyldimethylsilyl) -5'-*O*-(4,4'-dimethoxytrityl) uridine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (**16**, 507 µmol, 79%) as a white foam.

TLC: ethyl acetate + TEA 1%, R_f = 0.70
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.13 (d, 2H, arom. *H*, $^3J_{HH}$ = 8.5 Hz); 7.72 (d, 1H, C6*H*); 7.43 (d, 2H, arom. *H*, $^3J_{HH}$ = 7.5 Hz); 7.39 - 7.12 (m, 9H, arom. *H*); 6.82 (d, 4H, arom. *H*, $^3J_{HH}$ = 7.5 Hz); 5.98 (m, 1H, C1'*H*); 4.50 - 4.07 (m, 7H, C2'*H*, C3'*H*, C4'*H*, C*H*$_2$O, OC*H*$_2$C(O)); 4.05 - 3.81 (m, 1H, NC*H*); 3.77 (s, 6H, 2x OC*H*$_3$); 3.67 - 3.20 (m, 4H, C5'*H*$_2$, OC*H*$_2$); 2.95 (t, 2H, C*H*$_2$, $^3J_{HH}$ = 6.4 Hz); 2.64 (s, 1H, NC*H*); 2.32 (t, 1H, C*H*$_2$); 1.33 - 0.80 (m, 21H, 7x C*H*$_3$); 0.10 (s, 6H, 2x C*H*$_3$) ppm.
^{31}P-NMR (300 MHz, $CDCl_3$, 25°C): δ 151.97 (s); 150.45 (s).
ESI-MS: $[M+H]^+$ 1086.4367, $[M+Na]^+$ 1108.4169, $[M+K]^+$ 1124.3901 (calc. 1086.4426)



1.16 1,3-$^{15}N_2$-5-Carboxymethoxy uridine (18)

1,3-$^{15}N_2$-5-Methylcarboxymethoxy uridine (**10**, 200 mg, 602 µmol, 1.00 eq) was suspended in H_2O and stirred at room temperature. Thus, aq. NaOH (622 µl, 622 µmol, 1M, 1.10 eq) was added and the solution was stirred for 1 hour. After completion, the reaction mixture was neutralized with conc. HCl, concentrated, desalted over size exclusion column and dried under HV to give 191 mg pure 1,3-$^{15}N_2$-5-carboxymethoxy uridine (**18**, 602 µmol, quant.).

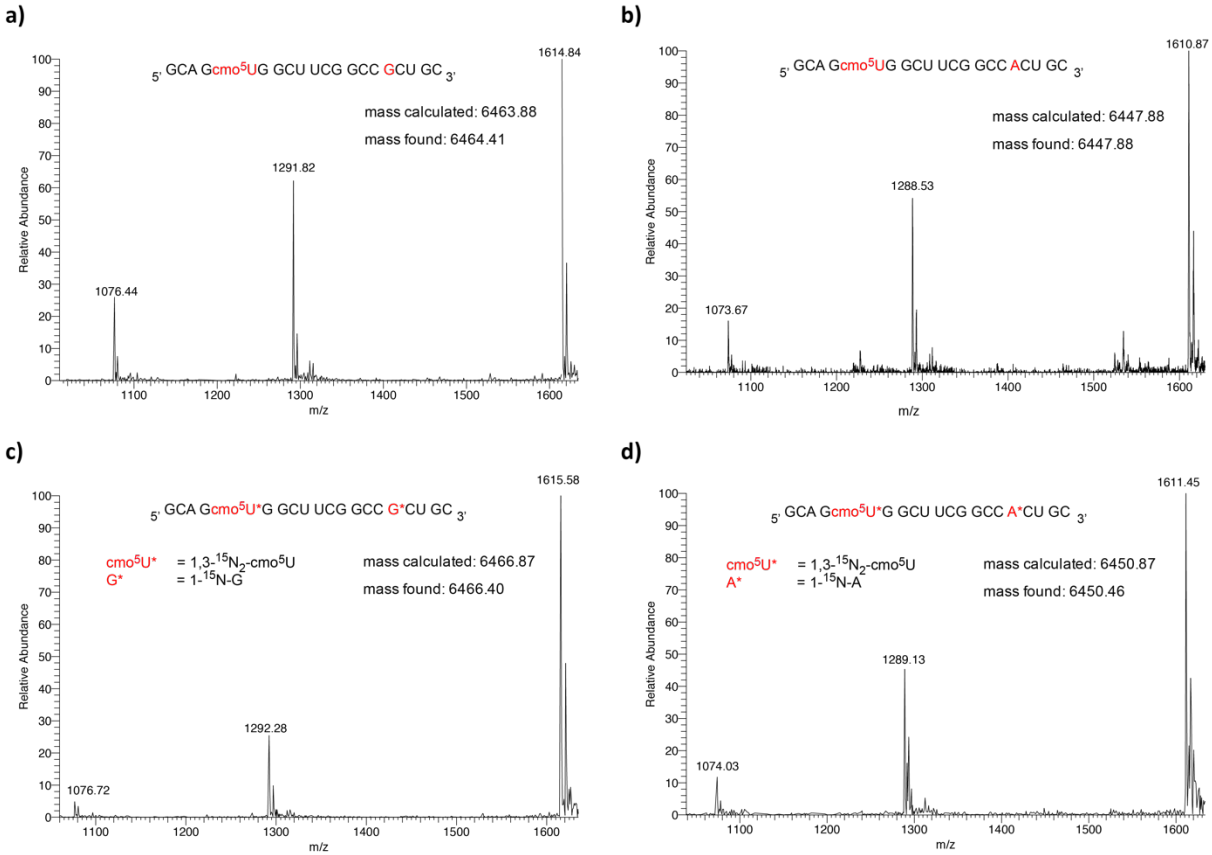
TLC: CH_2Cl_2/MeOH 8/2, R_f = 0.0
^{1}H-NMR (400 MHz, D_2O, 25°C): δ 7.80 (s, 1H, C6*H*); 6.05 (s, 1H, C1'*H*); 4.70 (s, 2H, C(O)CH_2O); 4.47 (m, 1H, C2'*H*); 4.41 (m, 1H, C3'*H*); 4.43 (m, 1H, C4'H); 4.08 (m, 1H, C5'H_2); 3.98 (m, 1H, C5'H_2) ppm.
^{13}C-NMR (400 MHz, D_2O, 25°C): δ 172.83 (O*C*(O)CH_2); 161.33 (*C4*); 150.09 (*C2*); 134.75 (*C5*); 122.42 (*C6*); 89.32 (*C1'*); 84.44 (*C4'*); 74.14 (*C2'*); 69.46 (*C3'*); 67.27 (C(O)*C*H_2O); 60.64 (*C5'*H_2) ppm.

