Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supporting Information

Chemical synthesis and NMR spectroscopy of long stable isotope labelled RNA

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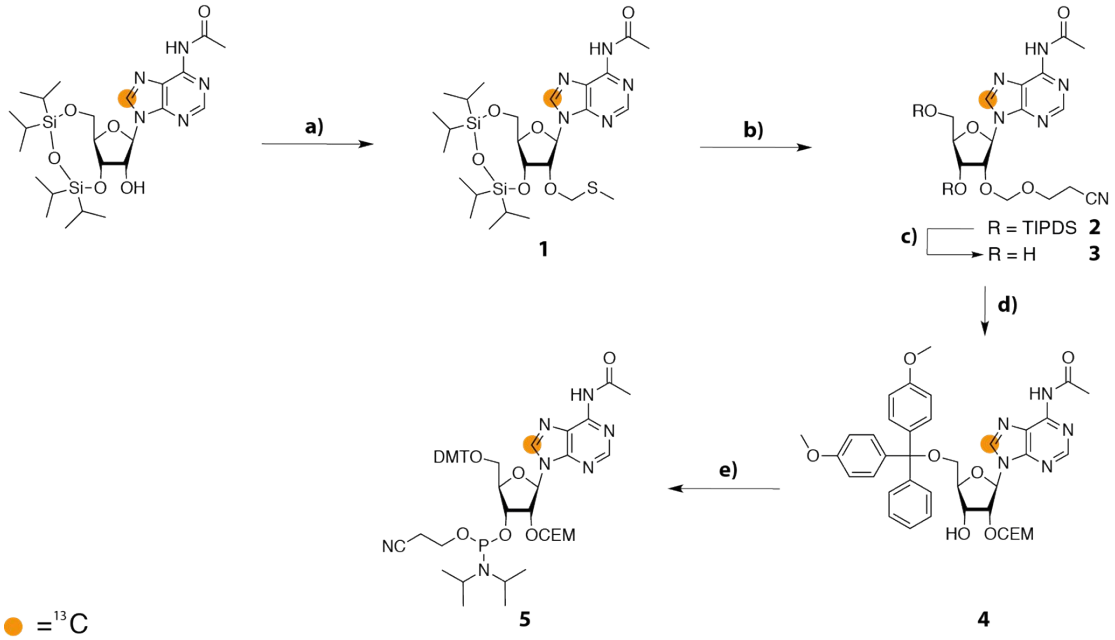
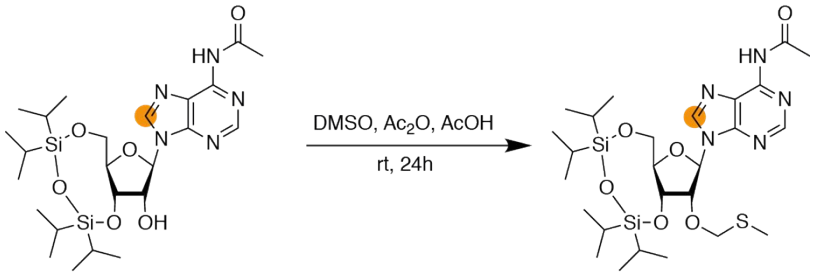
1 Synthetic procedures for $^{13}C/^{2}H$ labeled CEM RNA phosphoramidites

Synthesis of 2'-O-CEM-(8-^{13}C)-Adenosine 5´-O-DMT protected building block (compound 1 in manuscript)



Scheme 1: Synthetic route to 2'-O-CEM-(8-^{13}C)-Adenosine 5´-O-DMT protected building block; reagents and conditions: ***(a)****, DMSO, Ac_2O, AcOH, rt, 24 h, 54%;* ***(b)*** *3-hydroxypropionitrile, TfOH, NIS, Et_3N, in THF, -45°C, 1.5 h, 68%;* ***(c)*** *TEA·3HF, in THF, 45°C, 2 h, not purified;* ***(d)*** *DMT-Cl, in pyridine, rt, 1.5 h, 93%;* ***(e)*** *CEP-Cl, DIPEA, in CH_2Cl_2, rt, 2 h, 86%.*

N^6-Acetyl-2'-*O*-(methylthiomethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-(8-^{13}C)-adenosine (1)



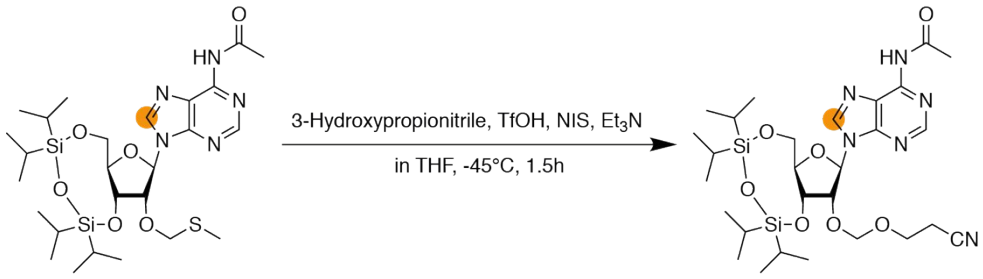
$C_{23}{}^{13}CH_{41}N_5O_6Si_2$
[552.78]

$C_{25}{}^{13}CH_{45}N_5O_6SSi_2$
[612.90]

N^6-Acetyl-3',5'-(tetraisopropyldisiloxane-1,3-diyl)-(8-^{13}C)-adenosine (2.50 g, 4.52 mmol, 1.00 eq.)[1] was dissolved in dimethyl sulfoxide (10.0 mL, 141 mmol, 31.1 eq.) and acetic anhydride (7.50 mL, 74.1 mmol, 16.4 eq.), then acetic acid (7.50 mL, 131 mmol, 29.0 eq.) were added. The solution was stirred at room temperature under argon atmosphere for 24 hours. Monitoring by TLC (EtOAc) showed a complete conversion, so the mixture was slowly poured into 60 mL of saturated sodium bicarbonate solution. It was extracted with n-hexane/EtOAc = 1/2 twice and the organic phase was washed with water. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The

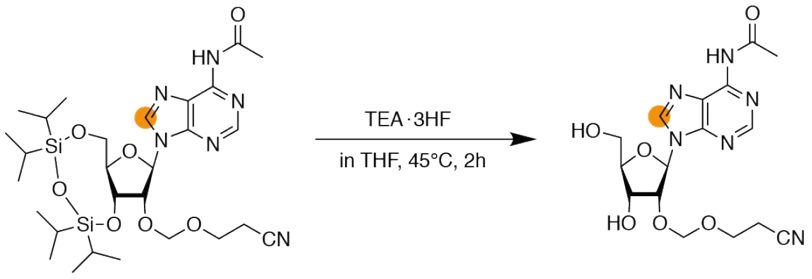
crude product was purified by column chromatography (50 g, SiO_2, n-hexane/EtOAc: 6/4 – 3/7) and pure compound **1** was obtained as a white foam after drying under high vacuum.
Yield: 1.49 g of a white foam (2.43 mmol, 54%)
TLC: (EtOAc); R_f = 0.51
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 9.41 (s, 1H, N^6*H*); 8.79 (s, 1H, C(2)*H*); 8.35 (d, 1H, C(8)*H*, $^1J_{CH}$ =212.24 Hz); 6.13 (s, 1H, C(1')*H*); 5.12 – 5.01 (2xd, 2H, -O-CH_2-S-, $^2J_{HH}$ = 11.40 Hz, $^2J_{HH}$ = 11.40 Hz); 4.77 – 4.70 (m, 2H, C(2')*H,* C(3')*H*); 4.31 – 4.05 (m, 1H, C(5')*H*; C(5'')*H*; C(4')*H*); 2.67 (s, 3H, NH-CO-CH_3); 2.24 (s, 3H, -S-CH_3) 1.14 – 0.98 (m, 28H, 4x Si-(C*H*)-(CH_3)$_2$; 4x Si-(CH)-(CH_3)$_2$)

N^6-Acetyl-2'-*O*-(2'cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-(8-^{13}C)-adenosine) (2)



Compound **1** (1.49 g, 2.43 mmol, 1.00 eq.) was dissolved in 15 mL anhydrous tetrahydrofuran, 1.48 g 4A molecular sieves and 3-hydroxypropionitrile (4.15 g, 58.3 mmol, 24.0 eq.) were added and the solution was cooled in a cold bath made of acetonitrile and liquid nitrogen (kept at -45°C), stirred under argon atmosphere for 30 minutes. Trifluoromethanesulfonic acid (0.86 g, 4.86 mmol, 2.00 eq.) was slowly dropped into the mixture, then *N*-iodosuccinimide (0.66 g, 2.92 mmol, 1.20 eq.) was added in one portion. The reaction mixture was stirred for 20 minutes at -45°C till triethylamine (0.75 mL 5.33 mmol, 2.19 eq.) was slowly added to quench the reaction and it was stirred for further 15 minutes. The mixture was diluted with ethyl acetate/n-hexane = 3/1 and the molecular sieves were filtered. The filtrate was washed with saturated, ice cold sodium thiosulfate solution twice, saturated sodium bicarbonate solution twice and saturated sodium chloride solution once. The aqueous phases were extracted with ethyl acetate/n-hexane = 3/1, the organic layers were combined and dried over sodium sulfate. After all solvents were removed under reduced pressure, the crude product was purified by column chromatography (40 g SiO_2, EtOAc/n-hexane: 6/4 – 10/0; EtOAc/MeOH: 9/1). The pure compound **2** was isolated as a white foam.
Yield: 1.04 g of a white foam (1.65 mmol, 68%)
TLC: (EtOAc); R_f = 0.32
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.69 (s, 1H, N^6*H*); 8.50 (s, 1H, C(2)*H*); 8.35 (d, 1H, C(8)*H*, $^1J_{CH}$ = 214.39 Hz); 6.14 (s, 1H, C(1')*H*); 5.11 (d, 1H, -O-CH_2'-O-, $^2J_{HH}$ = 7.06 Hz); 5.05 (d, 1H, -O-CH_2''-O-, $^2J_{HH}$ = 7.06 Hz); 4.71 (m, 1H, C(2')*H*); 4.53 (d, 1H, C(3')*H,* $^3J_{HH}$ = 4.33 Hz); 4.31 (d, 1H, C(5')*H*, $^2J_{HH}$ = 13.02 Hz); 4.21 (d, 1H, C(5'')*H*, $^2J_{HH}$ = 9.47 Hz); 4.13 – 4.05 (m, 2H, -O-CH_2-CH_2-); 3.88 (m, 1H, C(4')*H*); 2.73 – 2.60 (t, 2H, -CH_2-CH_2-CN, $^3J_{HH}$ = 6.43 Hz); 2.67 (s, 3H, NH-CO-CH_3); 1.30 – 0.98 (m, 28H, 4x Si-(C*H*)-(CH_3)$_2$; 4x Si-(CH)-(CH_3)$_2$)

N⁶-Acetyl-2'-*O*-(2'cyanoethoxymethyl)-(8-^{13}C)-adenosine (3)

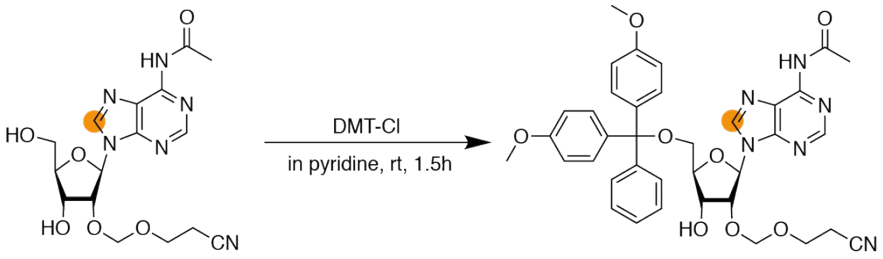


$C_{27}{}^{13}CH_{46}N_6O_7Si_2$
[635.87]

$C_{15}{}^{13}CH_{20}N_6O_6$
[393.36]

Compound **2** (1.04 g, 1.64 mmol, 1.00 eq.) was dissolved in 10 mL anhydrous tetrahydrofuran and TEA·3HF (0.29 g, 1.81 mmol, 1.10 eq.) was added. The solution was stirred under argon atmosphere at 45°C for 2 hours. TLC (CH_2Cl_2/MeOH = 10/1) showed a complete conversion, the solvent was removed in *vacuo* and crude compound **3** was obtained.
Further purification actions and a characterization with NMR spectroscopy were not carried out, crude product **3** was applied for the next reaction step.
Yield: Assumption: 643 mg of compound 3 (1.64 mmol, 100%)
TLC: (CH_2Cl_2/MeOH = 10/1), R_f = 0.16
^{1}H-NMR: not determined

N⁶-Acetyl-2'-*O*-(2'cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-(8-^{13}C)-adenosine (4)



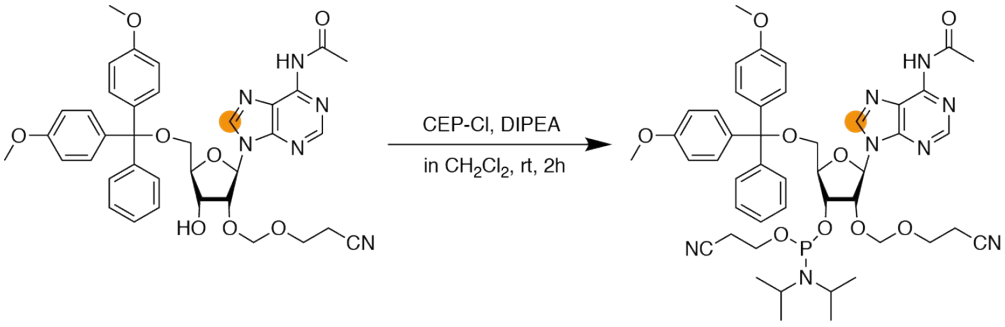
$C_{15}{}^{13}CH_{20}N_6O_6$
[393.36]

$C_{36}{}^{13}CH_{38}N_6O_8$
[695.74]

Crude compound **3** (643 mg, 1.63 mmol, 1 eq.) was co-evaporated with anhydrous pyridine thrice, then dissolved in 10 mL anhydrous pyridine. To the reaction mixture 4,4'-dimethoxytrityl chloride (0.67 g, 1.97 mmol, 1.20 eq.) was added in three equal portions in intervals of 30 minutes. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 95/5) and showed a complete conversion after 1.5 hours of stirring at room temperature under argon atmosphere. Pyridine was removed at the rotary evaporator, the residual oil was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution and 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate was evaporated to dryness and the residue was co-evaporated with toluene. The crude product was purified by column chromatography (35 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 90/10). Pure compound **4** was isolated as an off-white foam after drying under high vacuum.
Yield: 1.05 g of an off-white foam (1.51 mmol, 93%)
TLC: (CH_2Cl_2/MeOH = 95/5), R_f = 0.28
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.71 (s, 1H, N⁶*H*); 8.63 (s, 1H, C(2)*H*); 8.22 (d, 1H, C(8)*H*, $^1J_{CH}$ = 213,42 Hz); 7.37 – 7.21 (m, 9H, arom. C*H*); 6.85 – 6.81 (m, 4H, arom. C*H*-C-OCH_3); 6.20 (d, 1H, C(1')*H*, $^3J_{HH}$ = 4.40 Hz); 5.44 (d, 1H, C(3')O*H*, $^3J_{HOH}$ = 6.08 Hz); 4.93 (t, 1H,

C(2')*H*, $^{3}J_{HH}$ = 4.58 Hz); 4.81 (m, 2H, -O-CH_2-O-); 4.55 (dd, 1H, C(3')*H*, $^{3}J_{HH}$ = 5.36 Hz, $^{3}J_{HOH}$ = 10.41 Hz); 4.62 (m, 1H, C(4')*H*); 3.73 (s, 6H, 2x -OCH_3); 3.69 – 3.49 (m, 2H, -O-CH_2-CH_2-); 3.27 (m, 2H, C(5')*H*, C(5'')*H*); 2.73 – 2.60 (m, 2H, -CH_2-CH_2-CN, $^{3}J_{HH}$ = 5.95 Hz); 2.27 (s, 3H, NH-CO-CH_3)

N^{6}-Acetyl-2'-*O*-(2'cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-(8-^{13}C)-adenosine)-3'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite (5)



$C_{36}{}^{13}CH_{38}N_6O_8$
[695.74]

$C_{45}{}^{13}CH_{55}N_8O_9P$
[895.39]

Compound **4** (1.05 g, 1.51 mmol, 1.00 eq.) was dissolved with 10 mL of anhydrous methylene chloride and *N,N*-diisopropylethylamine (1.14 mL, 6.60 mmol, 4.37 eq.) was immediately added. After 20 minutes of stirring at room temperature under argon atmosphere, 2´cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.54 g, 2.27 mmol, 1.50 eq.) added and the solution was stirred for 2 hours. Monitoring by TLC (EtOAc/acetone = 95/5) showed a complete conversion, so 2 mL of anhydrous methanol were added, the reaction mixture was diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate, the filtrate was evaporated under reduced pressure and the resulting crude product was purified via column chromatography (40 g SiO_2, EtOAc/acetone: 5/1 + 1% triethylamine). Pure compound **5** was obtained as a white foam consisting of two diastereomeres.

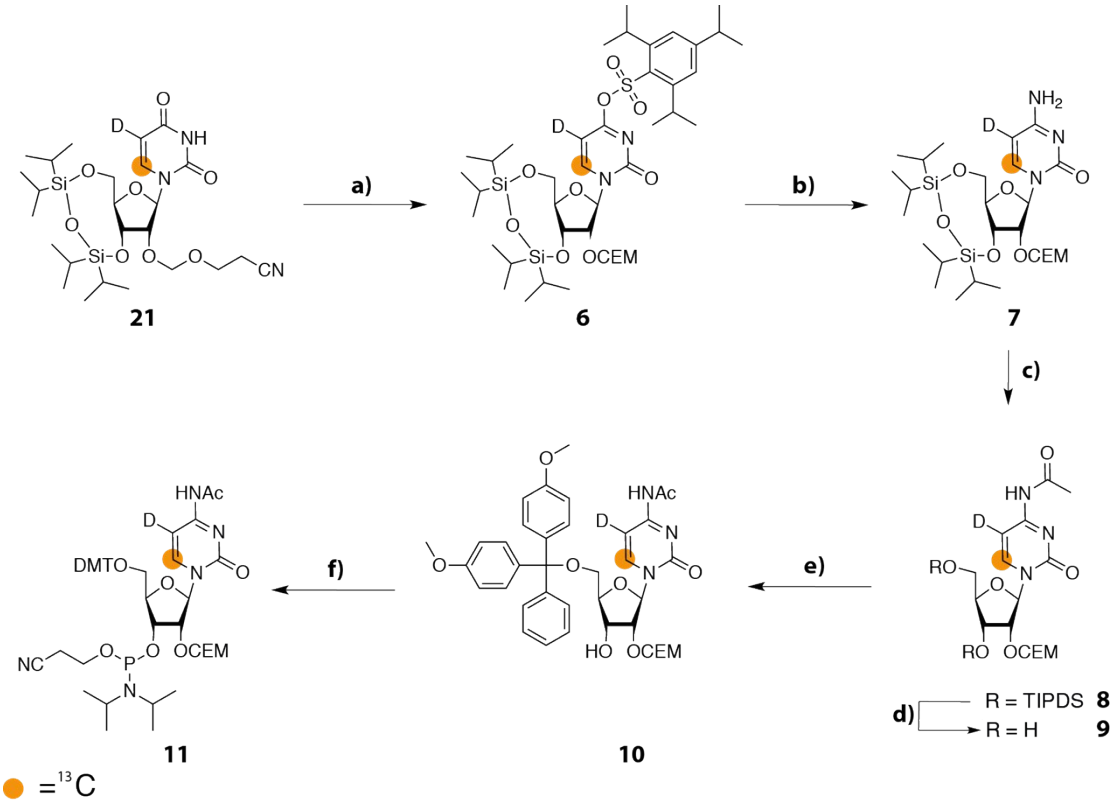
Yield: 1.16 g of an off-white foam (1.30 mmol, 86%)