2 RNA solid phase synthesis

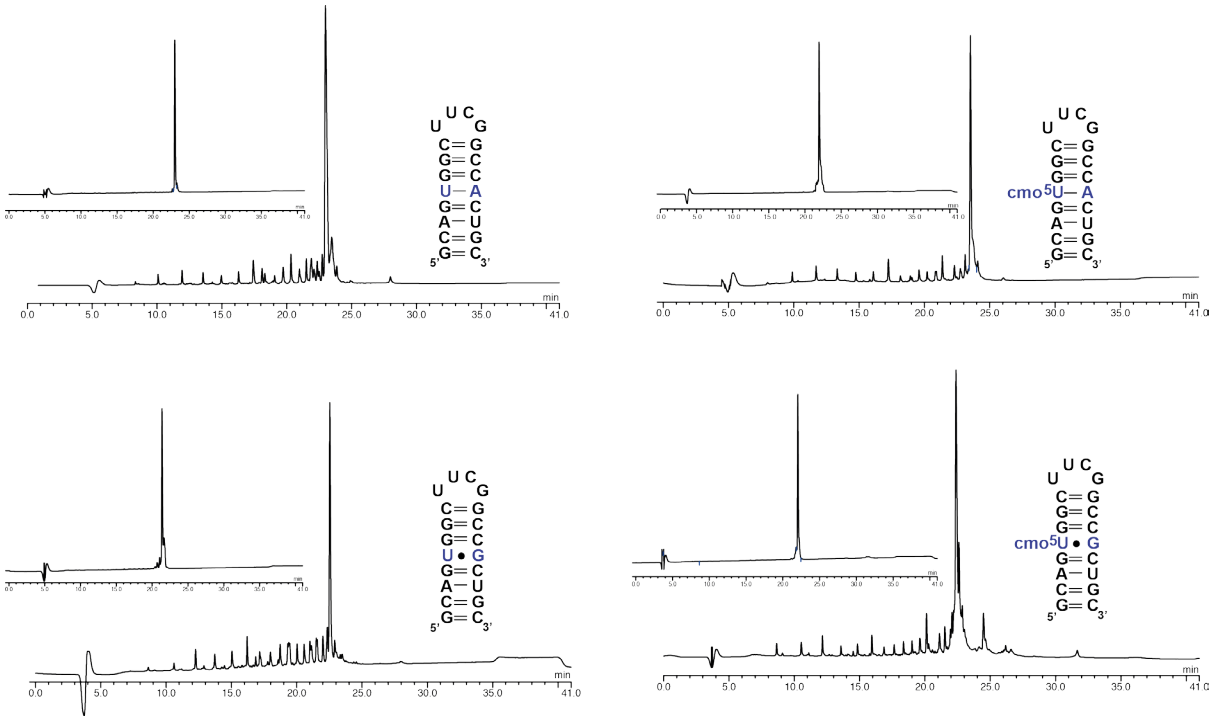
Standard and stable isotope labeled 2'-*O*-TBDMS phosphoramidites were used to assemble the described non-modified as well as cmo^{5}U-modified RNAs. TBDMS-protected controlled pore glass (CPG) solid support (1000 Å pore size, *ChemGenes*) with an average loading of 40 µmol g^{-1} was used. The sequences were synthesized on an ABI 391 PCR Mate using a self-written RNA/DNA synthesis cycle. Amidite (0.1 M) and activator (5-benzylthio-*1H*-tetrazole, 0.25 M) solutions were dried over freshly activated molecular sieves (3 Å) for at least 24 hours. The following reagent mixtures were used: *Cap A*: 5.7 g phenoxyacetic anhydride dissolved in 200 mL anhydrous tetrahydrofuran. *Cap B*: 20 mL *N*-methylimidazole, 20 mL 2,3,5-trimethylpyridine and 160 mL anhydrous tetrahydrofuran. *Oxidation solution*: 500 mg iodine dissolved in a mixture of 70 mL THF, 20 mL pyridine and 10 mL water. *Detritylation solution*: 4% dichloroacetic acid in 1,2-dichloroethane. After complete RNA synthesis, the solid support was dried in high vacuum. For cleavage of the cmo^{5}U-nitrophenylethyl (NPE) protective group, the column was treated with 20 mL freshly prepared 1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution in anhydrous acetonitrile for 30 minutes at room temperature. After washing with 50 mL acetonitrile, the solid support was dried in high vacuum and transferred into a 1.5 mL reaction tube. *Standard alkaline deprotection*: 650 µL aq. methylamine solution (40 %) and 650 µL aq. ammonia solution (28-30 %) were added to the solid support. The reaction tube was shaken vigorously and incubated at 37°C for 12-16 hours. The solid support was pelleted via centrifugation and the supernatant was filtered. The remaining solid support was washed three times with a mixture of THF/water (1/1), the liquid phases were filtered, combined with the first filtrate and evaporated to dryness in a 10 mL round bottom flask. The residue was dried in high vacuum for at least 1 hour. *2'-O-TBDMS deprotection:* 1.5 ml freshly prepared 1 M tetrabutylammonium fluoride (TBAF) solution in anhydrous THF were added to the flask. After the RNA has fully dissolved, the flask was sealed with a glass stopper and incubated at 37°C for 20-24 hours. Then, the deprotection solution was quenched with 1.5 ml 1 M triethyl ammonium acetate (TEAA). The THF was evaporated and the concentrated solution was applied to a HiPrep 26/10 desalting column (*GE Healthcare*) using a ÄKTA start system (*GE Healthcare*). The crude RNA was eluted using HPLC grade water and the RNA containing fractions (UV detection at 254 nm) were collected in a 50 mL round bottom flask. After evaporation, the crude RNA was dissolved in 1 mL HPLC grade water and transferred to a 1.5 mL reaction tube. The crude RNA was stored at -20°C. The quality of the crude RNAs was checked via anion exchange chromatography on an analytical Dionex