TLC: (EtOAc/acetone = 95/5), R_f = 0.59 + 0.63

^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.64 (s, 1H, N^{6}*H*); 8.47 (s, 1H, C(2)*H*); 8.24 (d, 1H, C(8)*H*, $^{1}J_{CH}$ = 213.69 Hz); 7.36 – 7.25 (m, 9H, arom. C*H*); 6.87 – 6.82 (m, 4H, arom. C*H*-C-OCH_3); 6.25 (d, 1H, C(1')*H*, $^{3}J_{HH}$ = 4.96 Hz); 5.08 (m, 2H, -O-CH_2-O-); 4.93 (d, 1H, C(2')*H*, $^{3}J_{HH}$ = 7.49 Hz); 4.81 (m, 1H, C(3')*H*); 4.72 (m, 1H, C(4')*H*); 3.82 (s, 6H, -OCH_3); 3.75 – 3.56 (m, 6H, -P-O-CH_2-CH_2-; -CH_2-O-CH_2-CH_2; C(5')*H*; C(5'')*H*); 2.73 – 2.60 (m, 2H, -CH_2-CH_2-CN, $^{3}J_{HH}$ = 5.95 Hz); 2.66 (s, 3H, NH-CO-CH_3) 2.53 – 2.48 (m, 4H, -P-O-CH_2-CH_2-CN, -CH_2-O-CH_2-CH_2-CN); 2.43 (triplettoid, 2H, 2x -N-C*H*-$(CH_3)_2$) 1.33 – 1.10 (m, 12H, 2x -N-CH-$(CH_3)_2$)

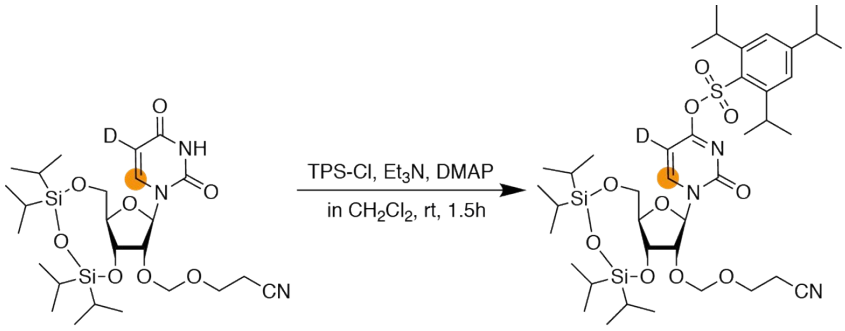
ESI-MS: $[M+H]^+$ 896.3621 (calc. 896.39)

Synthesis of 2'-O-CEM-(6-^{13}C-5-D)-cytidine 5´-O-DMT protected building block (compound 2 in manuscript)



Scheme 2: Synthetic route to 2'-O-CEM-(6-^{13}C-5-D)-Cytidine 5´-O-DMT protected building block; reagents and conditions: ***(a)*** *TPS-Cl, Et_3N, DMAP, in CH_2Cl_2, rt, 1.5h, not purified;* ***(b)*** *NH_4OH_{aq} (28%), in THF, rt, 18 h, 94%* ***(c)*** *Ac_2O, in DMF, rt, 15 h, 75%;* ***(d)*** *TEA·3HF, in THF, 45°C, 2 h, 81%;* ***(e)*** *DMT-Cl, in pyridine, rt, 1.5 h, 89%;* ***(f)*** *CEP-Cl, DIPEA, in CH_2Cl_2, rt, 5 h, 87%*

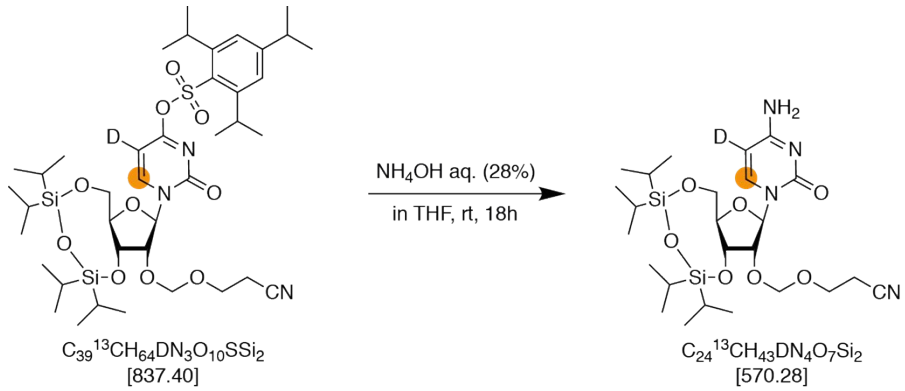
O^4-(2,4,6-triisopropylphenyl)sulfonyl-2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-D-6-^{13}C uridine (6)



Compound **21** (2.85 g, 4.99 mmol, 1.00 eq.) was dissolved in 53 mL anhydrous methylene chloride and triethylamine (6.93 g, 49.9 mmol, 10.0 eq.) was added immediately. A catalytic amount of 4-(dimethylamino)pyridine (78 mg, 0.53 mmol, 0.11 eq.) was added and the solution was stirred at room temperature under argon atmosphere for a few minutes. To the reaction mixture (2,4,6-triisopropylphenyl)sulfonyl chloride (2.31 g, 7.26 mmol, 1.53 eq.)

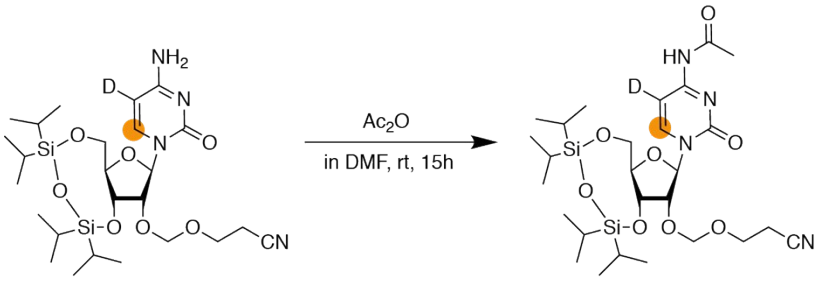
was added and stirred under equal conditions. After 1.5 hours monitoring by TLC (EtOAc/n-hexane = 7/3) showed complete conversion, the solution was diluted with methylene chloride and washed with saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the solvent was evaporated. After drying under high vacuum the crude compound **6** was obtained as an amber colored foam.
No further purification steps and characterization via NMR spectroscopy were carried out. The crude product **6** was used in the next step of synthesis.
Yield: Assumption: 4.18 g of compound 6 (4.99 mmol, 100%)
TLC: (EtOAc/n-hexane = 7/3), R_f = 0.93
^{1}H-NMR: not determined.
^{13}C-NMR: not determined.

2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-D-6-^{13}C-cytidine (7)



Crude compound **6** (4.18 g, 4.99 mmol, 1.00 eq.) was dissolved in 50 mL anhydrous tetrahydrofuran and 50 mL of aqueous ammonia (28,0%) was added. After the solution was stirred vigorously at room temperature for 18 hours, monitoring by TLC (CH_2Cl_2/MeOH = 9/1) showed a complete conversion. The solvent was removed under reduced pressure and the residue was shortly dried under high vacuum before it was resolved in methylene chloride and washed with saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate, evaporated to dryness and again dried under high vacuum. The residual white-brown foam was solved in methylene chloride and purified with column chromatography (60 g SiO_2, CH_2Cl_2/MeOH: 100/0 – 95/5). The pure compound **7** was obtained as a white solid.
Yield: 2.68 g of a white solid (4.68 mmol, 94%).
TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.50
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 7.79 (d, 1H, ^{13}C(6)*H*, $^{1}J_{CH}$ = 180.79 Hz); 7.42 (br, 1H, NH_2); 5.62 (singlettoid, 1H, C(1')*H*); 5.05 (d, 1H, -O-C*H*'$_2$-O-, $^{2}J_{HH}$ = 6.58 Hz); 4.91 (d, 1H, -O-C*H*''$_2$-O-, $^{2}J_{HH}$ = 6.58 Hz); 4.22 – 3.92 (m, 5H, C(2')*H*; C(3')*H*; C(4')*H*; C(5')*H*, C(5'')*H*); 3.79 (t, 2H ,-O-C*H*$_2$-CH$_2$-, $^{3}J_{HH}$ = 6.10 Hz); 2.83 – 2.78 (m, 2H, -O-CH$_2$-C*H*$_2$-); 1.08 – 1.00 (m, 28H, 4x- Si-C*H*-(CH$_3$)$_2$; 4x -Si-CH-(C*H*$_3$)$_2$).
^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 165.84 (*C*(4)); 154.64 (*C*(2)); 140.49 (^{13}*C*(6)); 122.18 (*C*(5)); 119.84 (-CH$_2$-*C*N); 94.10 (-O-*C*H$_2$-O-); 89.92 (*C*(1')); 81.67 (*C*(2')); 78.48 (*C*(3')); 68.64 (*C*(4')); 63.13 (-O-*C*H$_2$-CH$_2$-); 60.38 (*C*(5')); 18.81 (-O-CH$_2$-*C*H$_2$-); 18.20 – 12.87 (-Si-*C*H-(CH$_3$)$_2$; -Si-CH-(*C*H$_3$)$_2$).

N⁴-Acetyl-2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-D-6-^{13}C cytidine (8)



$C_{24}{}^{13}CH_{43}DN_4O_7Si_2$
[570.28]

$C_{26}{}^{13}CH_{45}DN_4O_8Si_2$
[612.30]

Compound **7** (2.68 g, 4.68 mmol, 1.00 eq.) was dissolved in 20 mL absolute *N,N*-dimethylformamide and acetic anhydride (450 µL, 4.86 mmol, 1.00 eq.) was added. The colorless solution was stirred at room temperature and under argon atmosphere for 15 hours. TLC (CH_2Cl_2/MeOH = 9/1) showed a complete conversion, so 1 mL of anhydrous methanol was added to quench the reaction. The mixture was diluted with methylene chloride and washed with saturated sodium bicarbonate solution and saturated sodium chloride solution twice. The organic layer was dried over sodium sulfate, the solvent was evaporated and the residual oil dried under high vacuum. The crude product was purified by column chromatography (40 g SiO_2, CH_2Cl_2/MeOH: 100/0 – 98/2) to give the pure compound **8** as a white solid.

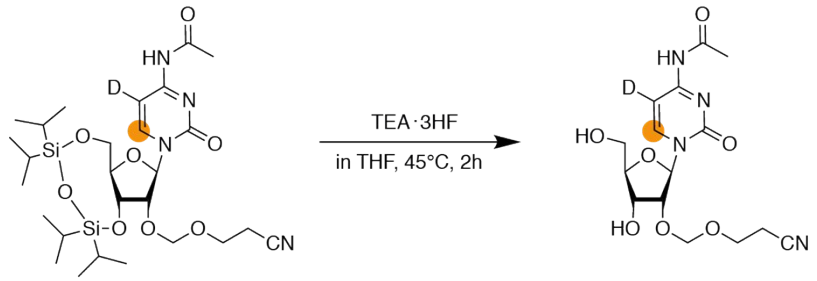
Yield: 2.15 g of a white solid (3.50 mmol, 75%).

TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.75

^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 9.80 (s, 1H, N⁴*H*); 8.32 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 183.94 Hz); 5.80 (singlettoid, 1H, C(1')*H*); 5.11 (d, 1H, -O-C*H*'$_2$-O-, $^2J_{HH}$ = 6.76 Hz); 5.03 (d, 1H, -O-C*H*''$_2$-O-, $^2J_{HH}$ = 6.87 Hz); 4.35 – 4.21 (m, 4H, C(2')*H*; C(3')*H*; C(4')*H*; C(5')*H*); 4.08 – 4.00 (m, 2H , C(5'')*H*; -O-C*H*'$_2$-CH_2); 3.91 – 3.84 (m, 1H , -O-C*H*''$_2$-CH_2); 2.76 – 2.70 (m, 2H, -O-CH_2-C*H*$_2$-); 2.30 (s, 3H, -NH-CO-C*H*$_3$); 1.13 – 1.02 (m, 28H, 4x- Si-C*H*-(CH_3)$_2$; 4x -Si-CH-(C*H*$_3$)$_2$).

^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 171.11 (NH-*C*O-CH_3); 163.40 (*C*(4)); 155.18 (*C*(2)); 144.41 (^{13}*C*(6)); 118.29 (*C*(5)); 94.83 (-O-*C*H_2-O-); 90.51 (*C*(1')); 82.33 (*C*(4')); 78.12 (*C*(2')); 68.25 (*C*(3')); 63.14 (-O-*C*H_2-CH_2-); 59.72 (*C*(5')); 25.31 (NH-CO-*C*H_3); 19.10 (-O-CH_2-*C*H_2-); 18.20 – 12.87 (-Si-*C*H-(CH_3)$_2$; -Si-CH-(*C*H$_3$)$_2$).

N⁴-Acetyl-2'-*O*-(2-cyanoethoxymethyl)-5-D-6-^{13}C cytidine (9)



$C_{26}{}^{13}CH_{45}DN_4O_8Si_2$
[612.30]

$C_{14}{}^{13}CH_{19}DN_4O_7$
[370.14]

Compound **8** (2.15 g, 3.50 mmol, 1.00 eq.) was dissolved in 20 mL anhydrous tetrahydrofuran and triethylamine trihydrofluoride (0.63 g, 3.85 mmol, 1.10 eq.) was added. The solution was stirred under argon atmosphere at 45°C for 2 hours. After 40 minutes the product already began to precipitate as a white solid, at the end of the reaction time almost

the whole mixture became solid. The flask was put on ice for 30 minutes, in which the precipitation was accomplished. The solid product was filtered and washed with cold tetrahydrofuran. The solid phase was allowed to dry under high vacuum and pure compound **9** was obtained as a white powder.

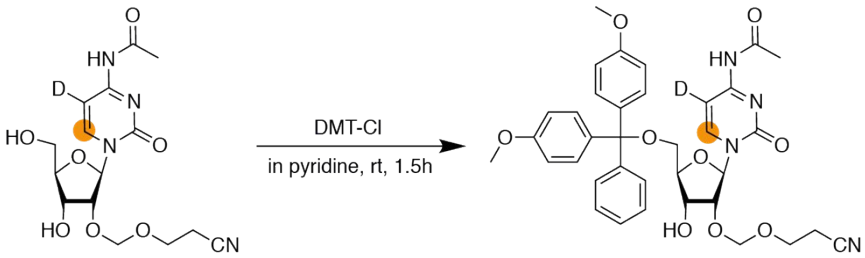
Yield: 1.05 g of a white powder (2.82 mmol, 81%).

TLC: (CH_2Cl_2/MeOH = 95/5) R_f = 0.06

^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.90 (s, 1H, N^4*H*); 8.46 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 182.73 Hz); 5.85 (singlettoid, 1H, C(1')*H*); 4.95 (d, 1H, -O-C*H*'$_2$-O-, $^2J_{HH}$ = 6.70 Hz); 4.84 (d, 1H, -O-C*H*''$_2$-O-, $^2J_{HH}$ = 6.82 Hz); 4.11 (m, 2H, C(2')*H*; C(3')*H*); 3.93 (m, 1H, C(4')*H*); 3.81- 3.61 (m, 4H, C(5')*H*; C(5'')*H*; -O-C*H*$_2$-CH$_2$); 2.78 (t, 2H, -O-CH$_2$-C*H*$_2$-, $^3J_{HH}$ = 6.13 Hz); 2.11 (s, 3H, -NH-CO-C*H*$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 171.87 (NH-*C*O-CH$_3$); 163.26 (*C*(4)); 155.38 (*C*(2)); 145.75 (^{13}*C*(6)); 120.02 (*C*(5)); 94.44 (-O-*C*H$_2$-O-); 89.59 (*C*(1')); 85.04 (*C*(4')); 79.63 (*C*(2')); 68.34 (*C*(3')); 63.19 (-O-*C*H$_2$-CH$_2$-); 60.24 (*C*(5')); 25.22 (NH-CO-*C*H$_3$); 18.86 (-O-CH$_2$-*C*H$_2$-).

N^4-Acetyl-2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-5-D-6-^{13}C cytidine (10)



$C_{14}{}^{13}CH_{19}DN_4O_7$
[370.14]

$C_{35}{}^{13}CH_{37}DN_4O_9$
[672.27]

Compound **9** (1.05 g, 2.82 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice and then dissolved in 20 mL anhydrous pyridine. To the reaction mixture 4,4'-dimethoxytrityl chloride (1.15 g, 3.38 mmol, 1.20 eq.) was added in three equal portions in intervals of 20 minutes. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 95/5) and showed a complete conversion after 1.5 hours of stirring at room temperature under argon atmosphere. The solvent was removed at the rotary evaporator, the residual oil was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution, then with 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate was evaporated to dryness and the residue dried under high vacuum.

The resulting foam was purified by column chromatography (40 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 97/3) and pure compound **10** was isolated as a white foam after drying under high vacuum.

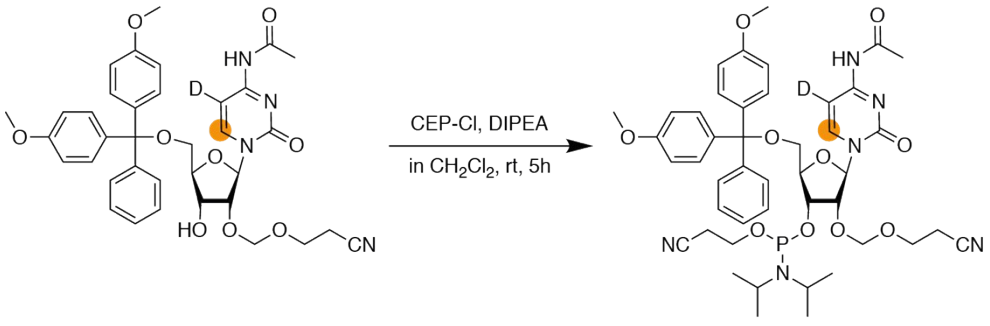
Yield: 1.70 g of a white foam (2.52 mmol, 89%).

TLC: (CH_2Cl_2/MeOH = 95/5), R_f = 0.25.

^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 9.01 (s, 1H, N^4*H*); 8.52 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 183.37 Hz); 7.47 – 7.34 (m, 9H, arom. C*H*); 6.92 – 6.89 (d, 4H, arom. C*H*-C-OCH$_3$, $^3J_{HH}$= 8.90 Hz); 6.00 (singlettoid, 1H, C(1')*H*); 5.27 (d, 1H, -O-C*H*'$_2$-O-, $^2J_{HH}$ = 6.72 Hz); 5.00 (d, 1H, -O-C*H*''$_2$-O-, $^2J_{HH}$ = 6.55 Hz); 4.52 (m, 1H, C(3')*H*); 4.33 (duplettoid, 1H, C(2')*H*); 4.14 (duplettoid, 1H, C(4')*H*); 3.93- 3.86 (m, 2H, -O-C*H*$_2$-CH$_2$); 3.85 (s, 6H, 2x -OC*H*$_3$); 3.69 – 3.56 (m, 2H, C(5')*H*; C(5'')*H*); 2.71 – 2.66 (m, 2H, -O-CH$_2$-C*H*$_2$-); 2.22 (s, 3H, -NH-CO-C*H*$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 171.87 (NH-*C*O-CH$_3$); 163.26 (*C*(4)); 155.38 (*C*(2)); 145.75 (^{13}*C*(6)); 120.02 (*C*(5)); 94.44 (-O-*C*H$_2$-O-); 89.59 (*C*(1')); 85.04 (*C*(4')); 79.63 (*C*(2')); 68.34 (*C*(3')); 63.19 (-O-*C*H$_2$-CH$_2$-); 60.24 (*C*(5')); 25.22 (NH-CO-*C*H$_3$); 18.86 (-O-CH$_2$-*C*H$_2$-).

N^4-Acetyl-2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-5-D-6-^{13}C cytidine 3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (11)



$C_{35}{}^{13}CH_{37}DN_4O_9$
[672.27]

$C_{44}{}^{13}CH_{54}DN_6O_{10}P$
[871.38]

Compound **10** (1.60 g, 2.37 mmol, 1.00 eq.) was dissolved with a prepared mixture of 15 mL anhydrous methylene chloride and *N,N*-diisopropylethylamine (1.81 mL, 10.4 mmol, 4.37 eq.) and the clear solution was stirred at room temperature under argon atmosphere for 20 minutes. 2´cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.73 g, 3.08 mmol, 1.30 eq.) was injected and the reaction mixture was stirred 3 more hours. The monitoring with TLC (EtOAc) showed a complete conversion, so 2 mL of anhydrous methanol were added to quench the reaction and the solution was stirred for 15 more minutes. It was diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the solvent was distilled at the rotary evaporator. The crude product was dried under high vacuum before it was purified via column chromatography (45 g SiO_2, n-hexane/ isopropyl acetate/ acetone: 2/1/1 – 1/2/2 + 1% triethylamine).

Pure compound **11** was isolated as a white foam consisting of two diastereomeres.

Yield: 1.80 g of a white foam (2.06 mmol, 87%).

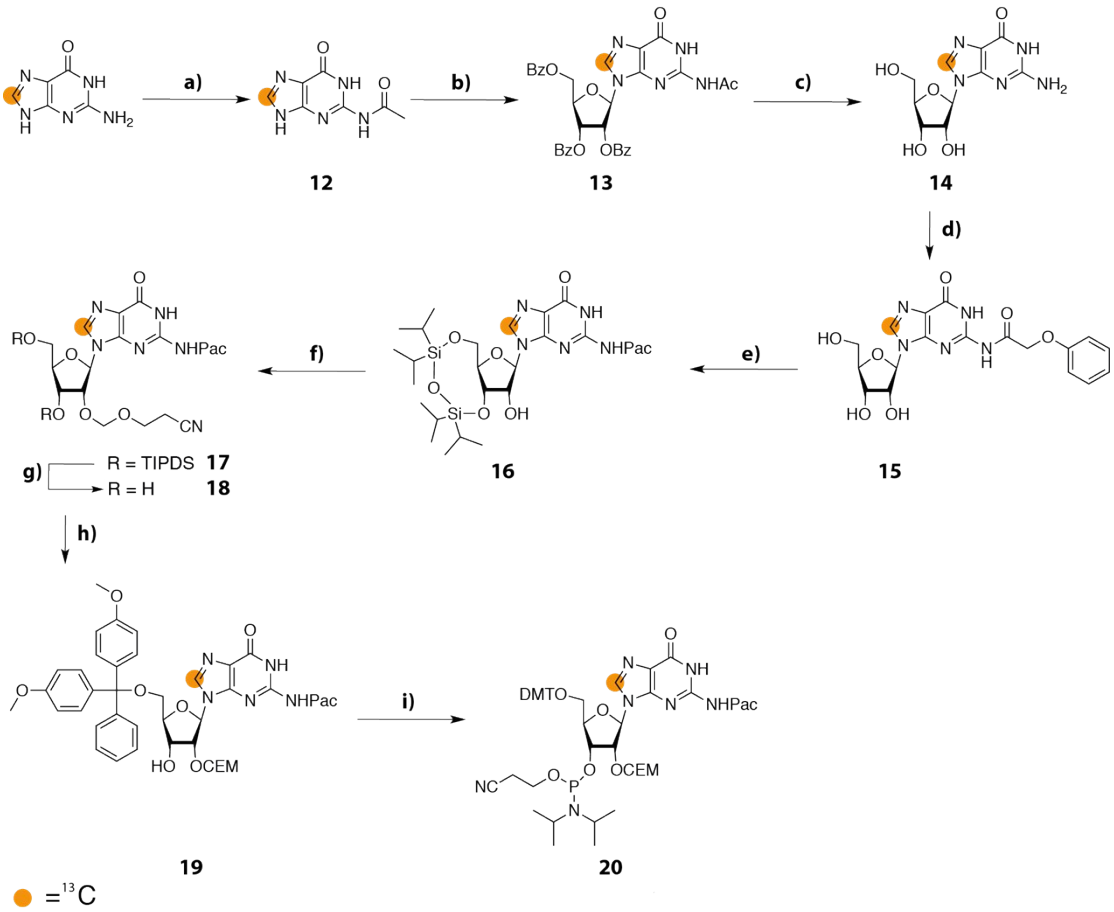
TLC: (EtOAc), R_f = 0.18 + 0.24.

^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.72 – 8.64 (s, 1H, N^4H); 8.57 – 8.51 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 182.65 Hz); 7.48 – 7.32 (m, 9H, arom. C*H*); 6.92 – 6.88 (m, 4H, arom. C*H*-C-OCH_3); 6.03 (singlettoid, 1H, C(1')*H*); 5.17 – 4.95 (m, 2H, -O-CH_2-O-); 4.57 – 4.50 (m, 1H, C(3')*H*); 4.42 – 4.36 (m, 1H, C(2')*H*); 4.34 – 4.27 (m, 1H, C(4')*H*); 4.18 – 4.04, (m, 1H, -P-O-C*H*'$_2$-CH_2-); 3.85 (s, 6H, 2x –OCH_3); 3.79 – 3.72 (m, 2H, -P-O-C*H*''$_2$-CH_2-; -CH_2-O-C*H*'$_2$-CH_2-); 3.60 – 3.48 (m, 3H, C(5)H_2; -CH_2-O-C*H*''$_2$-CH_2-); 2.86 – 2.63 (m, 3H, -P-O-CH_2-CH_2-; -CH_2-O-CH_2-C*H*'$_2$-); 2.46 – 2.42 (m, 1H, -CH_2-O-CH_2-C*H*''$_2$-); 2.25 – 2.23 (s, 3H, -NH-CO-CH_3); 1.28 – 1.03 (m, 14H 2x -N-C*H*-$(CH_3)_2$; 2x -N-CH-(CH_3)$_2$).

^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 145.14 (^{13}C(6)).

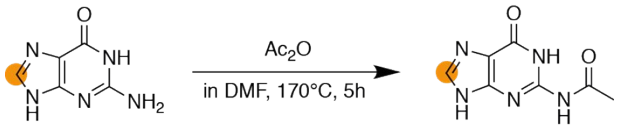
^{31}P-NMR (121 MHz, $CDCl_3$, 25°C): δ 152.13 (s); 150.10 (s)

Synthesis of 2'-O-CEM-(8-^{13}C)-Guanosine 5´-O-DMT protected building block (compound 3 in manuscript)



Scheme 3: Synthetic route to 2'-O-CEM-(8-^{13}C)-Guanosine 5´-O-DMT protected building block; reagents and conditions: ***(a)*** *Ac_2O, in DMF, 170°C, 5 h, 91%;* ***(b)*** *ATBR, BSA, TMSOTf, in 1,2-DCE, 110°C, 1.5 h, 55%;* ***(c)*** *in $MeNH_2$/EtOH, rt, 17 h, 88%;* ***(d)*** *TMS-Cl, Pac-Cl, in pyridine/CH_2CH_2, 0°C/rt, 22 h, 72%* ***(e)*** *TIPDSCl_2, in pyridine, rt, 2 h, 77%;* ***(f)*** *CEM, TfOH, NIS, Et_3N, in THF, -45°C, 1.5 h, not purified;* ***(g)*** *TEA·3HF, in THF, 35°C, 2 h, 96%;* ***(h)*** *DMT-Cl, in pyridine, rt, 2 h, 71%;* ***(i)*** *CEP-Cl, DIPEA, in CH_2Cl_2, rt, 2.5 h, 85%*

N^2-Acetyl-(8-^{13}C)-guanine (12)



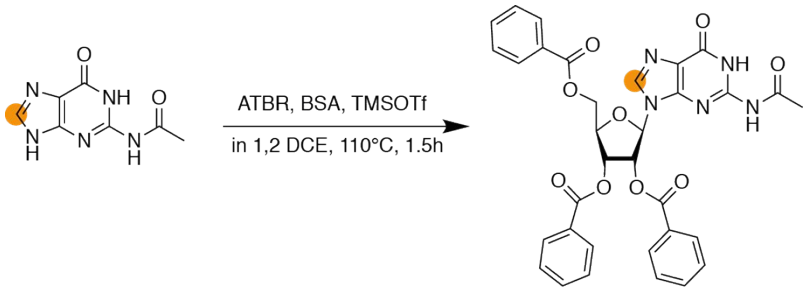
$C_4{}^{13}CH_5N_5O$
[152.12]

$C_6{}^{13}CH_7N_5O_2$
[194.16]

8-^{13}C-Guanine (8.20 g, 53.9 mmol, 1.00 eq.)[1] was suspended in anhydrous *N,N*-dimethylformamide (123 mL), acetic anhydride (15.5 mL, 161 mmol, 2.99 eq.) was added and the mixture was stirred at 170°C under argon atmosphere for 5 hours. The reaction was allowed to cool to room temperature and the solvent was removed under high vacuum. The reddish residue was suspended in a mixture of H_2O/EtOH = 1/1 (100 mL) and refluxed for 1 hour. After cooling to room temperature, the precipitate was filtered, the filtrate was concentrated under reduced pressure and cooled to 0°C. Additional solid product was filtered, the precipitates were combined and allowed to dry under high vacuum.

Yield: 9.54 g of a red-ocher solid (49.1 mmol, 91%).
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 13.20 (s, 1H, N(7)*H*); 12.00 (s, 1H, N(1)*H*); 11.55 (s, 1H, N^{2}*H);* 7.96 (d, 1H, ^{13}C (8)*H*, $^{1}J_{CH}$ = 212.33 Hz); 2.18 (s, 3H, -CO-CH_3).
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 138.52 (^{13}C(8)).
ESI-MS: 873.3425 (calc. 873.38)

N^{2}-Acetyl-2',3',5'-tri-*O*-benzoyl-(8-^{13}C)-guanosine (13)

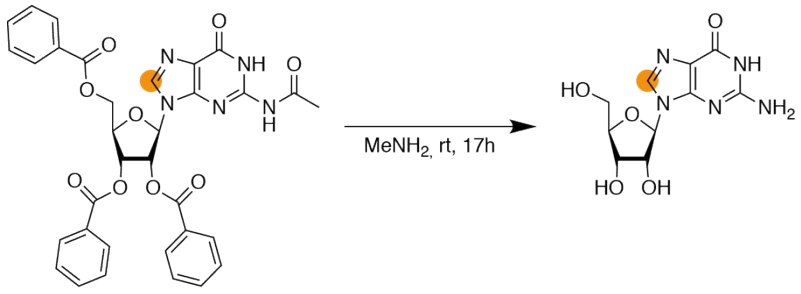


$C_6{}^{13}CH_7N_5O_2$
[194.16]

$C_{32}{}^{13}CH_{27}N_5O_9$
[638.60]

A mixture of compound **12** (4.00 g, 20.6 mmol, 1.00 eq.) and 1'-*O*-acetyl-2',3',5'-tri-*O*-benzoyl ribofuranoside (ATBR) (10.6 g, 20.6 mmol, 1.00 eq.) was suspended in 1,2-dichloroethane (120 mL). After the addition of *N,O*-bis(trimethylsilyl)acetamide (30.2 mL, 120 mmol, 5.84 eq.), the mixture was heated to 110°C and stirred for 20 minutes under argon atmosphere. Carefully trimethylsilyl trifluoromethane sulfonate (TMSOTf) (11.2 mL, 61.6 mmol, 3.00 eq.) was added and the reaction mixture was refluxed for 1 hour. After cooling to room temperature, it was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$-solution. The organic layer was dried over Na_2SO_4, the solvent was removed in *vacuo* and the residual foam was dried under high vacuum, before pure compound **13** was obtained after column chromatography (60 g SiO_2, EtOAc/n-hexane: 6/4 – 9/1).
Yield: 7.20 g of a yellow foam (11.3 mmol, 55%).
TLC: (EtOAc); R_f = 0.58
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 12.11 (s, 1H, N(1)*H*); 11.56 (s, 1H, N^{2}*H);* 8.31 (d, 1H, ^{13}C (8)*H*, $^{1}J_{CH}$ = 215.64 Hz); 8.01 – 7.42 (m, 15H, arom. *H*s (Bz)); 6.43 (triplettoid, 1H, C(1')*H*); 6.30 (dxd, 1H, C(2'), $^{3}J_{HH}$ = 5.76 Hz, $^{3}J_{HH}$ = 5.24 Hz); 6.11 (dxd, 1H, C(3'), $^{3}J_{HH}$ = 5.71 Hz, $^{3}J_{HH}$ = 5.58 Hz); 4.92 – 4.70 (m, 3H, C(4'), C(5'), C(5'')); 2.20 (s, 3H, -CO-CH_3).
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 139.14 (^{13}C(8))

(8-^{13}C)-Guanosine (14)

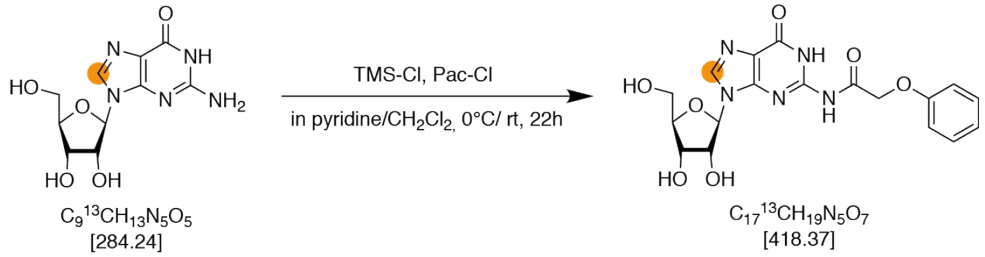


$C_{32}{}^{13}CH_{27}N_5O_9$
[638.60]

$C_9{}^{13}CH_{13}N_5O_5$
[284.24]

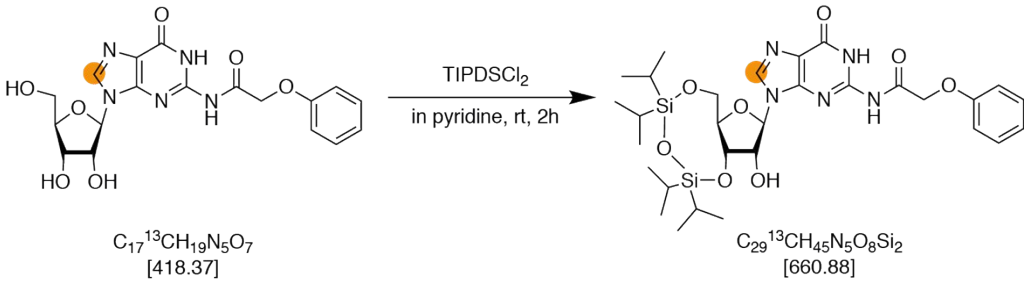
Compound **13** (7.40 g, 11.6 mmol, 1.00 eq.) was suspended in methylamine in ethanol (33%, 160 mL). While stirring for 15 hours at room temperature under argon atmosphere, the yellow suspension turned into an orange solution. The solvent was removed in *vacuo* and the residue was triturated with a mixture of diethyl ether and methylene chloride to remove side products. Compound **14** was filtered and dried under high vacuum.
Yield: 2.91 g of an off-white solid (10.2 mmol, 88%).
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.67 (br, 1H, N(1)*H*); 7.93 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 213.33 Hz); 6.45 (br, 2H, C(2')O*H*, C(3')O*H*); 5.70 (s, 1H, C(1')*H*); 5.37 (br, 1H, C(5')O*H*); 5.03 (s, 2H, N$^{2}H_2$); 4.40 (s, 1H, C(2')*H*); 4.09 (s, 1H, C(3')*H*); 3.88 (s, 1H, C(4')*H*); 3.63 – 3.51 (m, 2H, C(5')H_2).
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 136.69 (^{13}C(8))

N^{2}-Phenoxyacetyl-(8-^{13}C)-guanosine (15)



(8-^{13}C)-Guanosine (**14**) (2.91 g, 10.2 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine thrice and afterwards suspended in 50 mL anhydrous pyridine. The mixture was diluted with 200 mL methylene chloride, cooled to 0°C and trimethylsilyl chloride (30.3 mL, 92.2 mmol, 9.00 eq.) was slowly added under argon atmosphere. The ice bath was removed and the reaction mixture was allowed to stir at room temperature for 2 hours. After 30 minutes the suspension turned to a clear solution indicating a successful silylation. In accordance, TLC (CH_2Cl_2/MeOH = 7/3) showed a positive reaction and the solution was cooled on ice again. Phenoxyacetyl chloride (1.56 mL, 11.3 mmol, 1.10 eq.) was slowly dropped into the solution, a white solid precipitated and resolved quickly. After the total addition of Pac-Cl, the reaction mixture was stirred at 0°C for 3 hours. Methanol (41 mL) was added, the cold bath was removed and the mixture was stirred at room temperature for 18 more hours. The solution was evaporated to dryness and the solid residue was co-evaporated with toluene twice. The solid was suspended in 60 mL of ice cold water and the resulting white suspension was stirred at 0°C for 1 hour. The precipitate was filtered, washed with cold EtOH and dried under high vacuum to obtain pure compound **15**.
Yield: 3.10 g of an off-white solid (7.41 mmol, 72%).
TLC: (CH_2Cl_2/MeOH = 8/2); R_f = 0.36
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.84 (s, 1H, N(1)*H*); 11.80 (s, 1H, N^{2}H); 8.30 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 215.61 Hz); 7.33 (t, 2H, arom. C*H* (m), $^{3}J_{HH}$ = 7.52); 6.99 (m, 3H, arom. C*H* (o/p)); 5.83 (d, 1H, C(1')*H*, $^{3}J_{HH}$ = 5,63 Hz); 4.89 (s, 2H, -CO-CH_2-O-); 4.46 (t, 1H, C(2')*H*, $^{3}J_{HH}$ = 5,09 Hz); 4.16 (triplettoid; 1H, C(3')*H*); 3.93 (duplettoid, 1H, C(4')*H*); 3.68 – 3.56 (m, 2H, C(5')H_2)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 135.86 (^{13}C(8))

N^2-Phenoxyacetyl-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-(8-^{13}C)-guanosine (16)

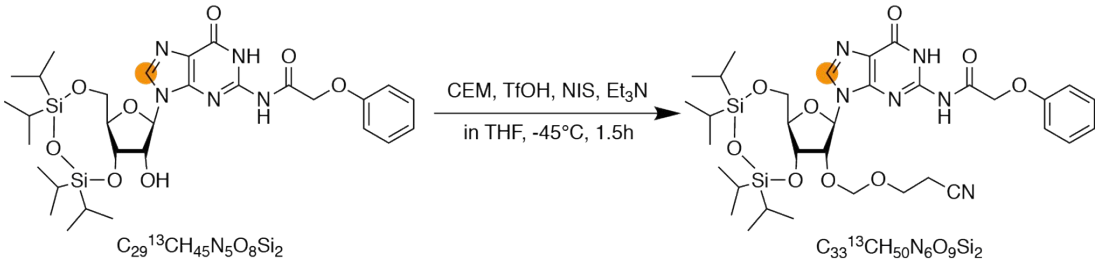


$C_{17}{}^{13}CH_{19}N_5O_7$
[418.37]

$C_{29}{}^{13}CH_{45}N_5O_8Si_2$
[660.88]

Compound **15** (3.10 g, 7.41 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice, then dissolved in 45 mL of anhydrous pyridine. To the solution 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.96 mL, 9.26 mmol, 1.25 eq.) was added and the reaction mixture was stirred under argon atmosphere at room temperature for 2 hours. After TLC (CH_2Cl_2/MeOH = 9/1) showed a complete reaction, 1 mL of methanol was added to quench the reaction and the solution was stirred for further 10 minutes. The solvent was removed under reduced pressure, the residue was dissolved in chloroform and washed with saturated sodium bicarbonate solution, then with 5% citric acid. The organic phase was dried over sodium sulfate and the solution was evaporated to dryness. After drying the residue under high vacuum, the crude product was purified via column chromatography (80 g SiO_2, EtOAc/n-hexane: 3/7 – 9/1) to obtain the clean compound **16**.
Yield: 3.76 g of a yellow solid foam (5.69 mmol, 77%).
TLC: (EtOAc); R_f = 0.66
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.84 (br, 2H, N(1)*H,* N^2*H*); 8.08 (d, 1H, ^{13}C(8)*H,* $^1J_{CH}$ = 214.83 Hz); 7.33 (t, 2H, arom. C*H* (m), $^3J_{HH}$ = 7.83); 7.00 (m, 3H, arom. C*H* (o/p)); 5.82 (s, 1H, C(1')*H*); 5.70 (s, 1H, C(2')O*H*); 4.88 (s, 2H, -CO-CH_2-O-); 4.41 – 4.37 (m, 2H, C(2')*H*, C(3')*H*); 4.17 – 3.95 (m, 3H, C(4')*H*, C(5')H_2); 1.08 – 1.02 (m, 28H, 4x -Si-(C*H*)-(CH_3)$_2$; 4x -Si-(CH)-(CH_3)$_2$)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 137.47 (^{13}C(8))

N^2-Phenoxyacetyl-2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-(8-^{13}C)-guanosine (17)



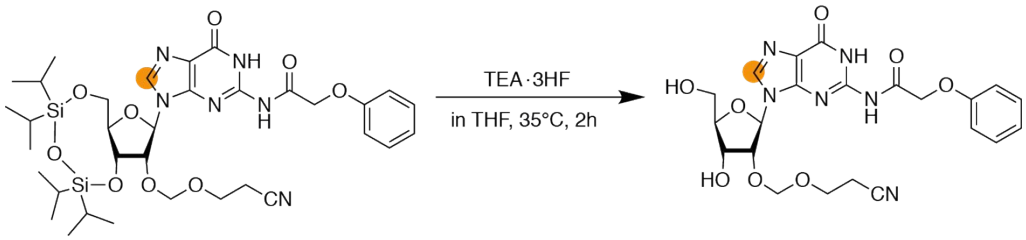
$C_{29}{}^{13}CH_{45}N_5O_8Si_2$
[660.88]