DNAPac PA-100 column (4x250 mm; *Eluent A*: 25 mM Tris.HCl, 6 M urea, pH 8.0; *Eluent B*: 25 mM Tris.HCl, 500 mM sodium perchlorate, 6 M urea, pH 8.0) and at elevated temperature (80 °C). Purification of the RNA sequences was achieved in a single run by applying the crude RNA on a preparative Dionex DNAPac PA-100 column (22x250 mm, eluents as before). The fractions containing the desired RNA were pooled and loaded on a C18 SepPak catridge (*Waters*) to remove HPLC buffer salts. The RNA sodium salt form was then eluted from the C18 column with water/acetonitrile (1/1, v/v), concentrated and transferred to a 1.5 ml reaction tube for concentration evaluation and mass spectrometric analysis. Sample concentrations were determined by measuring UV absorption at 260 nm on a NanoPhotometer (*Implen*).

3 MS of cmo^{5}U-modified RNAs and Anion exchange chromatographic analysis of RNAs



Supporting Figure 1. LC-ESI mass spectra of cmo^{5}U-modified 20 nt hairpin RNA incorporating the following base pairs: **a)** cmo^{5}U•G; **b)** cmo^{5}U-A; **c)** ^{15}N-labeled cmo^{5}U*•G*; **d)** ^{15}N-labeled cmo^{5}U*-A*.



Supporting Figure 2. Anion-exchange HPLC traces of all RNAs used in the study. The crude RNA and the purified RNA (inset) were injected. For details of HPLC conditions refer to the RNA solid phase synthesis section.

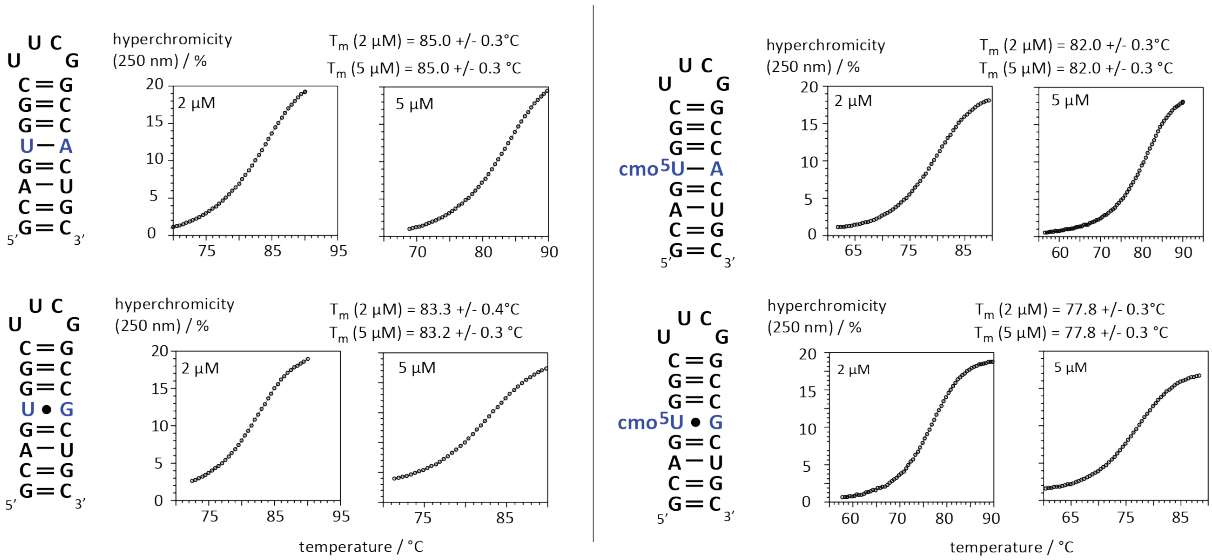
4 Measurement of UV melting curves and extraction of thermodynamic data

Appropriate amounts of unmodified and cmo^{5}U-modified RNAs were lyophilized and then dissolved in 800 µL melting curve buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCl) to give RNA concentrations of 2 and 5 µM. Absorbance versus temperature profiles were recorded at 250 nm and 260 nm on a Cary-100 spectrophotometer equipped with a multiple cell holder and a Peltier temperature-control device. Data were collected for five heating-cooling cycles at a rate of 0.7 °C/minute. Melting transitions were essentially the same with respect to the two different wavelengths and heating-cooling cycles. Melting point temperatures are reported as mean value of the five measurements.

The thermodynamic parameters of the monomolecular melting process were obtained by plotting the association degree α versus temperature and fitting the experimental data in *KaleidaGraph* (Synergy Software using the following equation:

$$\alpha = \frac{1}{1 + e^{\frac{\Delta H^0 - T\Delta S^0}{RT}}}$$

with α association degree and R ideal gas constant to give the enthalpy ΔH^0 and the entropy ΔS^0 of the melting transition.



Supporting Figure 3. UV melting curve data of all four RNA constructs for the extraction of the thermodynamic data.

5 NMR sample preparation

RNA samples were lyophilized as sodium salts and dissolved in the respective buffer: 15 mM sodium phosphate, 25 mM NaCl, 0.1 mM EDTA, pH 6.9 or 8.0, at 5 - 25 °C.

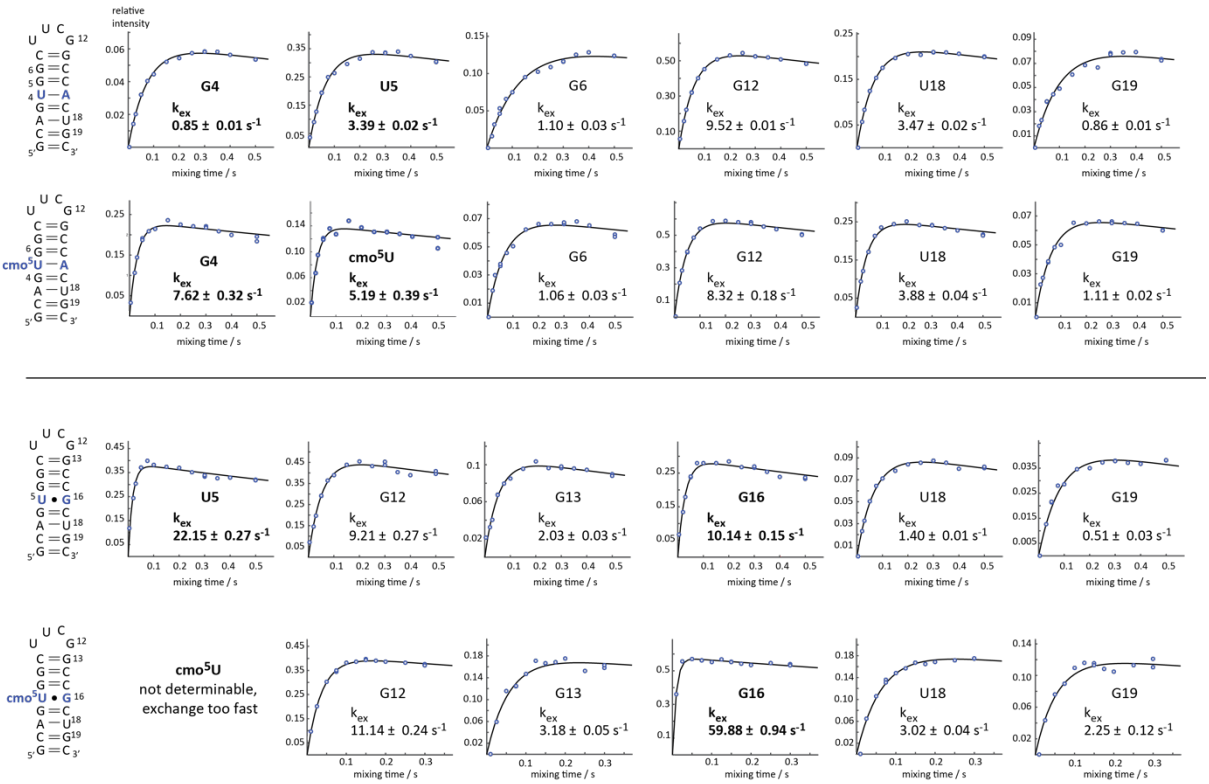
6 Determination of imino proton water exchange rates by CLEANEX-PM experiment