$C_{33}{}^{13}CH_{50}N_6O_9Si_2$
[743.97]

Compound **16** (3.76 g, 5.69 mmol, 1.00 eq.) was dissolved in 40 mL anhydrous tetrahydrofuran and 2-cyanoethyl methylthiomethylether (1.15 g, 8.88 mmol, 1.56 eq.) was added. The solution was cooled to -45°C and stirred under argon atmosphere for 30 minutes. Trifluoromethanesulfonic acid (1.34 g, 8.88 mmol, 1.56 eq.) was slowly dropped to the mixture, then *N*-iodosuccinimide (2.00 g, 8.88 mmol, 1.56 eq.) was added in one portion. The reaction mixture was stirred for 20 minutes at -45°C till triethylamine (1.38 mL 9.73 mmol, 1.71 eq.) was slowly added to quench the reaction and it was stirred for further 15 minutes. The mixture was diluted with cold ethyl acetate and washed with saturated, ice cold sodium thiosulfate solution twice, with saturated, ice cold sodium bicarbonate solution twice and with saturated ice cold sodium chloride solution twice. The aqueous phases were

extracted with ethyl acetate, the organic layers combined and dried over sodium sulfate. The solvent was removed in *vacuo* and crude compound **17** was obtained while drying under high vacuum. Further purification was not carried out, crude product **17** was used for the next reaction step.
Yield: Assumption: 4.23 g of compound **17** (5.69 mmol, 100%).
TLC: (EtOAc/n-hexane: 9/1); R_f = 0.68
^{1}H-NMR: not determined
^{13}C-NMR: not determined

N^2-Phenoxyacetyl-2'-*O*-(2-cyanoethoxymethyl)-(8-^{13}C)-guanosine (18)

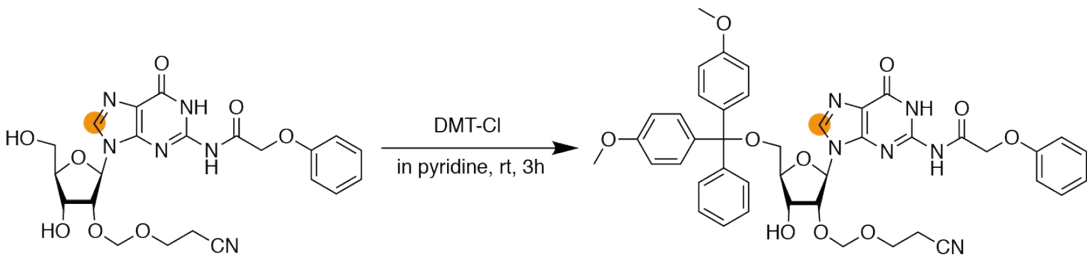


$C_{33}{}^{13}CH_{50}N_6O_9Si_2$
[743.97]

$C_{21}{}^{13}CH_{24}N_6O_8$
[501.46]

Crude compound **17** (4.23 g, 5.69 mmol, 1.00 eq.) was dissolved in anhydrous THF and to the solution triethylamine trihydrofluoride (TEA·3HF) (0.92 g, 5.69 mmol, 1.00 eq.) was added. It was stirred under argon atmosphere at 35°C for 2 hours. After 30 minutes, product started to precipitate. Monitoring the reaction by TLC (EtOAc) showed the desired conversion. The mixture was cooled in an ice bath, 2 mL of water were added and the reaction was stirred vigorously for 1 hour at 0°C until precipitation of the white solid was accomplished. 33 mL ethanol were added, the mixture was allowed to warm to room temperature and stirred for 30 more minutes. The pure compound **18** was filtered and dried under high vacuum.
Yield: 2.74 of a pale rose powder (5.46 mmol, 96%).
TLC: (EtOAc); R_f = 0.05
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.52 (br, 2H, N(1)*H,* N^2*H*); 8.27 (d, 1H, ^{13}C(8)*H,* $^1J_{CH}$ = 215.25 Hz); 7.32 (t, 2H, arom. C*H* (m), $^3J_{HH}$ = 7.92); 6.98 (m, 3H, arom. C*H* (o/p)); 5.97 (s, 1H, C(1')*H*); 5.34 (s, 1H, C(3')O*H*); 5.14 (s, 1H, C(5')O*H*); 4.84 (s, 2H, -CO-CH_2-O-); 4.79 – 4.72 (2xd, 2H, -O-CH_2-O-, $^2J_{HH}$ = 6.96 Hz, $^2J_{HH}$ = 7.10 Hz) 4.61 (m, 2H, C(2')*H*); 4.33 (singlettoid, 1H, C(3')*H*); 3.98 (duplettoid, 1H, C(4')*H*); 3.65 – 3.60 (m, 2H, C(5')H_2); 3.51 – 3.44 (m, 2H, -O-CH_2-CH_2-); 2.68 – 2.61 (m, 2H, -CH_2-CH_2-CN)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 138.61 (^{13}C(8))

N^2-Phenoxyacetyl-2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-(8-^{13}C)-guanosine (19)

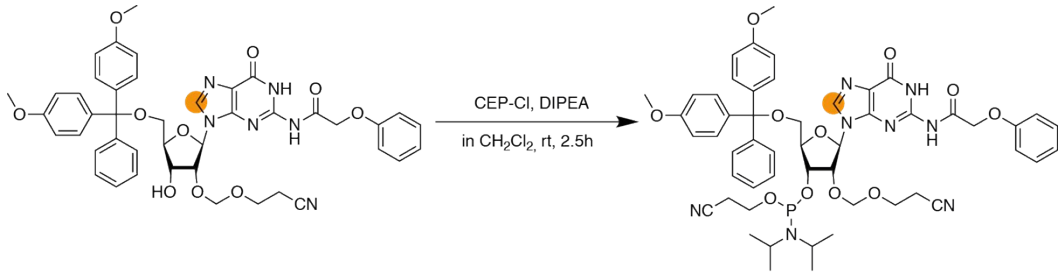


$C_{21}{}^{13}CH_{24}N_6O_8$
[501.46]

$C_{42}{}^{13}CH_{42}N_6O_{10}$
[803.83]

Compound **18** (2.74 g, 5.46 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine thrice, then dissolved in 35 mL anhydrous pyridine. To the reaction mixture 4,4'-dimethoxytrityl chloride (2.22 g, 6.55 mmol, 1.20 eq.) was added in three equal portions in intervals of 20 minutes. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 95/5) and showed complete conversion after 3 hours of stirring at room temperature under argon atmosphere. Pyridine was removed under reduced pressure, the residual oil was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution and 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate evaporated to dryness and the residue dried under high vacuum.
The crude product was purified by column chromatography (80 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 96/4) and compound **19** was isolated as a white foam.
Yield: 3.10 g of a white foam (3.86 mmol, 71%)
TLC: (CH_2Cl_2/MeOH = 95/5); R_f = 0.31
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.80 (s, 1H, N(1)*H*); 11.74 (s, 1H, N^2*H*); 8.15 (d, 1H, ^{13}C(8)*H*, $^1J_{CH}$ = 214.92 Hz); 7.38 -7.23 (m, 11H, 2x arom. C*H* (m,Pac), 9x arom. C*H* (trityl)); 7.01 (m, 3H, arom. C*H* (o/p,Pac)); 6.87 – 6.82 (triplettoid, 4H, arom. C*H*-C-OCH_3); 6.03 (singlettoid, 1H, C(1')*H*); 5.38 (d, 1H, C(3')O*H*, $^3J_{HH}$ = 5.61 Hz); 4.87 (s, 2H, -O-CH_2-O-); 4.81 (s, 2H, -CO-CH_2-O-); 4.70 (t, 1H, C(2')*H*, $^3J_{HH}$ = 4.91 Hz); 4,39 (m, 1H, C(3')*H*); 4.10 (singlettoid, 1H, C(4')*H*); 3.74 (s, 6H, 2x -OCH_3); 3.70 – 3.52 (m, 2H, -O-CH_2-CH_2-); 3.36 – 3.19 (m, 2H, C(5')H_2); 2.77 – 2.60 (m, 2H, -CH_2-CH_2-CN)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 138.69 (^{13}C(8))

N^2-Phenoxyacetyl-2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-(8-^{13}C)-guanosine-3'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (20)



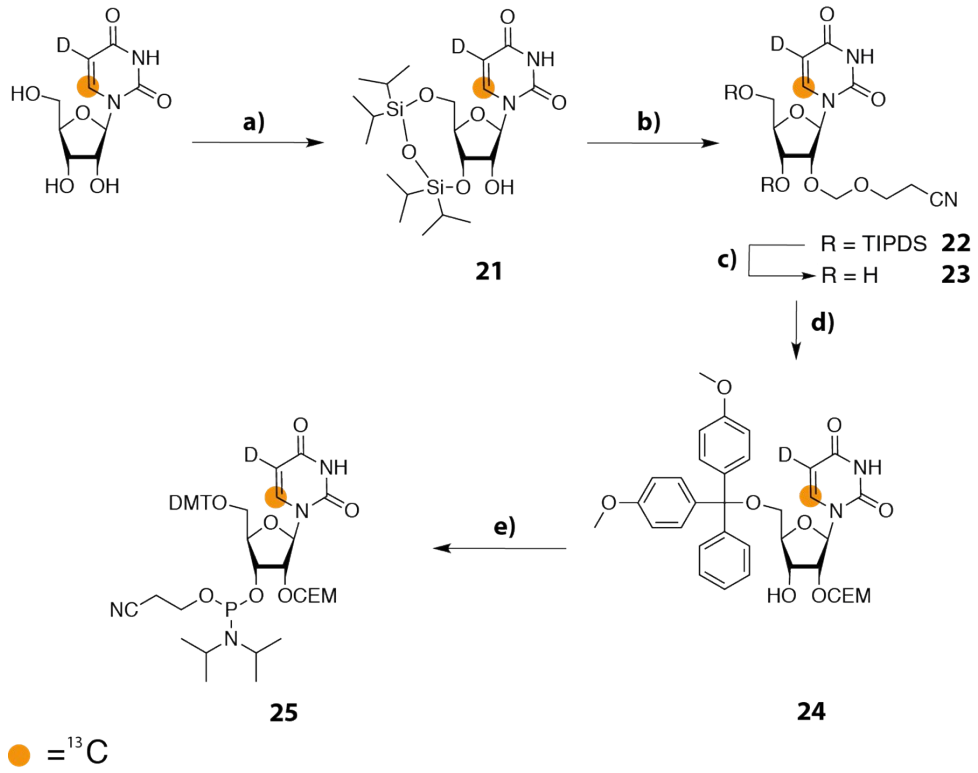
$C_{42}{}^{13}CH_{42}N_6O_{10}$
[803.83]

$C_{51}{}^{13}CH_{59}N_8O_{11}P$
[1003.41]

Compound **19** (3.10 g, 3.86 mmol, 1,00 eq.) was dissolved in 10 mL anhydrous methylene chloride and *N,N*-diisopropylethylamine (2.95 mL, 16.9 mmol, 4.37 eq.) was immediately added. After 20 minutes of stirring at room temperature under argon atmosphere, 2´cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.83 g, 7.72 mmol, 2.00 eq.) was added and the solution was stirred for 2.5 hours. Monitoring by TLC (EtOAc) showed complete conversion, so 2 mL anhydrous methanol were added to quench the reaction. The mixture was diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate, the filtrate was evaporated to dryness and dried under high vacuum. Via column chromatography (40 g SiO_2, 1. n-hexane/isopropyl acetate/acetone: 3/1/1 + 1% trimethylamine, eluted hydrolyzed phosphorylating reagent; 2. ethyl acetate/acetonitrile: 40/1 + 1% triehtylamine, eluted further impurities; 3. ethyl acetate/methanol: 9/1 + 1% trimethylamine, eluted product) the pure compound **20** was obtained as a white foam.
Yield: 3.29 g of a white foam (3.28 mmol, 85%)
TLC: (EtOAc + 1% NEt_3); R_f = 0.60 + 0.68

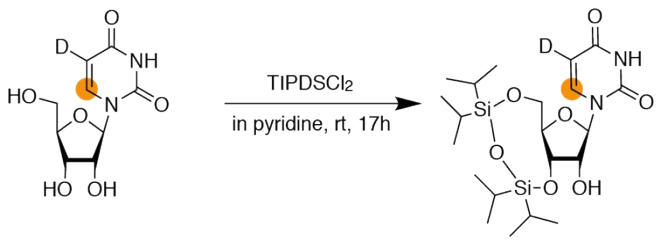
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 7.92 (s, 1H, N(1)*H*); 7.46 (s, 1H, N^{2}*H*); 7.42 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 214.92 Hz); 7.38 - 7.25 (m, 11H, 2x arom. C*H* (m, Pac); 9x arom. C*H* (trityl)); 6.99 – 6.92 (m, 3H, arom. C*H* (o/p, Pac)); 6.87 – 6.82 (d, 4H, arom. C*H*-C-OCH_3, $^{3}J_{HH}$ = 8.50); 6.11 – 6.05 (d, 1H, C(1')*H*, $^{3}J_{HH}$ = 6,50 Hz); 4.96 (s, 2H, -O-CH_2-O-); 4.88 – 4.75 (duplettoid, 1H, C(2')*H*); 4.68 – 4.60 (m, 3H, -CO-CH_2-O-; C(3')*H*,); 4.43 – 4.34 (singlettoid, 1H, C(4')*H*); 3.97 – 3.84 (m, 2H, -P-O-CH_2-CH_2-); 3.81 (s, 6H, 2x -OCH_3); 3.74 – 3.38 (m, 4H, -CH_2-O-CH_2-CH_2-; C(5')H_2); 2.79 – 2.68 (m, 4H, -CH_2-O-CH_2-CH_2-; -P-O-CH_2-CH_2-;); 2.50 – 2.39 (m, 2H, 2x -N-C*H*-$(CH_3)_2$), 1.31 – 1.08 (m, 12H, 2x -N-CH-(CH_3)$_2$)
^{31}P-NMR (121 MHz, $CDCl_3$, 25°C): 151.87 (s); 151.81 (s)
ESI-MS: 1004.3885 (calc. 1004.41)

Synthesis of 2'-O-CEM-(6-^{13}C-5-D)-uridine 5´-O-DMT protected building block (compound 4 in manuscript)



*Scheme 4: Synthetic route to 2'-O-CEM-(6-^{13}C-5-D)-Uridine 5´-O-DMT protected building block; reagents and conditions: **(a)** TIPDSCl_2, in pyridine, rt, 17 h, 52%; **(b)** CEM, TfOH, NIS, Et_3N, in THF, -45°C, 1.5 h, 78%; **(c)** NH_4F, in MeOH, 45°C, 5 h, not purified; **(d)** DMT-Cl, in pyridine, rt, 1.5 h, 41%; **(e)** CEP-Cl, DIPEA, in CH_2Cl_2, rt, 2 h, 81%*

3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-D-6-^{13}C-uridine (21)

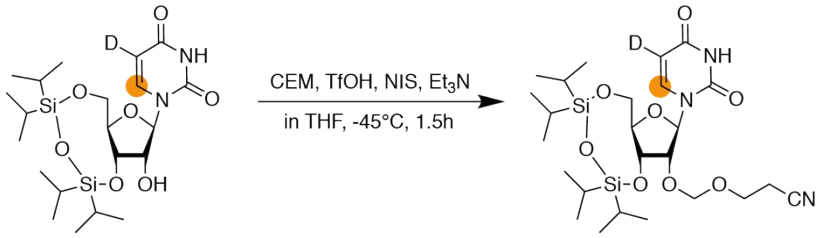


$C_8{}^{13}CH_{11}DN_2O_6$
[246.08]

$C_{20}{}^{13}CH_{37}DN_2O_7Si_2$
[488.23]

5-D-6-^{13}C-uridine (3.00 g, 12.2 mmol, 1.00 eq.)[1] was co-evaporated twice with anhydrous pyridine and dissolved in 70 mL anhydrous pyridine. 1,3-Dichloro-1,1,3,3-tetraisopropyl-disiloxane (4.82 g, 13.8 mmol, 1.13 eq.) was added and the solution was stirred at room temperature and under argon atmosphere. After 17 hours, the TLC (CH_2Cl_2/MeOH = 95/5) showed a complete conversion. Pyridine was removed at the rotary evaporator and the residue was dissolved in chloroform. It was washed with saturated sodium bicarbonate solution twice and 5% citric acid. The organic layer was dried over sodium sulfate, the filtrate was evaporated to dryness and the residue was dried under high vacuum. To isolate the pure compound **21**, the crude product was purified by column chromatography (60 g SiO_2, EtOAc/n-hexane: 1/1 – 7/3).
Yield: 3.11 g of an off-white foam (6.37 mmol, 52%)
TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.68
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.35 (s, 1H, N(3)*H*); 7.70 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 182.08 Hz); 5.56 (m, 2H, C(5)*H*; C(1')*H*); 4.19 – 4.11 (m, 3H, C(2')*H*; C(3')*H*; C(4')*H*); 4.00 – 3.96 (m, 2H, C(5')*H*; C(5'')*H*); 1.06 – 1.02 (m, 28H, 4x Si-C*H*-$(CH_3)_2$; 4x Si-CH-$(CH_3)_2$)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 140.62 (^{13}C(6))

2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-D-6-^{13}C-uridine (22)



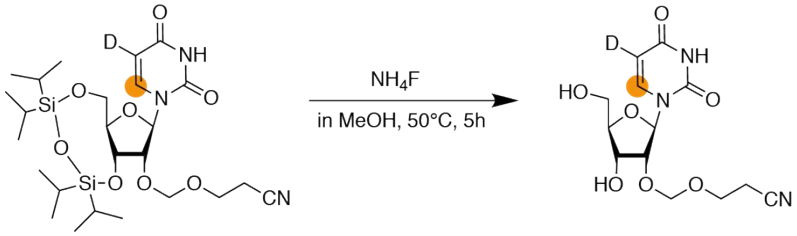
$C_{20}{}^{13}CH_{37}DN_2O_7Si_2$
[488.23]

$C_{24}{}^{13}CH_{42}DN_3O_8Si_2$
[571.27]

Compound **21** (3.11 g, 6.37 mmol, 1.00 eq.) was dissolved in 40 mL anhydrous tetrahydrofuran and 2-cyanoethyl methylthiomethylether (1.30 g, 9.93 mmol, 1.56 eq.) was added. The clear solution was cooled to -45°C and stirred under argon atmosphere for 30 minutes. Trifluoromethanesulfonic acid (1.50 g, 9.93 mmol, 1.56 eq.) was carefully dropped to the mixture and after 5 minutes of stirring *N*-iodosuccinimide (2.23 g, 9.93 mmol, 1.56 eq.) was added in one portion. The reaction mixture was stirred for 20 minutes at -45°C till triethylamine (1.52 mL, 10.9 mmol, 1.71 eq.) was slowly added to quench the reaction and it was stirred for further 15 minutes. The mixture was diluted with ethyl acetate and washed with saturated sodium thiosulfate solution twice, with saturated sodium bicarbonate solution twice and with saturated sodium chloride solution. The aqueous phases were extracted with ethyl acetate, the organic layers were combined and dried over sodium sulfate. The solvent was removed under reduced pressure and the crude oily product was obtained while drying under high vacuum. It was purified with column chromatography (40 g SiO_2, EtOAc/n-hexane: 1/9 – 1/1) and the pure compound **22** was obtained as a white foam.
Yield: 2.85 g of a white foam (4.99 mmol, 78%).
TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.73
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 9.55 (s, 1H, N(3)*H*); 7.92 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 183.39 Hz); 5.03 (m, 2H, -O-CH_2-O-); 4.32 – 4.15 (m, 4H, C(1')*H*; C(2')*H*; C(3')*H*; C(4')*H*); 4.07 – 3.99 (m, 2H ,-O-CH_2-CH_2-) 3.88 – 3.81 (m, 2H, C(5')*H*; C(5'')*H*); 2.75 – 2.65 (m, 2H, -O-CH_2-CH_2-); 1.13 – 1.06 (m, 28H, 4x- Si-C*H*-$(CH_3)_2$; 4x -Si-CH-$(CH_3)_2$).

^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 163.79 (*C*(4)); 150.64 (*C*(2)); 139.42 (^{13}*C*(6)); 118.35 (*C*(5)); 94.85 (-O-*C*H_2-O-); 82.19 (*C*(1')); 78.27 (*C*(2')); 68.63 (*C*(3')); 63.19 (*C*(5')); 61.24 (C(4')); 59.68 (-O-*C*H_2-CH_2-); 19.15 (-O-CH_2-*C*H_2-); 17.87 – 13.01 (-Si-*C*H-$(CH_3)_2$; -Si-CH-(*C*$H_3)_2$).

2'-*O*-(2-cyanoethoxymethyl)-5-D-6-^{13}C-uridine (23)

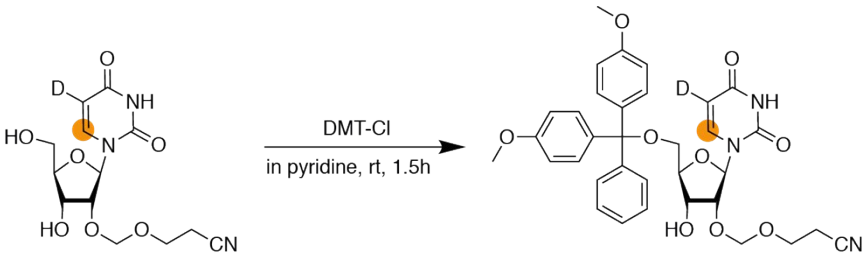


$C_{24}{}^{13}CH_{42}DN_3O_8Si_2$
[571.27]

$C_{12}{}^{13}CH_{16}DN_3O_7$
[329.12]

Compound **22** (6.96 g, 12.2 mmol, 1.00 eq.) was dissolved in 54 mL anhydrous methanol and ammonium fluoride (1.66 g, 44.5 mmol, 3.65 eq.) was added. The reaction mixture was heated to 50°C and stirred for 5 hours under argon atmosphere. After TLC (CH_2Cl_2/MeOH = 9/1) showed complete conversion, methanol was removed in *vacuo* and the residue was dissolved in acetonitrile. A white solid was precipitating, which was removed by filtration and washed with acetonitrile. The organic phase was extracted with hexane twice, the hexane layers were discarded and the acetonitrile phase was evaporated to dryness. The residual oil was isolated as crude compound **23** and dried under high vacuum.
No further purification steps were carried out and the crude product **23** was used in the next step of synthesis.
Yield: Assumption: 4.01 g of compound 3 (12.2 mmol, 100%).
TLC: (CH_2Cl_2/MeOH = 10/1), R_f = 0.21
^{1}H-NMR: not determined
^{13}C-NMR: not determined

2'-*O*-(2-cyanoethoxymethyl)- 5'-*O*-(4,4'-dimethoxytrityl)-5-D-6-^{13}C-uridine (24)



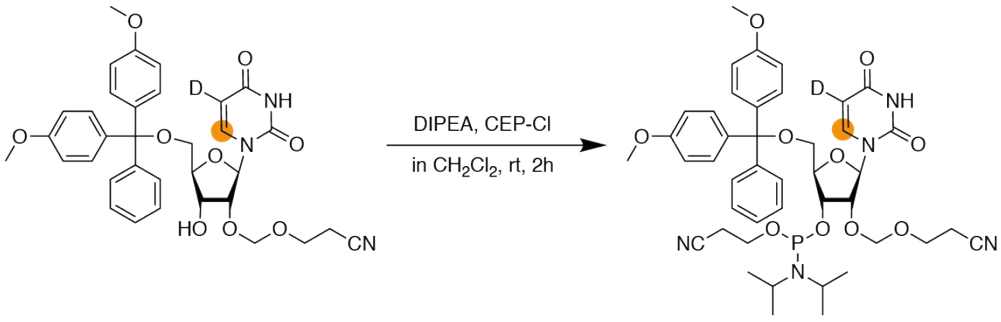
$C_{12}{}^{13}CH_{16}DN_3O_7$
[329.12]

$C_{33}{}^{13}CH_{34}DN_3O_9$
[631.25]

Crude compound **23** (4.01 g, 12.2 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice and then dissolved in 85 mL of anhydrous pyridine. To the reaction mixture 4,4'-dimethoxytrityl chloride (4.97 g, 14.6 mmol, 1.20 eq.) was added in three equal portions in intervals of 20 minutes. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 95/5) and showed complete conversion after 2 hours of stirring at room temperature under argon atmosphere. The solvent was removed at the rotary evaporator, the residual oil was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution

and with 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate was evaporated to dryness and dried under high vacuum.
The resulting foam was purified by column chromatography (50 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 97/3) and pure compound **24** was isolated as an off-white foam after drying under high vacuum.
Yield: 3.18 g of an off-white foam (5.04 mmol, 41%)
TLC: (CH_2Cl_2/MeOH = 95/5), R_f = 0.41
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.39 (s, 1H, N(3)*H*); 7.74 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 182.92 Hz) 7.41 – 7.26 (m, 9H, arom. C*H*); 6.93 – 6.90 (d, 4H, arom. C*H*-C-OCH_3, $^3J_{HH}$= 8.88 Hz); 5.84 (t, 1H, C(1')*H*; $^3J_{CH}$ = 2.96 Hz); 5.36 (d, 1H, C(3')O*H*, $^3J_{HH}$ = 6.25 Hz); 4.84 (s, 2H, -O-CH_2-O-); 4.30 – 4.23 (m, 2H, C(2')*H*; C(3')*H*); 4.00 (singlettoid, 1H, C(4')*H*); 3.76 (s, 6H, 2x -O*C*H_3); 3.71 – 3.67 (m, 2H, -O-*C*H_2-CH_2-); 3.32 – 3.27 (m, 2H, C(5')*H*; C(5'')*H*); 2.78 (dd, 2H, -O-CH_2-*C*H_2-, $^2J_{HH}$ = 10.28 Hz, $^3J_{HH}$ = 4.42 Hz)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 163.82 (*C*(4)); 159.03 (arom. *C*-OCH_3); 151.24 (*C*(2)); 145.48 (arom. *C*C); 140.97 (^{13}*C*(6)); 131.65 – 127.68 (arom. *C*H); 119.96 (*C*(5)); 114.15 (arom. *C*H-C-OCH_3); 94.77 (-O-*C*H_2-O-); 88.38 (*C*(1')); 83.43 (*C*(4')); 78.82 (*C*(3')); 69.41 (*C*(2')); 63.43 (*C*(5')); 63.28 (-O-*C*H_2-CH_2-); 55.85 (-O*C*H_3); 18.91 (-O-CH_2-*C*H_2-)

2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-5-D-6-^{13}C-uridine-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (25)



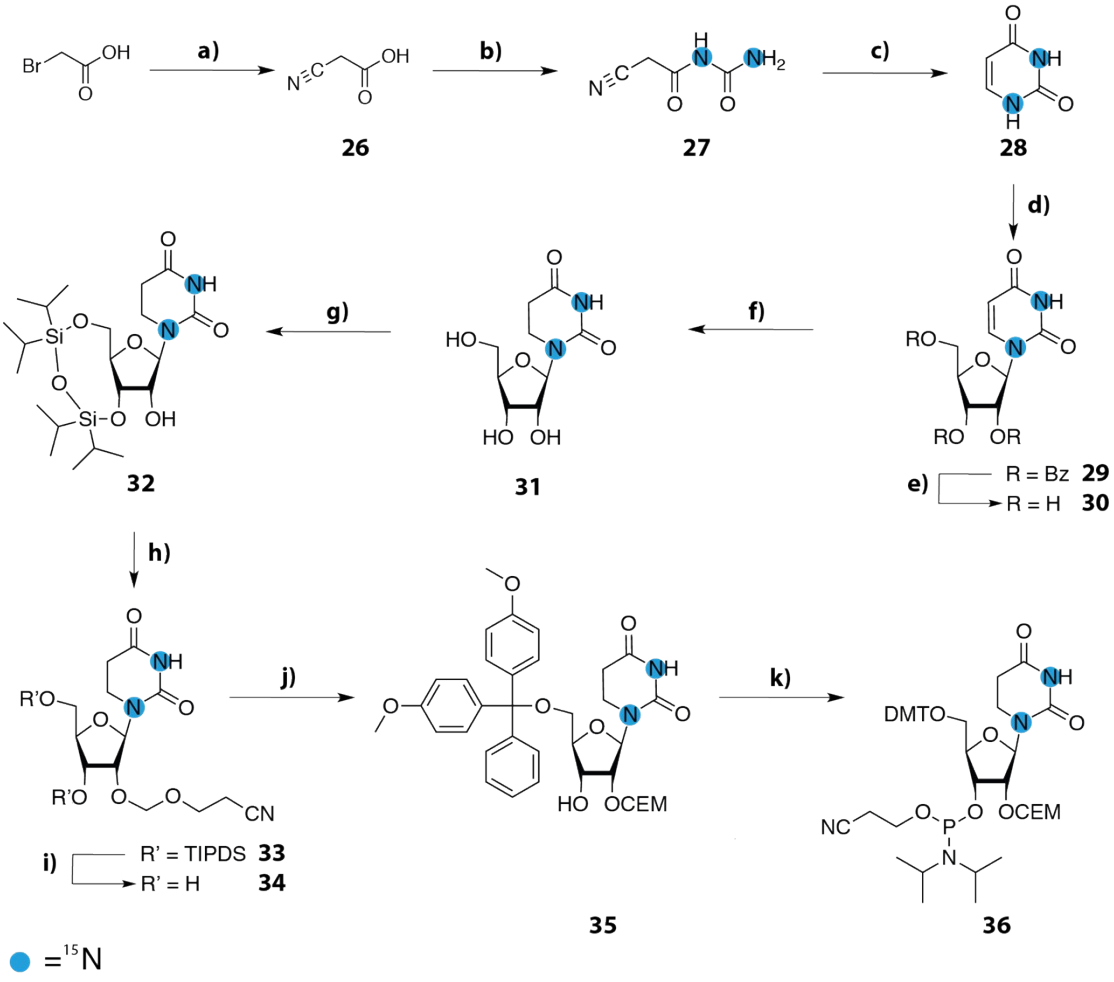
$C_{33}{}^{13}CH_{34}DN_3O_9$
[631.25]

$C_{42}{}^{13}CH_{51}DN_5O_{10}P$
[830.35]

N,N-Diisopropylethylamine (3.85 mL, 22.0 mmol, 4.37 eq.) was diluted with 30 mL of anhydrous methylene chloride and compound **24** (3.18 g, 5.04 mmol, 1.00 eq.) was dissolved in the prepared solvent mixture. After 20 minutes of stirring at room temperature 2´cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.92 g, 8.06 mmol, 1.60 eq.) was given to the reddish reaction mixture, the color changed to light yellow and the solution was stirred for 2 hours. Monitoring with TLC (EtOAc) showed a complete conversion, 2 mL of anhydrous methanol were added to the solution and it was stirred for 15 more minutes. The reaction mixture was diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the filtrate was evaporated to dryness at the rotary evaporator. The residual off-white foam was dried under high vacuum before it was purified via column chromatography (45 g SiO_2, EtOAc/n-hexane: 1/1 – 7/3 + 1% triethylamine). Pure compound **25** was obtained as a white foam consisting of a mixture of two diastereomeres.
Yield: 3.38 g of a white foam (4.07 mmol, 81%).
TLC: (EtOAc), R_f = 0.56 + 0.68
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 8.66 (br, 1H, N(3)*H*); 8.05 – 8.03 (d, 1H, ^{13}C(6)*H*, $^1J_{CH}$ = 182.57 Hz) 7.46 – 7.32 (m, 9H, arom. C*H*); 6.90 – 6.87 (d, 4H, arom. C*H*-C-OCH_3, $^3J_{HH}$= 8.88 Hz); 6.06 – 6.00 (m, 1H, C(1')*H*); 5.11 – 4.89 (m, 2H, -O-CH_2-O-); 4.65 – 4.57 (m, 2H, C(3')*H*); 4.48 – 4.39 (m, 1H, C(2')*H*); 4.29 – 4.21 (m, 1H, C(4')*H*); 4.06 – 3.94, (m, 1H, -P-O-C*H*'$_2$-CH_2-);

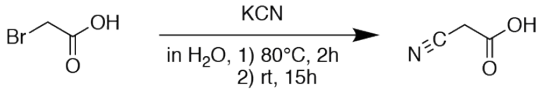
3.84 (s, 6H, 2x -OCH_3); 3.81 – 3.74 (m, 2H, -P-O-CH''_2-CH_2-; -CH_2-O-CH'_2-CH_2-); 3.71– 3.46 (m, 3H, C(5')H; C(5'')H; -CH_2-O-CH''_2-CH_2-); 2.75 – 2.64 (m, 3H, -P-O-CH_2-CH_2-; -CH_2-O-CH_2-CH'_2-); 2.50 – 2.45 (m, 1H, -CH_2-O-CH_2-CH''_2-); 1.33 – 1.05 (m, 14H 2x -N-CH-(CH_3)$_2$; 2x -N-CH-(CH_3)$_2$).
^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 140.22 (^{13}C(6)).
^{31}P-NMR: (121 MHz, $CDCl_3$, 25°C): 151.82 (s); 150.09 (s)
ESI-MS: 832.3461 (calc. 832.35)

Synthesis of 2'-O-CEM-(1,3-$^{15}N_2$)-dihydrouridine 5´-O-DMT protected building block (compound 5 in manuscript)



Scheme 5: Synthetic pathway to 2'-O-CEM-(1,3-^{15}N)-Dihydrouridine 5´-O-DMT protected building block; reagents and conditions: ***(a)*** *KCN, in H_2O, 1) 80°C, 2h, 2) rt, 15 h, 100%;* ***(b)*** *$^{15}N_2$-urea, in Ac_2O, 100°C, 30 min, 81%;* ***(c)*** *H_2, 5% Pd/$BaSO_4$ cat., in AcOH, 1) rt, 12 h, 2) 70°C, 1 h, 64%;* ***(d)*** *1)ATBR, BSA, 2) TMSOTf, in ACN, 60°C, 1 h, 82%;* ***(e)*** *in $MeNH_2$/EtOH, rt, 16 h, 86%;* ***(f)*** *H_2, Rh/C (5%) cat., in H_2O, rt, 66 h, 96%;* ***(g)*** *TIPDSCl_2, in pyridine, rt, 2 h, 63%;* ***(h)*** *CEM, TfOH, NIS, Et_3N, in THF, -45°C, 1.5 h, not purified;* ***(i)*** *TEA·3HF, in THF, rt, 2 h, not purified;* ***(j)*** *DMT-Cl, in pyridine, rt, 12 h, 75%;* ***(k)*** *CEP-Cl, DIPEA, in CH_2Cl_2, rt, 2 h, 85%*

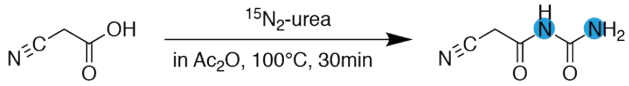
2-Cyanoacetic acid (26)



$C_2H_3BrO_2$ [137.93] $C_3H_3NO_2$ [85.02]

Bromoacetic acid (6.99 g, 50.3 mmol, 1.00 eq.) was dissolved in 20 mL water and a saturated solution of sodium carbonate was added until the reaction mixture reached pH 11. Potassium cyanide (3.27 g, 50.3 mmol, 1.00 eq.) was dissolved in 20 mL water and poured into the reaction mixture. The clear, colorless solution was heated to 80°C and stirred for 2 hours. The heating was removed, the mixture allowed to cool to room temperature and stirred for 15 more hours. Concentrated hydrochloric acid (10 mL) was added to adjust the pH value to 1, the solvent was removed at the rotary evaporator and the white, solid residue was dried under high vacuum for 30 minutes. The product was extracted with 500 mL diethyl ether, the remaining salt was filtered and washed with additional diethyl ether. The filtrate was evaporated and pure compound **26** was dried under high vacuum.
Yield: 4.28 g of an off-white solid (50.3 mmol, 100%).
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 3.86 (s, 2H, NC-C*H*$_2$-CO-).

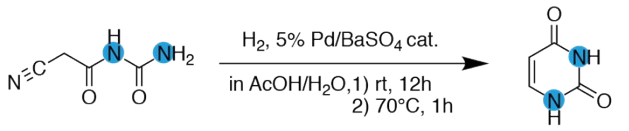
4,7-$^{15}N_2$-Cyanoacetyl urea (27)



$C_3H_3NO_2$ [85.02] $C_4H_5N^{15}N_2O_2$ [129.03]

Compound **26** (4.28 g, 50.3 mmol, 1.00 eq.) was mixed with $^{15}N_2$-urea (4.04 g, 67.3 mmol, 1.34 eq.) and then suspended in acetic anhydride (8.67 mL, 91.7 mmol, 1.82 eq.). The white suspension was stirred at 100°C for 30 minutes and a yellow solid began to precipitate. 20 mL water were added and the reaction mixture was stirred for 5 more minutes until it was allowed to cool to room temperature. The precipitate was filtered and washed with ice cold water. After drying under high vacuum, pure compound **27** was obtained as a white solid.
Yield: 5.20 g of a white solid (40.9 mmol, 81%).
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.38 (d, 1H, -CO-^{15}N*H*-CO-, $^1J_{NH}$ = 90,00 Hz); 7.36 (d, 2H, -CO-^{15}N*H*$_2$, $^1J_{NH}$ = 90.00 Hz); 3.92 (s, 2H, NC-C*H*$_2$-CO-).

1,3-$^{15}N_2$-Uracil (28)

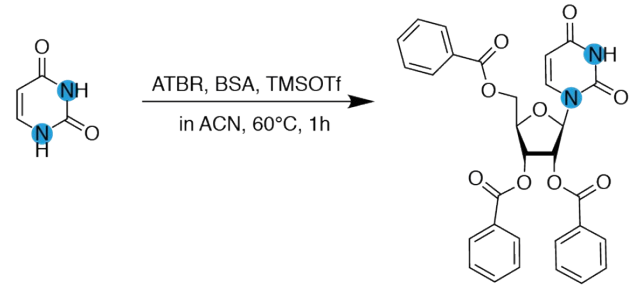


$C_4H_5N^{15}N_2O_2$ [129.03] $C_4H_4{}^{15}N_2O_2$ [114.02]

A mixture of acetic acid and water (1/1; 35 mL) was prepared to suspend 2.62 g of 5% palladium on barium sulfate in a 1 L round bottom flask. The reaction vessel was evacuated and flushed with hydrogen several times. The reduced palladium colored the suspension

black. Compound **27** (5.20 g, 40.9 mmol, 1.00 eq.) was separately suspended in 130 mL with the 1/1-mixture of acetic acid and water and heated to 100°C to dissolve all solid. The solution was poured to the catalyst suspension and the flask was evacuated and flushed with hydrogen several times again. The reaction mixture was stirred vigorously for 12 hours, heated to 70°C and stirred for another hour. Palladium on barium sulfate was filtered over celites and washed with water. The filtrate was concentrated under reduced pressure until the product began to precipitate. The residual mixture was cooled to 4°C over night to accomplish the precipitation. After filtration, pure compound **28**, a white solid, was obtained.
Yield: 2.97 g of a white solid (26.1 mmol, 64%).
TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.31
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.27 – 9.97 (br, 2H, ^{15}N(1)*H*; ^{15}N(3)*H*); 7.40 (dd, 1H, C(6)*H*, $^3J_{HH}$ = 7.42 Hz, $^3J_{NH}$ = 3.20 Hz); 5.45 (m, 1H, C(5)*H*)

2',3',5'-*O*-Tribenzoyl-1,3-$^{15}N_2$-uridine (29)

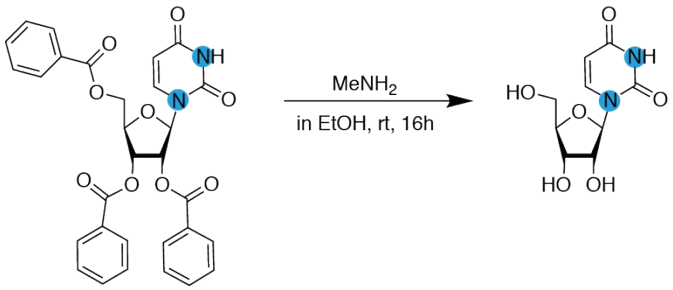


$C_4H_4{}^{15}N_2O_2$
[114.02]

$C_{30}H_{24}{}^{15}N_2O_9$
[558.14]

1,3-$^{15}N_2$-uracil (**28**) (2.97 g, 26.1 mmol, 1.00 eq.) was suspended in 100 mL acetonitrile, 1'-*O*-acetyl-2',3',5'-tri-*O*-benzoyl-β-D-ribofuranose (ATBR) (13.1 g, 26.1 mmol, 1.00 eq.) and *N,O*-bis(trimethylsilyl)acetamide (BSA) (19.1 mL, 78.2 mmol, 3.00 eq.) were added and the mixture was stirred at 60°C under argon atmosphere. After 30 minutes trimethylsilyl trifluoromethanesulfonate (TMSOTf) (16.7 mL, 91.2 mmol, 3.50 eq.) was injected and the reaction mixture was stirred for another 30 minutes until TLC (EtOAc/n-hexane = 1/1) showed a complete conversion. The solvent was removed at the rotary evaporator and the oily residue was diluted with methylene chloride. The solution was washed with saturated sodium bicarbonate solution. After drying the organic layer over sodium sulfate, the solvent was evaporated and the resulting solid turned into a powder while drying under high vacuum. The crude product purified by column chromatography (50 g SiO_2, CH_2Cl_2/MeOH: 100/0 – 96/4) to obtain pure compound **29**.
Yield: 11.9 g of an off-white powder (21.3 mmol, 82%)
TLC: (EtOAc/n-hexane = 1/1), R_f = 0.62
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.52 (d, 1H, ^{15}N(3)*H*, $^1J_{NH}$ = 89.02 Hz); 8.04 – 7.43 (m, 16H, C(6)*H*; arom. C*H*); 6.19 (d, 1H, C(1')*H*, $^3J_{HH}$ = 3.21 Hz), 5,. 9 – 5.91 (m, 2H, C(5)*H*; C(2')*H*); 5.70 (m, 1H, C(3')*H*); 4.76 (m, 1H, C(4')*H*); 4.72 – 4.63 (m, 2H, C(5')*H*; C(5'')*H*).