The CLEANEX-PM NMR experiments were carried out on unlabeled RNAs in the standard buffer (15 mM sodium phosphate, 25 mM NaCl, 0.1 mM EDTA, pH 6.9) at 25 °C. The same buffer stock solution was used for all samples to rule out changes in imino proton - bulk water exchange rates due to differences in the buffer composition. The CLEANEX-PM pulse sequence is available from the Bruker standard experiment collection (*zgcxesgp*) with an excitation sculpting water suppression element. A standard excitation sculpting water suppression experiment (*zgesgp*) was used as the reference experiment. For the determination of the water T_1 relaxation times under the experimental conditions a saturation recovery experiment was used and the longitudinal water relaxation time ranged between 3.00 and 3.33 s ($R_{1,water}$ = 0.333 - 0.309 s^{-1}). The following experimental parameters were used: spectral width 24 ppm, o1p: 4.7 ppm, number of scans 1024, dummy scans 32, interscan delay 1.5 s. Shaped pulse parameters were set via the *getprosol* command and the hard 90° 1H pulse. The mixing times were set to 5, 20, 30, 50, 50, 75, 100, 150, 200, 250, 300, 300, 350, 400, 500 and 500 milliseconds. for the U-A, U•G and cmo^5U-A construct. For the cmo^5U•G the CLEANEX mixing times were set to 10, 25, 50, 75, 75, 100, 125, 150, 150, 175, 200, 250, 300 and 300 milliseconds. The NMR spectra were processed in TOPSPIN 3.5 pl7 using a line broadening factor of 10 Hz (lb value set to 10). The absolute peak intensities of the CLEANEX-PM experiments and the reference experiments were determined and used to obtain the relative intensities.

The data was then exported to MATLAB and the build-up curves were fitted to the following equation:

$$I/I_0 = \frac{k}{(R_{1A}+k-R_{1,water})} \mathrm{X}\left\{e^{-R_{1,water}\tau_{mix}} - e^{(-R_{1,A}+k)\tau_{mix}}\right\}$$

with I/I_0 relative peak intensity, k imino proton- bulk water exchange rate / s^{-1}, $R_{1,water}$ longitudinal water exchange rate, τ_{mix} CLEANEX-PM mixing times / s and $R_{1,A}$ is a combination of the longitudinal and transverse relaxation rate of the imino proton resonance (floating parameter during fitting). Errors in exchange rates were estimated from replicate experiments and obtained from 1000 Monte-Carlo runs.



Supporting Figure 4. Relative intensities versus mixing times from CLEANEX-PM NMR experiments. Fitting of the experimental data gave imino proton- bulk water exchange rates. Significant changes in exchange rates are highlighted in bold. For a detailed discussion see main text.

Supporting Table 1. Imino proton water exchange rates of the A-U hp, G•U hp, cmo^{5}U-A hp and cmo^{5}U•G hp from CLEANEX-PM experiments.

base pair	X-Y base pair U5-A16	X-Y base pair cmo^{5}U5-A16	X-Y base pair U5•G16	X-Y base pair cmo^{5}U5•G16
G1-C20	-	-	-	-
C2-G19	0.86 ± 0.01	1.11 ± 0.02	0.51 ± 0.03	2.25 ± 0.12
A3-U18	3.47 ± 0.02	3.88 ± 0.04	1.40 ± 0.01	3.02 ± 0.04
G4-C17	0.85 ± 0.01	7.62 ± 0.32	-	-
X5-Y16	3.39 ± 0.02	5.19 ± 0.39	22.15 ± 0.27/10.14 ± 0.15	*/59.88 ± 0.94
G6-C15	1.10 ± 0.03	1.06 ± 0.03	-	-
G7-C12	-	-	-	-
C8-G13	-	-	2.03 ± 0.03	3.18 ± 0.05
U9-G12	-/9.53 ± 0.01	-/8.32 ± 0.18	-/9.21 ± 0.27	-/11.14 ± 0.24

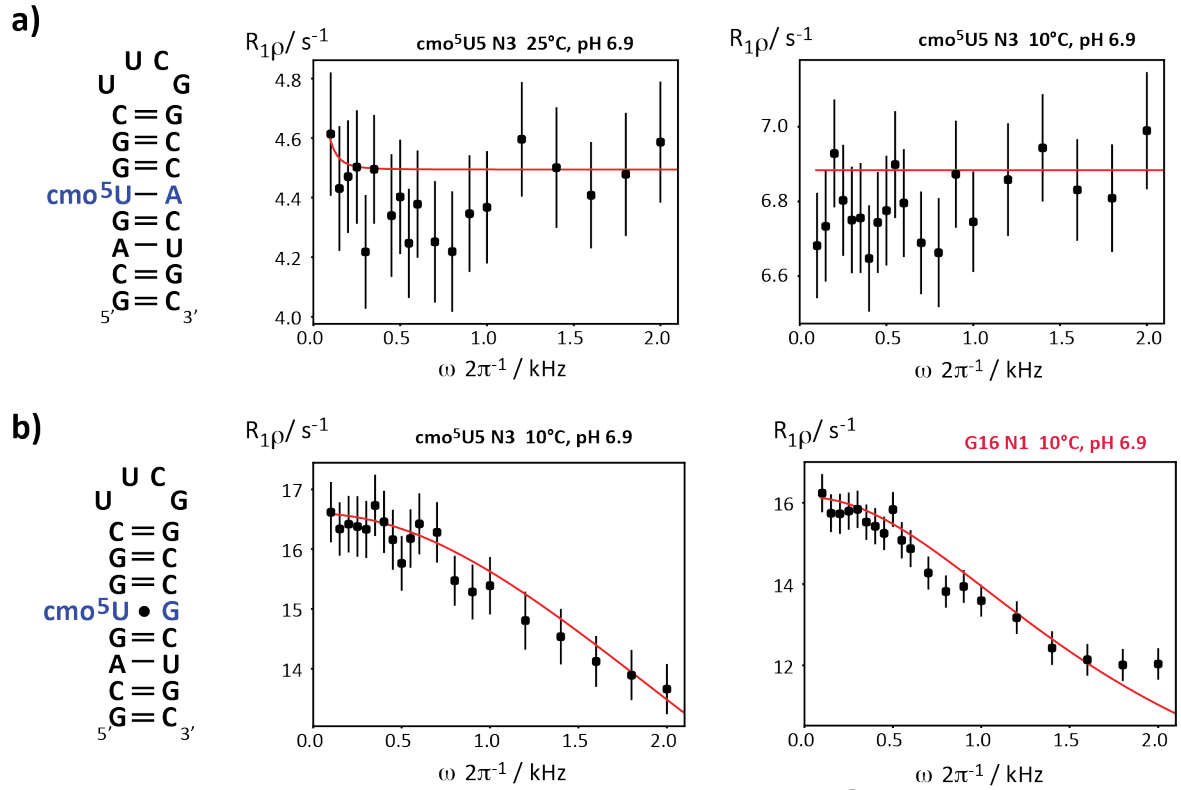
Exchange rates given in s^{-1}. Some exchange rates were not determinable either due to too slow exchange (e.g. G7), or the imino proton not being observable at 25 °C (e.g. G1), or resonance overlap (e.g. G4/6 in the hairpins with wobble mismatch). *cmo^{5}U5 NH^3 imino proton exchange broadened beyond detection at 25 °C.