1,3-$^{15}N_2$-uridine (30)



$C_{30}H_{24}{}^{15}N_2O_9$ [558.14]

$C_9H_{12}{}^{15}N_2O_6$ [246.06]

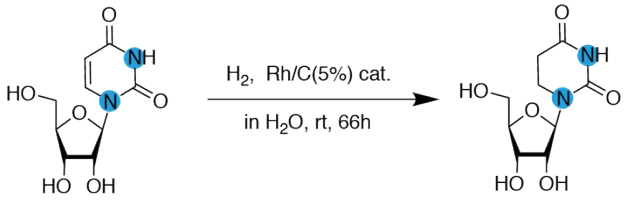
Compound **29** (11.9 g, 21.3 mmol, 1.00 eq.) was dissolved in 70 mL methylamine in absolute ethanol (33 wt. %) and stirred at room temperature under argon atmosphere for 16 hours. The solvent was removed at the rotary evaporator and the remaining oil was diluted with water. It was washed with methylene chloride, the aqueous layer was evaporated to dryness and the residual off-white foam was recrystallized from ethanol to obtain the pure product **30** as a white powder.

Yield: 4.47 g of a white powder (18.2 mmol, 86%).

TLC: (CH_2Cl_2/MeOH = 9/1), R_f = 0.11.

^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 11.52 (br, 1H, ^{15}N(3)*H*); 7.89 (dd, 1H, C(6)*H*, $^3J_{HH}$ = 8.02 Hz, $^2J_{NH}$ = 1.80 Hz), 5.79 (d, 1H, C(5)*H*, $^3J_{HH}$ = 5.73 Hz) 5.65 (m, 1H, C(1')*H*); 5.13 (br, 3H, C(2')O*H*; C(3')O*H*; C(5')O*H*); 4.02 (triplettoid, 1H, C(2')*H*); 3.97 (triplettoid, 1H, C(3')*H*); 3.85 (d, 1H, C(4')*H*, $^3J_{HH}$ = 3.37 Hz); 3.63 (dd, 1H, C(5')*H*, $^2J_{HH}$ = 12.17 Hz, $^3J_{HH}$ = 3.02 Hz); 3.55 (dd, 1H, C(5'')*H*, $^2J_{HH}$ = 11.84 Hz, $^3J_{HH}$ = 3.06 Hz).

1,3-$^{15}N_2$-dihydrouridine (31)



$C_9H_{12}{}^{15}N_2O_6$ [246.06]

$C_9H_{14}{}^{15}N_2O_6$ [248.08]

1,3-$^{15}N_2$-uridine (**30**) (4.40 g, 18.2 mmol, 1.00 eq.) was dissolved in 100 mL water and 0.95 g 5% rhodium on activated charcoal were added to the solution. The 1 L round bottom flask was evacuated and flushed with hydrogen several times to ensure a pure hydrogen atmosphere. The mixture was stirred vigorously at room temperature for 66 hours. Reaction control via NMR spectroscopy showed the successful reduction of the double bound between C(5) and C(6). The catalyst was removed by filtration over celites and washed with water. The filtrate was evaporated to dryness and the residue was co-evaporated three times with methanol. After drying under high vacuum, pure compound **31** was obtained as a white foam.

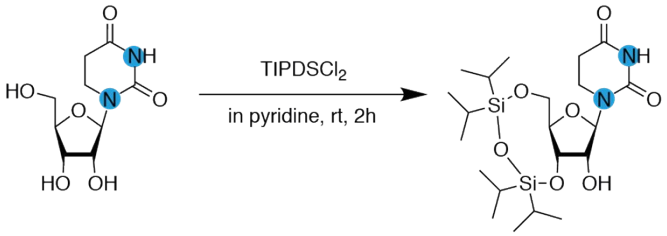
Yield: 4.23 g of a white foam (17.1 mmol, 95%).

TLC: (CH_2Cl_2/MeOH = 4/1), R_f = 0.75

^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.00 (br, 1H, ^{15}N(3)*H*); 5.68 (d, 1H, C(1')*H*, $^3J_{HH}$ = 6.01 Hz); 5.07 (s, 1H, C(2')O*H*); 4.95(s, 1H C(3')O*H*); 4.81 (s, 1H, C(5')O*H*); 4.02 (dd, 1H, C(2')*H*, $^3J_{HH}$ = 5.17 Hz, $^3J_{HH}$ = 5.04 Hz); 3.97 (dd, 1H, C(3')*H*, $^3J_{HH}$ = 5.01 Hz, $^3J_{HH}$ = 3.58 Hz); 3.85 (m, 1H,

C(4')*H*); 3.55 – 3.45 (m, 2H, C(5')H_2); 3.39 – 3.27 (m, 2H, (C(6)H_2); 2.55 (hidden under solvent signal, 2H, (C(5)H_2).

3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-1,3-$^{15}N_2$-dihydrouridine (32)

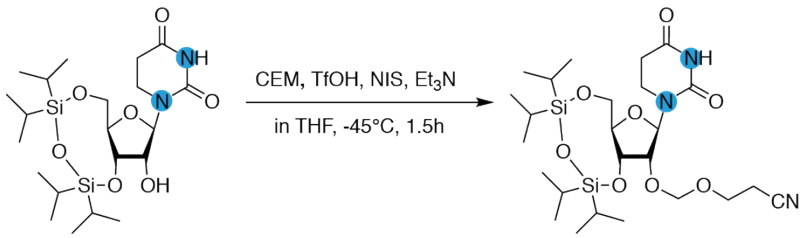


$C_9H_{14}{}^{15}N_2O_6$
[248.08]

$C_{21}H_{40}{}^{15}N_2O_7Si_2$
[490.23]

Compound **31** (4.23 g, 17.1 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice, then dissolved in 100 mL anhydrous pyridine. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (6.04 g, 19.1 mmol, 1.12 eq.) was added and the solution was stirred at room temperature under argon atmosphere for 2 hours. Monitoring by TLC (EtOAc) showed complete conversion (detection with anisaldehyde staining solution (90 mL absolute ethanol, 5 mL anisaldehyde, 5 mL concentrated sulfuric acid and 1 mL acetic acid)). The solvent was removed under reduced pressure and the residue was dissolved in chloroform. It was washed with saturated sodium bicarbonate and 5% citric acid. The organic layer was dried over sodium sulfate and the filtrate was evaporated to dryness. The yellow oily residue, was dried under high vacuum, then purified via column chromatography (60 g SiO_2, n-hexane/EtOAc: 6/4) to obtain pure compound **32** as a white solid.
Yield: 5.24 g of a white solid (10.7 mmol, 63%).
TLC: (EtOAc) R_f = 0.72
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.27 (d, 1H, ^{15}N(3)*H*, $^1J_{NH}$ = 90.01 Hz); 5.57 (s, 1H, C(1')*H*); 5.11 (d, 1H, C(2')O*H*, $^3J_{HH}$ = 4.65 Hz); 4.15 – 4.07 m, 2H, C(2')*H*; C(3')*H*); 3.99 (dd, 1H, C(5')*H*, $^2J_{HH}$ = 12.60 Hz, $^3J_{HH}$ = 9.57 Hz); 3.88 (dd, 1H, C(5'')*H*, $^2J_{HH}$ = 12.79 Hz, $^3J_{HH}$ = 10.32 Hz) 3.76 – 3.73 (m, 1H, C(4')*H*); 3.38 – 3.29 (m, 2H, (C(6)H_2); 2.55 (hidden under solvent signal, 2H, (C(5)H_2); 1.08 – 1.03 (m, 28H, 4x -Si-C*H*-$(CH_3)_2$; 4x -Si-CH-(CH_3)$_2$).
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 171.04 (*C*(4)); 153.90 (*C*(2)); 90.83 (*C*(1')); 80.76 (*C*(4')); 72.84 (*C*(3')); 71.05 (*C*(2')); 61.55 (*C*(5')); 37.34 (*C*(6)); 31.52 (*C*(5)); 18.19 – 12.90 (-Si-*C*H-$(CH_3)_2$; -Si-CH-(CH_3)$_2$).

2'-*O*-(2-cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-1,3-$^{15}N_2$-dihydrouridine (33)



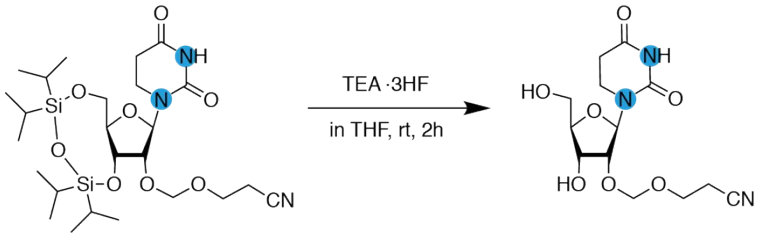
$C_{21}H_{40}{}^{15}N_2O_7Si_2$
[490.23]

$C_{25}H_{45}N^{15}N_2O_8Si_2$
[573.27]

Compound **32** (5.24 g, 10.7 mmol, 1.00 eq.) was dissolved in 75 mL anhydrous tetrahydrofuran and 2-cyanoethyl methylthiomethylether (2.19 g, 16.7 mmol, 1.56 eq.) was added. The solution was cooled to -45°C and stirred under argon atmosphere for 30

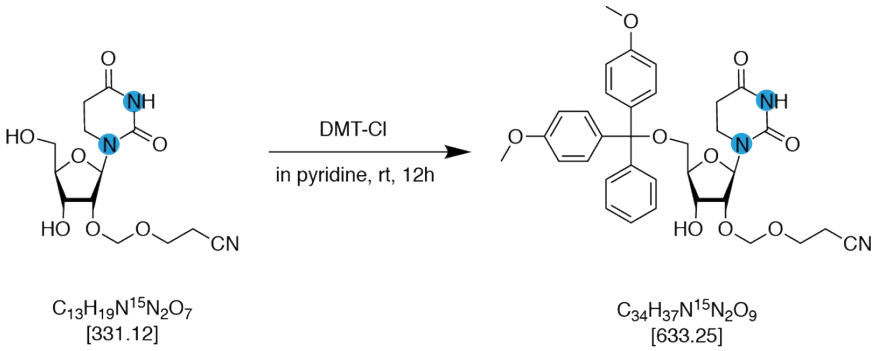
minutes. Trifluoromethanesulfonic acid (2.52 g, 16.7 mmol, 1.56 eq.) was slowly dropped to the mixture and 5 minutes later *N*-iodosuccinimide (3.75 g, 16.7 mmol, 1.56 eq.) was added in one portion. The reaction mixture was stirred for 20 minutes at -45°C till triethylamine (2.55 mL, 18.3 mmol, 1.71 eq.) was slowly added over a period of 10 minutes to quench the reaction and it was stirred for further 20 minutes. The mixture was diluted with ethyl acetate and washed with saturated, ice cold sodium thiosulfate solution twice, saturated, ice cold sodium bicarbonate solution twice and saturated sodium chloride solution. The aqueous phases were extracted with ethyl acetate, the organic layers were combined and dried over sodium sulfate. The solvent was evaporated and the crude product, a yellow viscous oil, was obtained while drying under high vacuum.
Further purification actions and a NMR spectroscopic characterization were not carried out, crude compound **33** was used in the next reaction step
Yield: Assumption: 6.13 g (10.7 mmol, 100%).
TLC: (EtOAc), R_f = 0.85
^{1}H-NMR: not determined
^{13}C-NMR: not determined

2'-*O*-(2-cyanoethoxymethyl)-1,3-$^{15}N_2$-dihydrouridine (34)



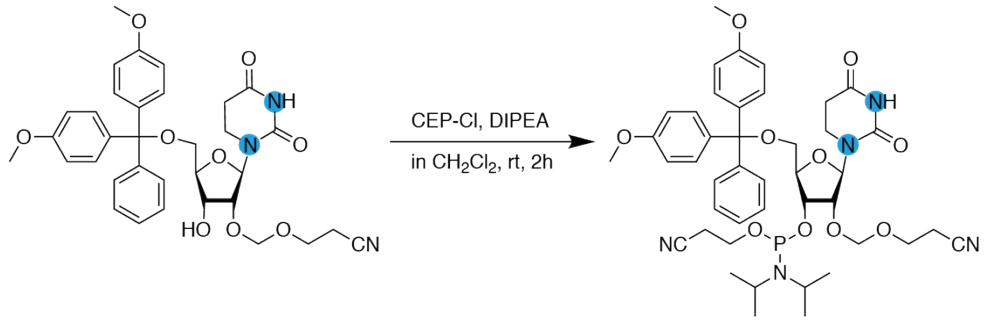
Crude compound **33** (6.13 g, 10.7 mmol, 1.00 eq.) was dissolved in 75 mL anhydrous tetrahydrofuran and triethylamine trifluorohydride (1.90 g, 11.8 mmol, 1.10 eq.) was added. The solution was stirred under argon atmosphere at room temperature for 2 hours. After TLC (EtOAc) showed complete conversion, the solvent was evaporated and the remaining oil was obtained as crude product **34**.
Further purification actions and a characterization with NMR spectroscopy were not carried out, the crude compound was applied for the next step of synthesis.
Yield: Assumption: 3.54 g of compound **34** (10.7 mmol, 100%).
TLC: (EtOAc), R_f = 0.08
^{1}H-NMR: not determined
^{13}C-NMR: not determined

2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-1,3-$^{15}N_2$-dihydrouridine (35)



Crude compound **34** (3.54 g, 10.7 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice, then dissolved in 75 mL anhydrous pyridine. To the reaction mixture 4,4'-dimethoxytrityl chloride (4.36 g, 12.8 mmol, 1.20 eq.) was added in three equal portions in intervals of 20 minutes. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 95/5) and showed a complete conversion after 12 hours of stirring at room temperature under argon atmosphere. Pyridine was removed at the rotary evaporator, the residual oil was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution and 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate was evaporated to dryness and the remaining foam was dried under high vacuum.
The crude product was purified by column chromatography (60 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 96/4) and the analytical pure compound **35** was isolated as an off-white foam after drying under high vacuum.
Yield: 5.04 g of an off-white foam (7.96 mmol, 75%).
TLC: (CH_2Cl_2/MeOH = 95/5) R_f = 0.29
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 10.31 (d, 1H, ^{15}N(3)*H*, $^1J_{NH}$ = 89.22 Hz); 7.41 – 7.25 (m, 9H, arom. C*H*); 6.93 – 6.90 (d, 4H, arom. C*H*-C-OCH_3, $^3J_{HH}$= 8.76 Hz); 5.83 (s, 1H, C(1')*H*, $^3J_{HH}$ = 5.00 Hz); 5.18 (d, 1H, C(3')O*H*, $^3J_{HH}$ = 5.64 Hz); 4.80 (s, 2H, -O-CH_2-O-); 4.13 – 4.07 m, 2H, C(2')*H*; C(3')*H*) 3.87 (m, 1H, C(4')*H*); 3.75 (s, 6H, 2x -OCH_3); 3.73 – 3.70 (m, 2H, -O-CH_2-CH_2-); 3.41 – 3.35 (m, 2H, (C(6)H_2); 3.16 (m, 2H, C(5')*H*; C(5'')*H*); 2.81 -2.77 (m, 2H, -O-CH_2-CH_2-) 2.46 (m, 2H, (C(5)H_2).
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 170.95 (*C*(4)); 158.97 (arom. *C*-OCH_3); 145.60 (*C*(2)); 136.37 – 136.27 (arom. *C*C); 130.58 – 127.58 (arom. *C*H); 120.08 (-*C*N); 114.09 (arom. *C*H-C-OCH_3); 94.84 (-O-*C*H_2-O-); 87.08 (*C*(1')); 82.83 (*C*(4')); 76.57 (*C*(3')); 70.13 (*C*(2')); 64.46 (*C*(5')); 63.33 (-O-*C*H_2-CH_2-); 55.82 (-O*C*H_3); 36.67 (C(6); 31.65 (C(5); 18.95 (-O-CH_2-*C*H_2-)

2'-*O*-(2-cyanoethoxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)-1,3-$^{15}N_2$-dihydrouridine-3'-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (36)



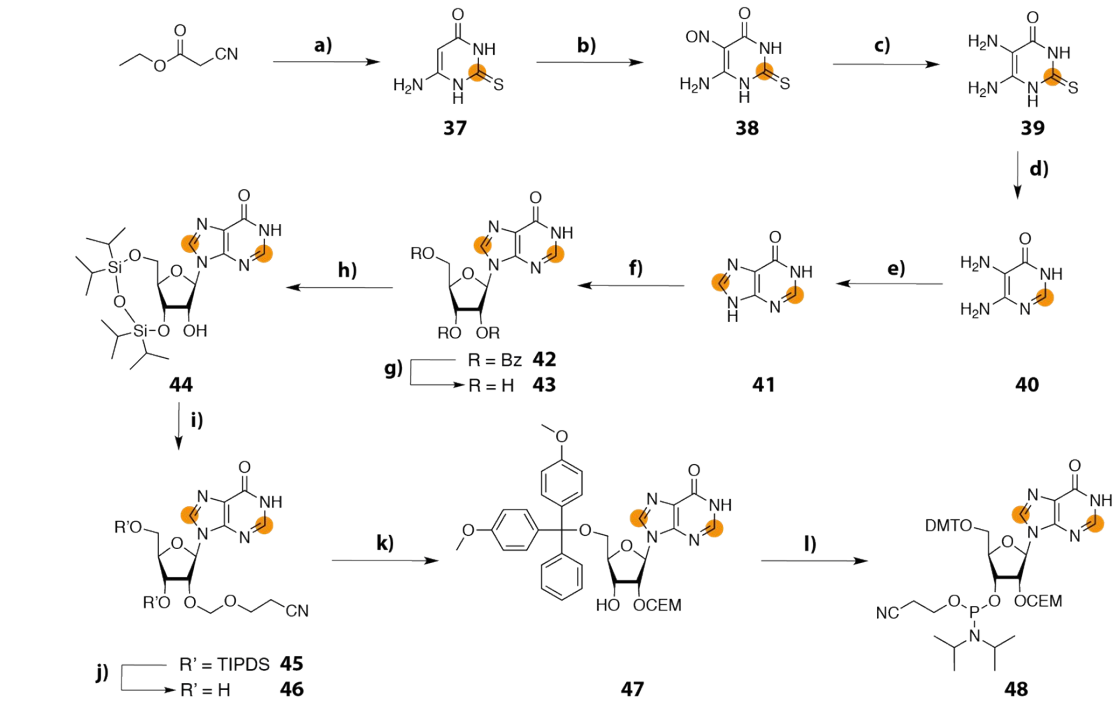
$C_{34}H_{37}N^{15}N_2O_9$
[633.25]

$C_{43}H_{54}N_3{}^{15}N_2O_{10}P$
[831.36]

N,N-diisopropylethylamine (6.08 mL, 34.8 mmol, 4.37 eq.) was diluted with 50 mL of anhydrous methylene chloride and compound **35** (5.04 g, 7.96 mmol, 1.00 eq.) was dissolved in the prepared solvent mixture. After 20 minutes of stirring at room temperature under argon atmosphere, 2´cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.32 g, 5.54 mmol, 1.10 eq.) was given to the reaction mixture and the solution was stirred for 1.5 hours. Monitoring with TLC (EtOAc/n-hexane = 8/2) showed a complete conversion, so 2 mL anhydrous methanol were added and it was stirred for 20 more minutes. The reaction mixture was diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the filtrate was evaporated to dryness at the rotary evaporator. The resulting white foam was dried under high vacuum before it was purified via column chromatography (60 g SiO_2, EtOAc/n-hexane:

4/6 – 7/3 + 1% triethylamine). The pure compound **36** was obtained as a white foam consisting of two diastereomeres.
Yield: 5.63 g of a white foam (6.76 mmol, 85%).
TLC: (EtOAc), R_f = 0.45 + 0.60
^{1}H-NMR (300 MHz $CDCl_3$, 25°C): δ 10.31 (d, 1H, ^{15}N(3)*H*, $^1J_{NH}$ = 90.59 Hz); 7.44 – 7.26 (m, 9H, arom. C*H*); 6.91 – 6.85 (d, 4H, arom. C*H*-C-OCH_3, $^3J_{HH}$= 8.50 Hz); 6.07 -6.06 (m, 1H, C(1')*H*); 5.04 – 4.85 (m, 2H, -O-CH_2-O-); 4.58 -4.50 (m, 1H, C(3')*H*); 4.38 – 4.35 (m, 1H, C(2')*H*); 4.19 – 4.11 (m, 1H, C(4')*H*); 3.96 – 3.94 (m, 2H, -P-O-C*H'*$_2$-CH_2-; -CH_2-O-C*H'*$_2$-CH_2-); 3.83 (s, 6H, 2x -OCH_3); 3.80 – 3.72 (m, 2H, -P-O-C*H''*$_2$-CH_2-; -CH_2-O-C*H''*$_2$-CH_2-); 3.71 -3.59 (m, 2H, C(6)H_2); 3.56 – 3.29 (m, 2H, C(5')*H*; C(5'')*H*); 2.73 – 2.60 (m, 4H, -P-O-CH_2-CH_2-; -CH_2-O-CH_2-CH_2-); 2.50 – 2.41 (m, 1H, C(5)H_2); 1.33 – 1.05 (m, 14H, 2x -N-C*H*-$(CH_3)_2$; 2x -N-CH-$(CH_3)_2$).
^{31}P-NMR (121 MHz, $CDCl_3$, 25°C): δ 151.16 (s); 150.03 (s).
ESI-MS: 834.3458 (calc. 834.35)

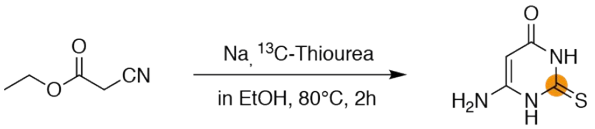
Synthesis of 2'-O-CEM-(2,8-$^{13}C_2$)-inosine-5´-O-DMT protected building block (compound 6 in manuscript)



● = ^{13}C

Scheme 6: Synthetic pathway to 2'-O-CEM-(2,8-^{13}C)-Inosine 5´-O-DMT protected building block; reagents and conditions: ***(a)*** *Na, ^{13}C-thiourea, in EtOH, 80°C, 2 h, 92%;* ***(b)*** *$NaNO_2$, in HCl_{aq}, 0°C 5 h, 90%;* ***(c)*** *$Na_2S_2O_4$, in $NaHCO_3$, 0°C, 6 h 100%;* ***(d)*** *H_2, RANEY®-Ni, in $NH_{3\ aq}$ 5%, 100°C, 2 h, 92%;* ***(e)*** *H_2SO_4, ^{13}C-formic acid, in H_2O, 100°C, 19 h, 54%;* ***(f)*** *ATBR, BSA, TMSOTf, in tolune, 100°C, 1.5 h, 68%;* ***(g)*** *in $MeNH_2$/EtOH, rt, 18 h, 78%;* ***(h)*** *TIPDSCl_2, in pyridine, rt, 18 h, 53%;* ***(i)*** *CEM, TfOH, NIS, Et_3N, in THF, -45°C, 1.5 h, 48%;* ***(j)*** *TEA·3HF, in THF, rt, 2 h, not purified;* ***(k)*** *DMT-Cl, in pyridine, rt, 4 h, 76%;* ***(l)*** *BTT, CTIP, in ACN, rt, 4 h, 68%*

(2-^{13}C)-6-Amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (37)



$C_5H_7NO_2$
[113.12]

$C_3{}^{13}CH_5N_3OS$
[144.16]

Sodium (1.60 g, 69.6 mmol, 1.05 eq.) was dissolved in absolute ethanol (50 mL) under argon atmosphere, then ethyl cyanoacetate (7.50 g, 66.3 mmol, 1.00 eq.) was added. To the resulting suspension ^{13}C-thiourea (5.10 g, 66.1 mmol, 1.00 eq.) was added and the mixture was refluxed for 2 hours. The solvents were removed in *vacuo* and the solid residue was dissolved with water (90 mL) to give a yellow solution. Acetic acid was added until pH 7 to precipitate the desired product. The solid was filtered, successively washed with water, ethanol and acetone and pure compound **37** was obtained as a white powder after drying under high vacuum.

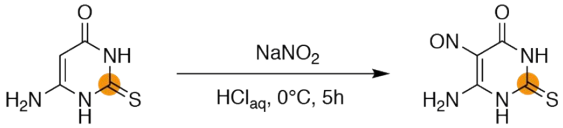
Yield: 8.77 g of a white powder (60.8 mmol, 92%)

TLC: not determined

^{1}H-NMR (300 MHz, DMSO-d$_6$, 25°C): δ 11.58 (s, 1H, N(3)*H*); 11.49 (s, 1H, N(1)*H*); 6.35 (s, 2H, N^6*H*$_2$); 4.71 (s, 1H, C(5)*H*)

^{13}C-NMR (75 MHz, DMSO-d$_6$, 25°C): δ 175.31 (^{13}*C*(2))

(2-^{13}C)-6-Amino-5-nitroso-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (38)



$C_3{}^{13}CH_5N_3OS$
[144.16]

$C_3{}^{13}CH_4N_4O_2S$
[173.15]

Compound **37** (8.77 g, 60.8 mmol, 1.00 eq.) was suspended in 1 N hydrochloric acid (175 mL) and a solution of sodium nitrite (4.41 g, 63.9 mmol, 1.05 eq.) in water (53 mL) was added to the reaction mixture at 0°C. It was stirred for 5 hours, until the red precipitate was filtered and successively washed with cold water and ethanol to give pure compound **38**.

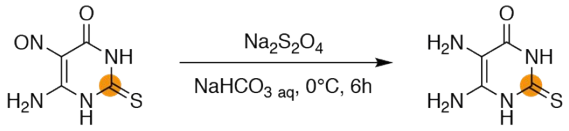
Yield: 9.43 g of a red powder (54.5 mmol, 90%)

TLC: not determined

^{1}H-NMR (300 MHz, DMSO-d$_6$, 25°C): δ 12.57 (s, 1H, N(3)*H*); 11.23 (s, 1H, N(1)*H*); 7.71 (s, 2H, N^6*H*$_2$)

^{13}C-NMR (75 MHz, DMSO-d$_6$, 25°C): δ 176.85 (^{13}*C*(2))

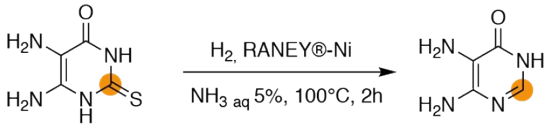
(2-^{13}C)-5,6-Diamino-2-thioxo-1,2-dihydro-4(1*H*)-pyrimidinone (39)



$C_3{}^{13}CH_4N_4O_2S$ [173.15] → $C_3{}^{13}CH_6N_4OS$ [159.17]

Compound **38** (9.43 g, 54.5 mmol, 1.00 eq.) was suspended in saturated sodium bicarbonate solution (225 mL) and solid sodium dithionite (24.7 g, 142 mmol, 2.60 eq.) was added in 4 equal portions at 0°C. The reaction mixture was stirred for 6h, then acetic acid was added until pH 6 was reached. The evolving precipitate was filtered, successively washed with ice cold water and ethanol. Pure compound **39** was dried under high vacuum.
Yield: 8.68 g of a pale yellow solid (54.5 mmol, 100%)
TLC: not determined
^{1}H-NMR (500 MHz, DMSO-d$_6$, 25°C): δ 7.71 (s, 4H, N^5*H*$_2$, N^6*H*$_2$)
^{13}C-NMR (75 MHz, DMSO-d$_6$, 25°C): δ 168.44 (^{13}*C*(2))

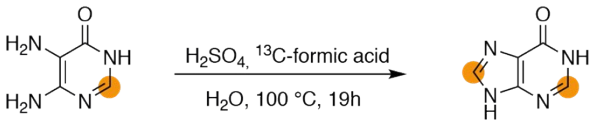
(2-^{13}C)-5,6-Diamino-4(3*H*)-pyrimidinone (40)



$C_3{}^{13}CH_6N_4OS$ [159.17] → $C_3{}^{13}CH_6N_4O$ [127.11]

Compound **39** (9.43 g, 54.5 mmol, 1.00 eq.) was dissolved in 5% aqueous ammonia (235 mL) and RANEY® nickel (45 mL of a 50% slurry in water) was added under hydrogen atmosphere. The reaction mixture was refluxed for 2 hours with vigorous stirring. The hot reaction mixture was filtered over celites and the filter cake was washed with boiling water several times. The filtrate was concentrated under reduced pressure and the yellowish residue, compound **40**, was co-evaporated with ethanol before drying under high vacuum.
Yield: 6.37 g of a pale yellow solid (50.1 mmol, 92%)
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d$_6$, 25°C): δ 7.39 (d, 1H, ^{13}C(2)*H*, $^1J_{CH}$ = 202.15 Hz); 5.48 (s, 4H, , N^5*H*$_2$, N^6*H*$_2$)
^{13}C-NMR (75 MHz, DMSO-d$_6$, 25°C):

(2,8-$^{13}C_2$)-Hypoxanthine (41)

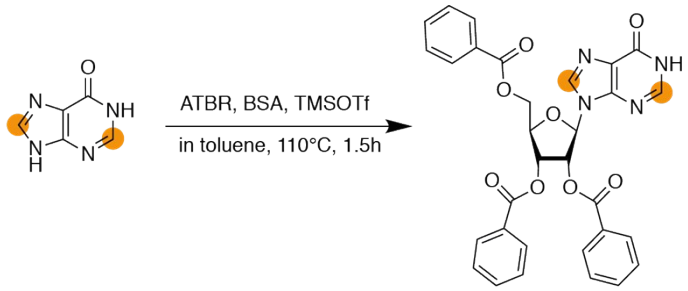


$C_3{}^{13}CH_6N_4O$ [127.11] → $C_3{}^{13}C_2H_4N_4O$ [138.10]

Compound **40** (3.95 g, 30.8 mmol, 1.00 eq.) was suspended with water (12 mL), then concentrated sulfuric acid (1.65 mL, 30.8, 1.00 eq) and ^{13}C-formic acid (1.75 mL, 46.3 mmol, 1.50 eq.) were successively added to the pale-yellow mixture. The reaction was refluxed 24 hours under argon atmosphere. It was allowed to cool to room temperature, then

neutralized with ammonia (28%) and acetic acid. The red precipitate was filtered, washed with water, ethanol and acetone and dried under high vacuum.
Yield: 2.31 g of a red solid (16.7 mmol, 54%)
TLC: not determined
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 12.65 (br, 2H, N(1)*H*, N(7)*H*); 8.12 (d, 1H, ^{13}C(2)*H*, $^{1}J_{CH}$ = 209.07 Hz); 7.98 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 204.54 Hz)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 145.16 (^{13}*C*(2)); 140.74 (^{13}*C*(8))

2',3',5'-tri-*O*-Benzoyl-(2,8-$^{13}C_2$)-inosine (42)

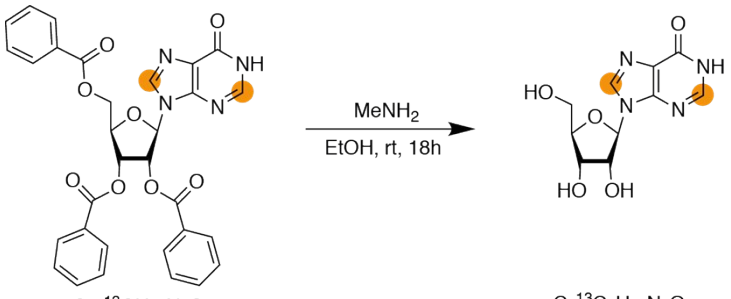


$C_3{}^{13}C_2H_4N_4O$
[138.10]

$C_{32}{}^{13}CH_{27}N_5O_9$
[638.60]

A mixture of solid compound **41** (3.36 g, 24.3 mmol, 1.00 eq.) and 1'-*O*-acetyl-2',3',5'-tri-*O*-benzoyl ribofuranoside (ATBR) (12.5 g, 24.3 mmol, 1.00 eq.) was co-evaporated with toluene twice, then suspended in toluene (150 mL). After addition of *N,O*-bis(trimethylsilyl)acetamide (36.7 mL, 146 mmol, 6.01 eq.), the mixture was heated to 110°C and stirred for 30 minutes under argon atmosphere. The reaction was allowed to cool to room temperature, trimethylsilyl trifluoromethane sulfonate (TMSOTf) (13.5 mL, 74.7 mmol, 3.00 eq.) was added and the mixture was refluxed for 30 more minutes. Monitoring by TLC (CH_2Cl_2/MeOH = 9/1) showed complete conversion. Toluene was removed *in vacuo* and the residue was dissolved with methylene chloride. The solution was washed with saturated sodium bicarbonate solution twice, the aqueous phase was extracted with diethyl ether twice, the organic layers were combined and dried over sodium sulfate. After evaporating the solvents, the crude product was purified by column chromatography (100 g SiO_2, CH_2Cl_2/MeOH: 100/0 – 94/6) to obtain compound **42** as a yellow foam.
Yield: 10.6 g of a yellow foam (16.6 mmol, 68%)
TLC: (CH_2Cl_2/MeOH = 95/5); R_f = 0.35
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 12.70 (s, 1H, N(1)*H*); 8.01 (d, 1H, ^{13}C(2)*H*, $^{1}J_{CH}$ = 214.05 Hz); 7.93 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 217.80 Hz); 7.64 – 7.38 (m, 15H, arom. *H* (Bz)); 6.43 – 6.36 (m, 2H, C(1')*H*, C(2')*H*); 6.22 (triplettoid, 1H, C(3')*H*); 4.97 – 4.72 (m, 3H, C(4')*H*, C(5')H_2)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 145.53 (^{13}*C*(2)); 139.39 (^{13}*C*(8))

(2,8-$^{13}C_2$)-Inosine (43)

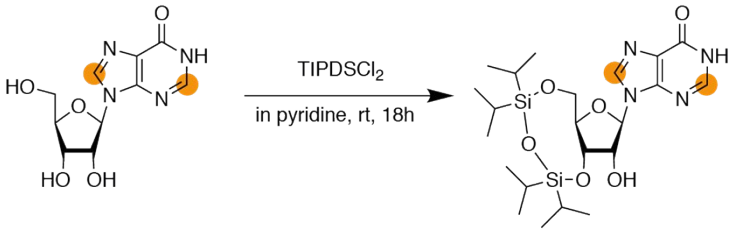


$C_{32}{}^{13}CH_{27}N_5O_9$
[638.60]

$C_8{}^{13}C_2H_{12}N_4O_5$
[270.21]

Compound **42** (10.6 g, 16.6 mmol, 1.00 eq.) was dissolved in a methylamine solution in ethanol (33% wt., 150 mL). The mixture was stirred for 18 hours at room temperature under argon atmosphere, then the solvent was removed *in vacuo* and the brown residue was dissolved with methanol. After adding diethyl ether, the product precipitated as a white solid. It was filtered and refluxed in diethyl ether afterwards for 30 minutes to remove residual benzamides. Pure compound **43** was filtered and dried under high vacuum.
Yield: 3.06 g of an off-white solid (11.32 mmol, 78%).
TLC: (CH_2Cl_2/MeOH = 7/3); R_f = 0.45
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 8.31 (d, 1H, ^{13}C(2)*H*, $^1J_{CH}$ = 214.23 Hz); 8.06 (d, 1H, ^{13}C(8)*H*, $^1J_{CH}$ = 204.96 Hz); 5.87 (triplettoid, 1H, C(1')*H*); C(2')*H*); 6.22 (triplettoid, 1H, C(3')*H*); 4.97 – 4.72 (m, 3H, C(4')*H*, C(5')H_2)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 145.53 (^{13}*C*(2)); 139.39 (^{13}*C*(8))

3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-(2,8-$^{13}C_2$)-inosine (44)



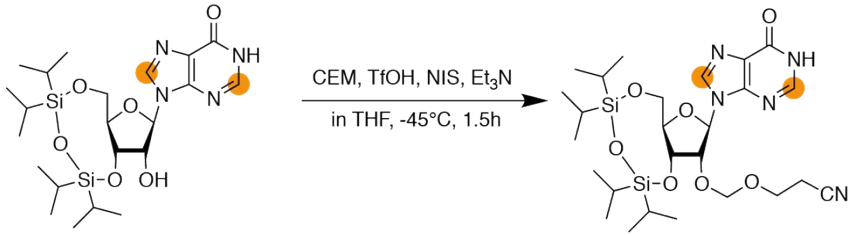
$C_8{}^{13}C_2H_{12}N_4O_5$
[270.21]

$C_{20}{}^{13}C_2H_{38}N_4O_6Si_2$
[512.72]

Compound **43** (1.00 g, 3.70 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice and then dissolved in 20 mL of anhydrous pyridine. 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.33 mL, 4.14 mmol, 1,12 eq.) was added and the reaction mixture was stirred under argon atmosphere at room temperature for 18 hours. Monitoring by TLC (CH_2Cl_2/MeOH = 9/1) showed a complete reaction, 2 mL methanol were added to quench the reaction and the solvent was removed under reduced pressure. The residue was dissolved in methylene chloride and washed with saturated sodium bicarbonate solution, then with 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate was evaporated under reduced pressure and the residual crude product was purified via column chromatography (75 g SiO_2, CH_2Cl_2/MeOH: 100/0 – 95/5).
Yield: 1.00 g of a yellow foam (1.95 mmol, 53%)
TLC: (CH_2Cl_2/MeOH = 9/1); R_f = 0.71
^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 12.38 (s, 1H, N(1)*H*); 8.16 (d, 1H, ^{13}C(2)*H*, $^1J_{CH}$ = 214.59 Hz); 7.99 (d, 1H, ^{13}C(8)*H*, $^1J_{CH}$ = 205.44 Hz); 5.87 (s, 1H, C(1')*H*); 5.68 (d, 1H, C(2')O*H*, $^3J_{HH}$ =

4.80); 4.61 – 4.57 (m, 1H, C(2')*H*); 4.45 (dd, 1H, C(3')*H*, $^{3}J_{HH}$ = 4.71 Hz, $^{3}J_{HH}$ = 5.28 Hz); 4.11 – 3.93 (m, 3H, C(4')*H*, C(5')H_2); 1.08 – 1.00 (m, 28H, 4x -Si-(C*H*)-$(CH_3)_2$; 4x -Si-(CH)-(CH_3)$_2$)
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 146.61 (^{13}*C*(2)); 139.05 (^{13}*C*(8))

2'-*O*-(2-Cyanoethoxymethyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-(2,8-$^{13}C_2$)-inosine (45)

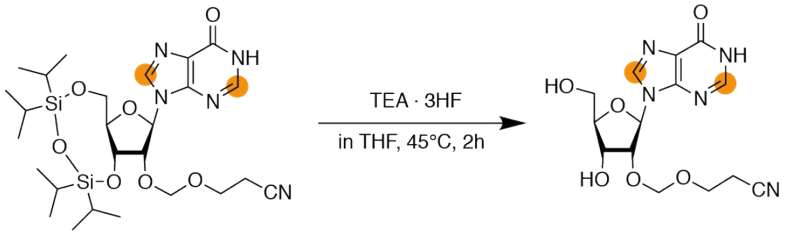


$C_{20}{}^{13}C_2H_{38}N_4O_6Si_2$
[512.72]

$C_{24}{}^{13}C_2H_{43}N_5O_7Si_2$
[595.81]

Compound **44** (1.00 g, 1.95 mmol, 1.00 eq.) was dissolved in 15 mL anhydrous tetrahydrofuran, 2-cyanoethyl methylthiomethylether (0.40 g, 3.04 mmol, 1.56 eq.) was added and the solution was cooled to -45°C. It was stirred under argon atmosphere for 30 minutes, then trifluoromethanesulfonic acid (0.46 g, 3.04 mmol, 1.56 eq.) was slowly dropped to the mixture. *N*-iodosuccinimide (0.69 g, 3.04 mmol, 1.56 eq.) was added in one portion and the reaction mixture was stirred for 20 minutes at -45°C till triethylamine (0.47 mL, 3.33 mmol, 1.71 eq.) was slowly added over 15 minutes to quench the reaction. The mixture was diluted with cold ethyl acetate and washed successively with saturated, ice cold sodium thiosulfate solution twice, saturated, ice cold sodium bicarbonate solution twice and saturated ice cold sodium chloride solution. The aqueous phases were extracted with ethyl acetate, the organic layers were combined and dried over sodium sulfate. The solvent was removed *in vacuo* and the pure compound **45** was obtained after column chromatography (40 g SiO_2; CH_2Cl_2/MeOH: 100/0 – 95/5).
Yield: 560 mg of a yellow foam (0.94 mmol, 48%)
TLC: (CH_2Cl_2/MeOH: 95/5); R_f = 0.29
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 12.18 (s, 1H, N(1)*H*); 8.23 (d, 1H, ^{13}C(2)*H*, $^{1}J_{CH}$ = 216.69 Hz); 8.09 (d, 1H, ^{13}C(8)*H*, $^{1}J_{CH}$ = 206.19 Hz); 6.07 (s, 1H, C(1')*H*); 5.11- 5.04 ((2xd, 2H, -O-CH_2-O-, $^{2}J_{HH}$ = 7.05 Hz, $^{2}J_{HH}$ = 7.05 Hz); 4.68 – 4.64 (m, 1H, C(2')*H*); 4.43 (duplettoid, 1H, C(3')*H*, $^{3}J_{HH}$ = 4.33 Hz); 4.20 (m, 1H, C(4')*H*); 4.09 – 4.00 (m, 2H, C(5')H_2); 3.92-3.85 (m, 2H, -O-CH_2-CH_2-); 2.73 – 2.60 (t, 2H, -CH_2-CH_2-CN, $^{3}J_{HH}$ = 5.69 Hz); 1.15 – 1.03 (m, 28H, 4x Si-(C*H*)-$(CH_3)_2$; 4x Si-(CH)-(CH_3)$_2$)
^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 145.10 (^{13}*C*(2)); 138.40 (^{13}*C*(8))

2'-*O*-(2-Cyanoethoxymethyl)-(2,8-$^{13}C_2$)-inosine (46)



$C_{24}{}^{13}C_2H_{43}N_5O_7Si_2$
[595.81]

$C_{12}{}^{13}C_2H_{17}N_5O_6$
[353.30]

Compound **45** (560 mg, 0.94 mmol, 1.00 eq.) was dissolved in anhydrous THF and triethylamine trihydrofluoride (TEA·3HF) (0.20 g, 0.94 mmol, 1.00 eq.) was added. The solution was stirred under argon atmosphere at 45°C for 2 hours. Monitoring by TLC (CH_2Cl_2/MeOH = 9/1) showed the desired conversion, so the solvent was removed *in vacuo*. The yellow crude compound **46** was not further purified and directly used for the next step of synthesis.