7 $R_{1\rho}$ relaxation dispersion experiments

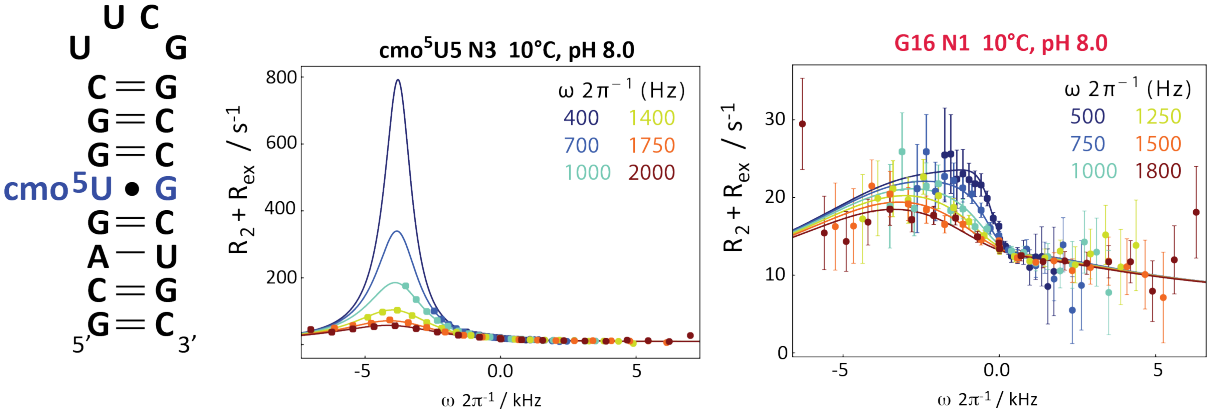
All RD data was collected on a 700 MHz Bruker Avance spectrometer equipped with cryogenic HCN probes using a ^{15}N $R_{1\rho}$ experiment as described previously.[1] Peaks from 1D experiments were fit to a Lorentzian peak shape, to extract peak intensities as a function of delay time, which were then fit to an exponential decay to get the $R_{1\rho}$ value for a given spin lock power offset combination. On- and off-resonance $R_{1\rho}$ measurements were performed using spinlock powers ranging from 100 to 2,000 Hz, with the absolute offset frequencies (Ω $2\pi^{-1}$ Hz) ranging from 0 – 3.5 × the applied spinlock power. Errors in $R_{1\rho}$ were calculated using a Monte-Carlo approach as described previously.[2] Exchange parameters of interest were obtained by fitting the measured $R_{1\rho}$ values using numerical Bloch-McConnell simulations. For a given choice of exchange parameters, the Bloch-McConnell equations were used to find the corresponding dependence of $R_{1\rho}$ on spin-lock power and offset. The combination of parameters that minimized the residual sum of squares of the experimental and simulated $R_{1\rho}$ data was then found. Errors in the exchange parameters were computed based on the standard error of the fit. Model selection (2-state vs. 3-state fitting) was performed by calculating the Akaike's and Bayesian information criteria for each model as described previously (16).

Supplementary Table 2. Extracted parameters from fitting the off-resonance ^{15}N-$R_{1\rho}$-relaxation dispersion profiles at 10°C and various pH values.

	cmo^{5}U5-N3, pH 6.9		**G16-N1, pH 6.9**	
	Fit value	Error	Fit value	Error
p_B (enolic) / %	**0.16**	0.03	0.16	0.03
p_C (anionic) / %	**0.24**	0.03	0.24	0.03
$\Delta\omega_B$ (enolic) / ppm	16.0	4.8	40.4	4.0
$\Delta\omega_C$ (anionic) / ppm	55.4	2.9	-0.02	3.52
$k_{ex,GS\text{-}ESB}$ (enolic) / s^{-1}	5949	709	5949	709
$k_{ex,GS\text{-}ESC}$ (anionic) / s^{-1}	976	683	976	683
$k_{ex,ESB\text{-}ESC}$ /s^{-1}	38222	8416	38222	8416
R_1 / s^{-1}	1.57	0.05	1.45	0.05
R_2 / s^{-1}	6.26	0.17	6.27	0.26
red. X^2	0.56		0.56	
	cmo^{5}U5-N3, pH 8.0		**G16-N1, pH 8.0**	
	Fit value	Error	Fit value	Error
p_B (enolic) / %	**0.14**	0.04	0.14	0.04
p_C (anionic) / %	**0.85**	0.21	0.85	0.21
$\Delta\omega_B$ (enolic) / ppm	10.3	5.7	42.3	4.5
$\Delta\omega_C$ (anionic) / ppm	55.8	0.75	1.3	1.1
$k_{ex,GS\text{-}B}$ (enolic) / s^{-1}	8059	4243	8059	4243
$k_{ex,GS\text{-}C}$ (anionic) / s^{-1}	581	662	581	662
$k_{ex,B\text{-}C}$ /s^{-1}	24771	3901	24771	3901
R_1 / s^{-1}	1.7	0.06	1.7	0.08
R_2 / s^{-1}	6.97	0.24	6.35	1.53
red. X^2	0.52		0.52	



Supporting Figure 5. On-resonance relaxation dispersion profiles. **a)** In the cmo^{5}U-A RNA flat dispersion profiles were obtained indicative of "static base pairing" at 25°C or 10°C and pH 6.9. **b)** In the cmo^{5}U•G RNA non-flat dispersion profiles were obtained indicative of exchange between alternative base pairing states at 10°C and pH 6.9.



Supporting Figure 6. Off-resonance relaxation dispersion profiles at pH 8.0.

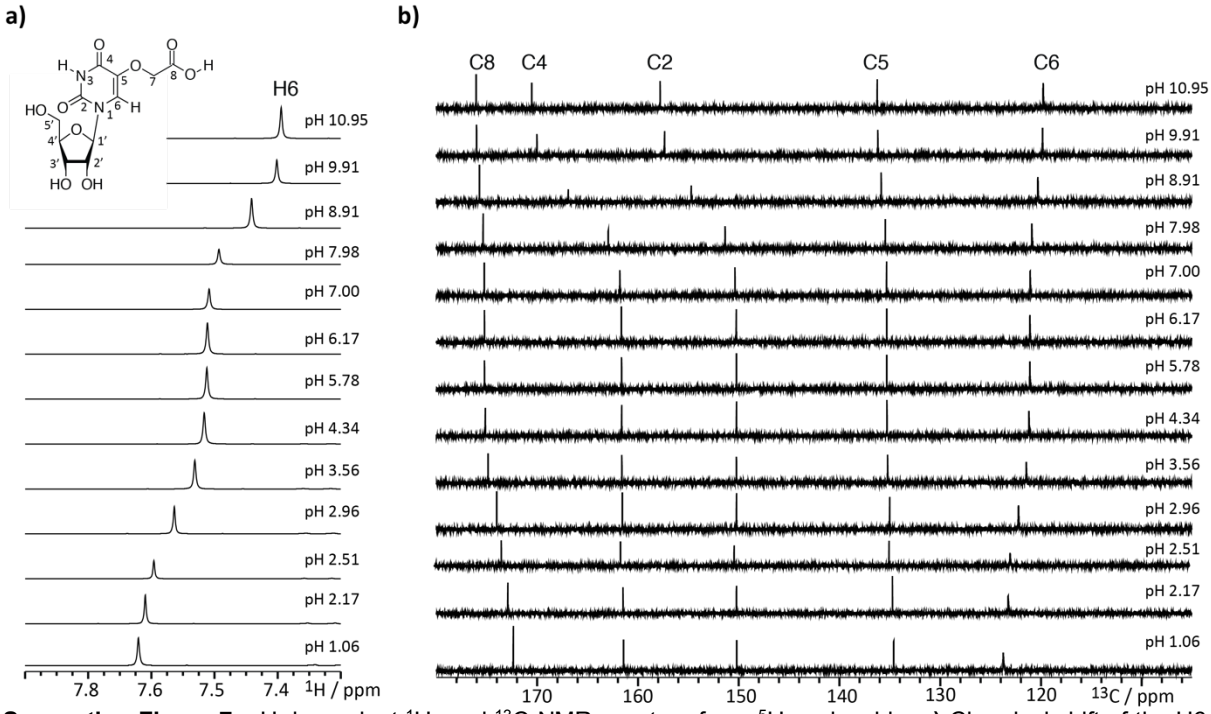
8 pH-dependent ^{13}C-NMR spectra of cmo^{5}U nucleoside and pK$_a$ fitting

For the determination of the pK$_a$ values of the cmo^{5}U nucleoside pH dependent NMR experiments were carried out. For this purpose, 25 mg of cmo^{5}U nucleoside were dissolved in the standard buffer and the pH of the sample was determined directly in the 5 mm NMR tube using a Sigma-Aldrich micro pH combination glass electrode. The pH was gradually adjusted by the addition of microliter amounts of 100 mM hydrochloric acid and 100 mM sodium hydroxide solutions, respectively. The pH of the solution was checked before and after the measurement. While the NMR spectrum was collected, the electrode was submerged in the pH 7 (for data between pH 10.95 and 5.778) or pH 4 (for data below pH 5.78)

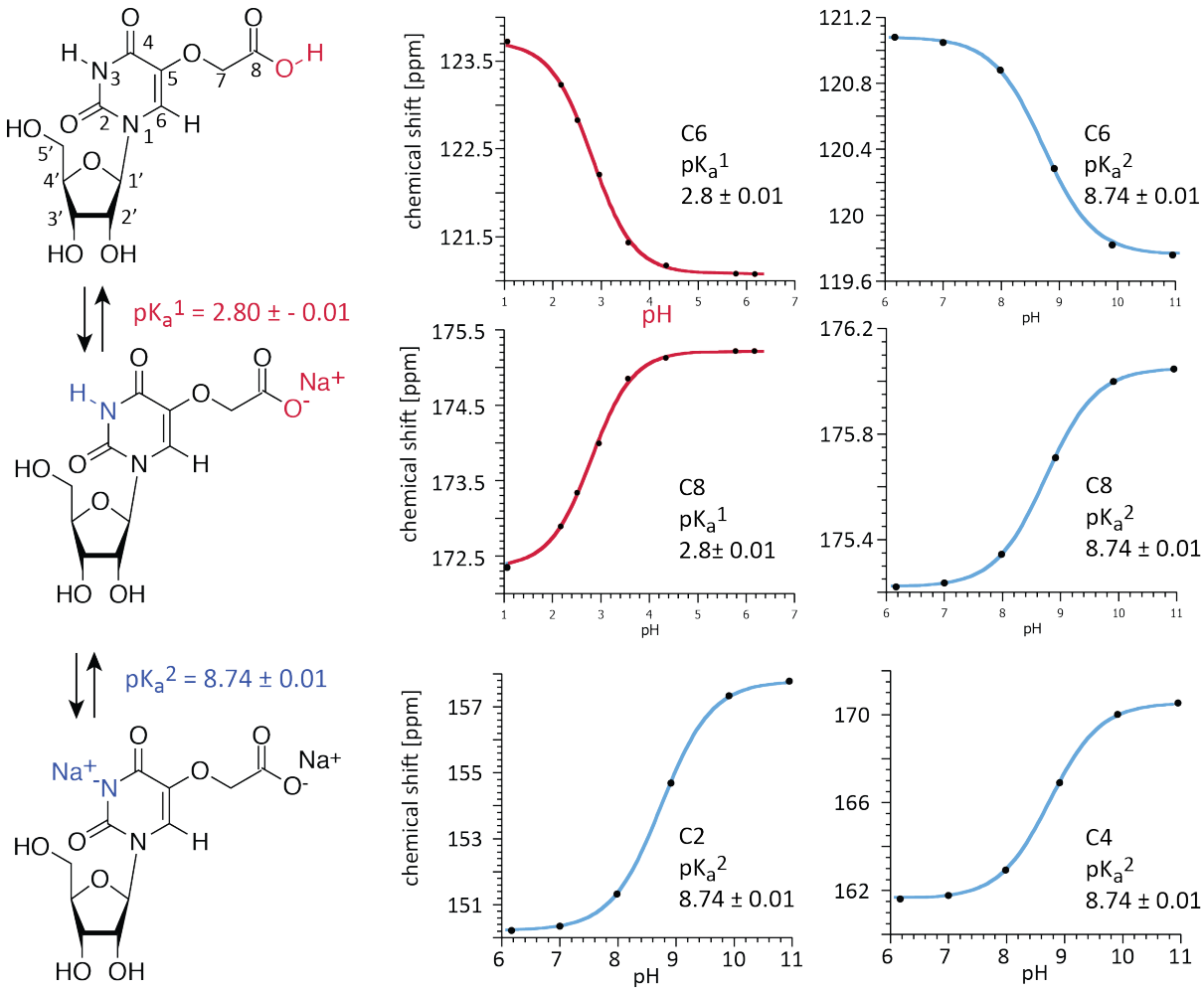
standard buffer. At each pH value, a 1D ^{1}H- NMR spectrum (*zgesgp*) and a ^{13}C-NMR spectrum at natural abundance with power gated decoupling (zgpg30) were collected. The NMR spectra were processed in TOPSPIN 3.5 pl7 and the peak positions as a function of pH were exported to *KaleidaGraph* (Synergy Software). The two pK_a values were obtained by fitting the data to the following equation:

$$\delta_{obs} = \frac{\delta_{deprotonated} \times 10^{(pH-pK_a)} + \delta_{protonated}}{1 + 10^{(pH-pK_a)}}$$

with δ_{obs} observed ^{13}C chemical shift at the respective pH value, $\delta_{deprotonated}$ ^{13}C chemical shift of deprotonated species and $\delta_{protonated}$ ^{13}C chemical shift of protonated species.



Supporting Figure 7. pH dependent ^{1}H- and ^{13}C-NMR spectra of cmo^{5}U nucleoside. **a)** Chemical shift of the H6 resonance as a function of pH. **b)** ^{13}C chemical shifts of C2, C4, C5, C6 and C8 as a function of pH. The ^{13}C-spectra were obtained at natural abundance.



Supporting Figure 8. Acid/base equilibria in the cmo^{5}U nucleoside. A selection of pH dependent ^{13}C- chemical shift data used for fitting the pK_a values of the cmo^{5}U nucleoside. The pK_a values were obtained fitting the NMR data according to the equation described above.