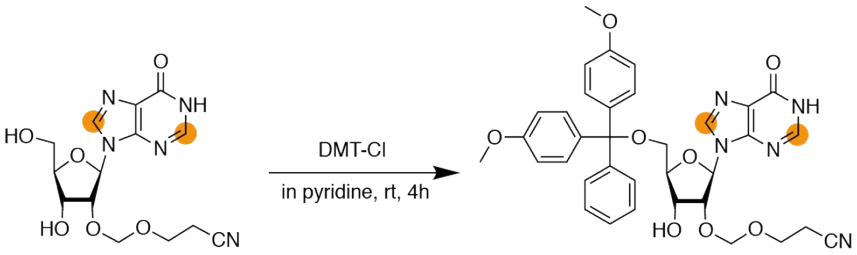
Yield: Assumption: 332 mg of compound 46 (0.94 mmol, 100%)

TLC: (CH_2Cl_2/MeOH: 9/1); R_f = 0.10

^{1}H-NMR: not determined

^{13}C-NMR: not determined

2'-*O*-(2-Cyanoethoxymethyl)-5'-*O*-(4,4'dimethoxytrityl)-(2,8-$^{13}C_2$)-inosine (47)



$C_{12}{}^{13}C_2H_{17}N_5O_6$
[353.30]

$C_{33}{}^{13}C_2H_{35}N_5O_8$
[655.68]

Crude compound **46** (332 mg, 0.94 mmol, 1.00 eq.) was co-evaporated with anhydrous pyridine twice, then dissolved in 35 mL anhydrous pyridine. 4,4'-dimethoxytrityl chloride (1.43 g, 4.23 mmol, 4.50 eq.) was added progressively until TLC (CH_2Cl_2/MeOH = 95/5) showed a complete conversion after 4 hours of stirring at room temperature under argon atmosphere. The reaction as quenched by adding 1 mL methanol, solvents were removed under reduced pressure and the residual oil was dissolved in methylene chloride. It was washed with saturated sodium bicarbonate solution and 5% citric acid. The organic phase was dried over sodium sulfate, the filtrate evaporated to dryness and the residue dried under high vacuum.

The crude product was purified by column chromatography (30 g SiO_2, CH_2Cl_2/MeOH = 100/0 – 96/4) and compound **47** was isolated as a yellow foam after drying under high vacuum.

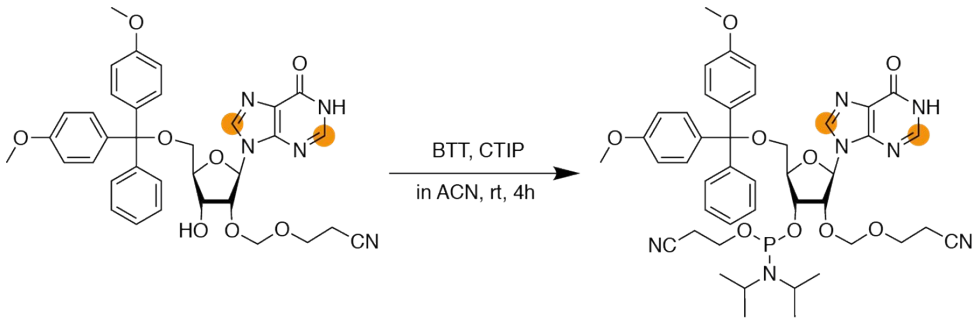
Yield: 470 mg of a yellow foam (0.72 mmol, 76%)

TLC: (CH_2Cl_2/MeOH: 95/5); R_f = 0.28

^{1}H-NMR (300 MHz, DMSO-d_6, 25°C): δ 12.41 (s, 1H, N(1)*H*); 8.21 (d, 1H, ^{13}C(2)*H*, $^1J_{CH}$ = 214.05 Hz); 8.00 (d, 1H, ^{13}C(8)*H*, $^1J_{CH}$ = 205.71 Hz); 7.38 – 7.23 (m, 9H, arom. C*H*); 6.93 – 6.83 (m, 4H, arom. C*H*-C-OCH_3); 6.07 (s, 1H, C(1')*H*); 5.40 (d, 1H, C(3')O*H*, $^3J_{HH}$ = 6.12 Hz) 4.86 – 4.75 (m,

2H, -O-CH_2-O-, C(2')*H*); 4.43 (m, 1H, C(3')*H*); 4.10 (m, 1H, C(4')*H*); 3.74 (s, 6H, 2x -OCH_3); 3.69 – 3.51 (m, 2H, C(5')H_2); 3.27 -3.22 (m, 2H, -O-CH_2-CH_2-); 2.74 – 2.63 (m, 2H, -CH_2-CH_2-CN);
^{13}C-NMR (75 MHz, DMSO-d_6, 25°C): δ 146.76 (^{13}*C*(2)); 139.66 (^{13}*C*(8))

2'-*O*-(2-Cyanoethoxymethyl)-5'-*O*-(4,4'dimethoxytrityl)-(2,8-$^{13}C_2$)-inosine-3'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (48)



$C_{33}{}^{13}C_2H_{35}N_5O_8$
[655.68]

$C_{42}{}^{13}C_2H_{52}N_7O_9P$
[855.36]

Compound **47** (470 mg, 0.72 mmol, 1,00 eq.) was dissolved in 5 mL anhydrous acetonitrile, 5-benzyl-1*H*-thiotetrazole (0.15 g, 0.79 mmol, 1.10 eq.) and 500 mg of 3A molecular sieves were added and stirred for 2 hours at room temperature under argon atmosphere. 2´Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane (543 mg, 1.80 mmol, 2.50 eq.) was added and the solution was stirred for 2 hours. Monitoring by TLC (CH_2Cl_2/MeOH = 95/5) showed complete conversion, so the molecular sieves were filtered, the filtrate diluted with methylene chloride and washed with half-saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate, the filtrate was evaporated and the residue dried under high vacuum. After column chromatography (40 g SiO_2, 1. n-hexane/isopropyl acetate/acetone: 3/1/1 + 1% triethylamine to elute the hydrolyzed phosphorylating reagent; 2. ethyl acetate/methanol: 9/1 + 1% triethylamine to elute product) the pure compound **48** was obtained as a white foam.
Yield: 420 mg of a white foam (0.49 mmol, 68%)
TLC: (EtOAc/acetone); R_f = 0.63 + 0.71
^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ 8.41 (s, 1H, N(1)*H*); 8.21 (d, 1H, ^{13}C(2)*H*, $^1J_{CH}$ = 214.05 Hz); 7.94 (d, 1H, ^{13}C(8)*H*, $^1J_{CH}$ = 214.92 Hz); 7.48 - 7.25 (m, 9H, arom. C*H*); 6.88 – 6.84 (m, 4H, arom.C*H*-C-OCH_3); 6.18 (m, 1H, C(1')*H*); 5.01 – 4.86 (m, 2H, -O-CH_2-O-); 4.82 – 4.78 (m, 1H, C(2')*H*); 4.65 – 4.39 (m, 1H, C(3')*H*,); 4.44 – 4.39 (m, 1H, C(4')*H*); 4.20 – 3.89 (m, 2H, -P-O-CH_2-CH_2-); 3.82 (s, 6H, 2x -OCH_3); 3.71 – 3.40 (m, 4H, -CH_2-O-CH_2-CH_2-; C(5')H_2); 2.70 – 2.41 (m, 6H, -CH_2-O-CH_2-CH_2-; -P-O-CH_2-CH_2, 2x -N-C*H*-$(CH_3)_2$), 1.32 – 1.21 (m, 12H, 2x -N-CH-$(CH_3)_2$)
^{13}C-NMR (75 MHz, $CDCl_3$, 25°C): δ 145.31 (^{13}*C*(2)); 139.35 (^{13}*C*(8))
^{31}P-NMR (121 MHz, $CDCl_3$, 25°C): δ 151.97; 151.71
ESI-MS. 856.3383 (calc. 856.36)

2 Solid phase synthesis of long SI-labeled RNAs

Standard and stable isotope labeled CEM phosphoramidites were used to assemble the described SI-modified RNAs. TBDMS protected controlled pore glass (CPG) solid support (1000Å 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-1*H*-tetrazole, 0.25 M) solutions

were dried over freshly activated molecular sieves (3Å) overnight. 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 2,6-lutidine and 160 mL anhydrous tetrahydrofuran
Oxidation solution: 500 mg iodine dissolved in THF (70 mL) and pyridine (20 mL) then 10 mL water was added.
Detritylation solution: 4% dichloroacetic acid in 1,2-dichloroethane.
After the RNA synthesis, the solid support was dried in high vacuum and then transferred into a 1.5 mL reaction tube to carry out the deprotection steps.
Standard alkaline deprotection: 300 µL absolute ethanol and 900 µL aqueous ammonia solution (28%) were added to the solid support. The reaction tube was shaken vigorously and incubated at 37°C overnight. The solid support was pelleted via centrifugation and the supernatant was filtered. The remaining solid support was washed three times with a mixture of methanol/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 1-2h.
Alkaline treatment for dihydrouridine-containing RNAs: The standard alkaline treatment could not be applied as a side reaction leading to ring opening of the nucleobase occurs under these conditions. Thus, a mild alkaline deprotection method using 1.2 mL 2 M ammonia in methanol at 30°C for 16 to 20h was applied. The solid support was pelleted via centrifugation and the supernatant was filtered. The remaining solid support was washed three times with a mixture of methanol/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 1-2h.
Removal of CEM protecting group: The CEM protecting group was removed by dissolving the partially deprotected RNA in 1 mL anhydrous DMSO followed by the addition of 10 µL nitromethane and 1 mL anhydrous 1M tetrabutylammonium fluoride (TBAF) solution in DMSO. The 10 mL round bottom flask was incubated at 30°C for 16h. The deprotection solution was directly applied to a HiPrep 26/10 desalting column (GE Healthcare) using a ÄKTA start system (GE Healthcare). The crude RNAs 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.

3 Quality control and purification of RNAs via anion-exchange chromatography

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 nucleic acid 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) and lyophilized. The integrity of the DNAs was further checked by mass spectrometry on a Finnigan LCQ Advantage MAX ion trap instrumentation connected to an Amersham Ettan micro LC (GE Healthcare) or on a 7T FTICR-mass spectrometer.

4 Mass spectrometry:

RNAs were desalted using Vivaspin 500 PES centrifugal concentrators (MWCO 5000) (Sartorius). Sample concentrations were determined by measuring UV absorption at 260 nm

on a NanoPhotometer (Implen). MS and MS/MS experiments were performed on a 7 Tesla Fourier transform ion cyclotron resonance (FT-ICR) instrument (Bruker) equipped with an electrospray ionization (ESI) source and a collision cell for collisionally activated dissociation (CAD). For ESI, a 3-4 µM solution of each RNA in 1:1 H_2O/CH_3OH (v/v) with piperidine and imidazole as additives (100 mM each) was prepared. Using polyethylene glycol 1000 as internal calibrant, exact mass values (most abundant isotopic peak) were determined. CAD of $(M\text{-}nH)^{n-}$ ions produced characteristic $\boldsymbol{c}$- and $\boldsymbol{y}$-type fragment ions from phosphodiester backbone cleavage that were used to confirm the correct sequence, and to locate the SI labels within the RNA sequences.

5 NMR sample preparation

RNA samples were lyophilized as the sodium salts and dissolved in the respective buffer:
63 nt MLV PK RNA
0.2 mM, 15 mM sodium phosphate buffer, 25 mM NaCl, pH 6.5, 9/1 H_2O/D_2O.
72 nt ssR26 RNA apo
0.2 mM, 15 mM sodium phosphate buffer, 25 mM NaCl, pH 6.5, 9/1 H_2O/D_2O.
72 nt ssR26 RNA holo
0.2 mM ssR26 RNA, 0.44 mM substrate RNA, 15 mM sodium phosphate buffer, 25 mM NaCl, pH 6.5, 9/1 H_2O/D_2O.
76 nt DHU only modified yeast $tRNA^{Phe}$
0.15 mM, 15 mM sodium cacodylate buffer, 25 mM NaCl, pH 6.5, 9/1 H_2O/D_2O.

6 NMR spectroscopy

NMR experiments on SI-modified RNA sequences were conducted on a Bruker 600 MHz Avance II+ with a Prodigy TCI™ probe.

63 nt MLV PK RNA
The imino proton spectrum was acquired using a selective excitation pulse sequence with the ^1H selective excitation centered at 12 ppm and an excitation band-width of 6 ppm and with the following parameters: spectral width 24 ppm, transmitter frequency offset 4.7 ppm, number of scans 256, interscan delay 200 ms, acquisition time 60 seconds. The 2D ^1H-^{13}C correlation spectrum was acquired using a ^1H-^{13}C-BEST TROSY pulse sequence (provided by Bernhard Brutscher, IBS Grenoble) with the ^1H selective excitation centered at 8.5 ppm and an excitation band-width of 5 ppm and the following acquisition parameters: ^1H spectral width 10 ppm, ^{13}C spectral width 12 ppm, time domain data 2048x48 data points, ^1H transmitter frequency offset 4.7 ppm, ^{13}C transmitter frequency offset 139 ppm, number of scans 128, interscan delay 300 ms, acquisition time 1h.

72 nt ssR26 RNA apo & holo
The imino proton spectra were acquired using a selective excitation pulse sequence with the ^1H selective excitation centered at 12 ppm and an excitation band-width of 6 ppm and with the following parameters: spectral width 24 ppm, transmitter frequency offset 4.7 ppm, number of scans 512, interscan delay 200 ms, acquisition time 120 seconds.
The 2D ^1H-^{15}N correlation spectra were acquired using a ^1H-^{15}N-SOFAST pulse sequence (provided by Bernhard Brutscher, IBS Grenoble) with the ^1H selective excitation centered at 12.5 ppm and an excitation band-width of 5 ppm and the following acquisition parameters: ^1H spectral width 24 ppm, ^{15}N spectral width 20 ppm, time domain data 2048x64 data points, ^1H transmitter frequency offset 4.7 ppm, ^{15}N transmitter frequency offset 150 ppm, number of scans 128, interscan delay 300 ms, acquisition time 1h. The HNN COSY spectrum was acquired using the *na_hnncosygpphspwg* pulse sequence from the standard Bruker

pulse program library with the following acquisition parameters: ^{1}H spectral width 24 ppm, ^{15}N spectral width 120 ppm, time domain data 1024x128 data points, ^{1}H transmitter frequency offset 4.7 ppm, ^{15}N transmitter frequency offset 188 ppm, number of scans 64, interscan delay 1.2 s, acquisition time 3h.

76 nt DHU only modified yeast tRNA^{Phe}

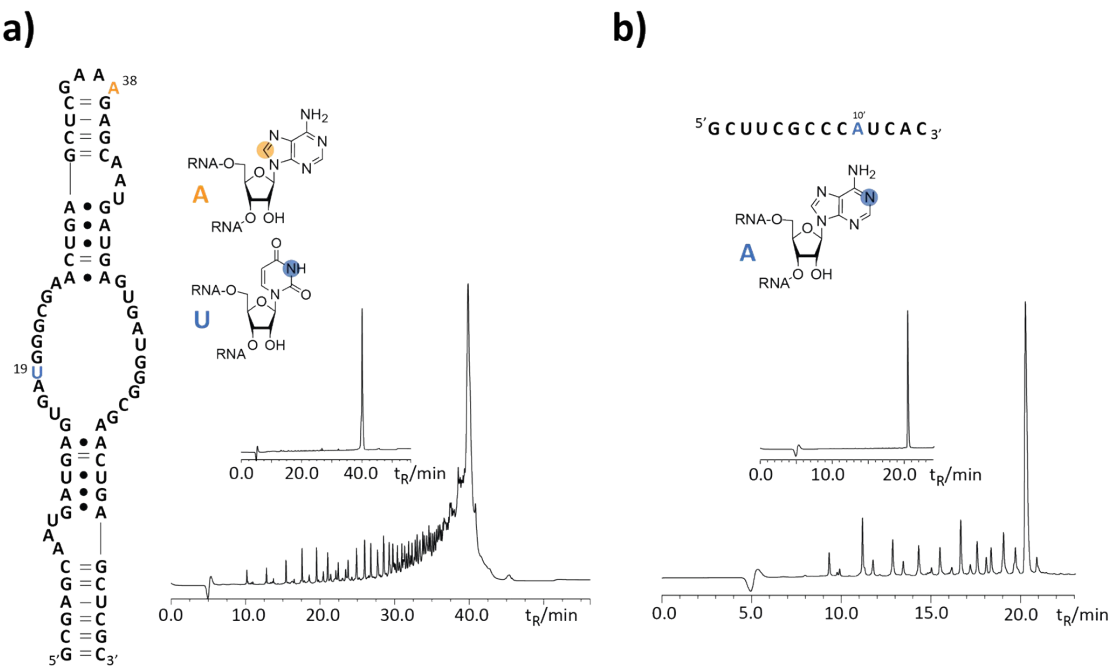
The imino proton spectra were acquired using a selective excitation pulse sequence with the ^{1}H selective excitation centered at 12 ppm and an excitation band-width of 6 ppm and with the following parameters: spectral width 24 ppm, transmitter frequency offset 4.7 ppm, number of scans 512, interscan delay 200 ms, acquisition time 120 seconds.

The 2D ^{1}H-^{15}N correlation spectra were acquired using a ^{1}H-^{15}N-SOFAST pulse sequence with the ^{1}H selective excitation centered at 11 ppm and an excitation band-width of 5 ppm and the following acquisition parameters: ^{1}H spectral width 24 ppm, ^{15}N spectral width 20 ppm, time domain data 2048x64 data points, ^{1}H transmitter frequency offset 4.7 ppm, ^{15}N transmitter frequency offset 150 ppm, number of scans 128, interscan delay 300 ms, acquisition time 2h.

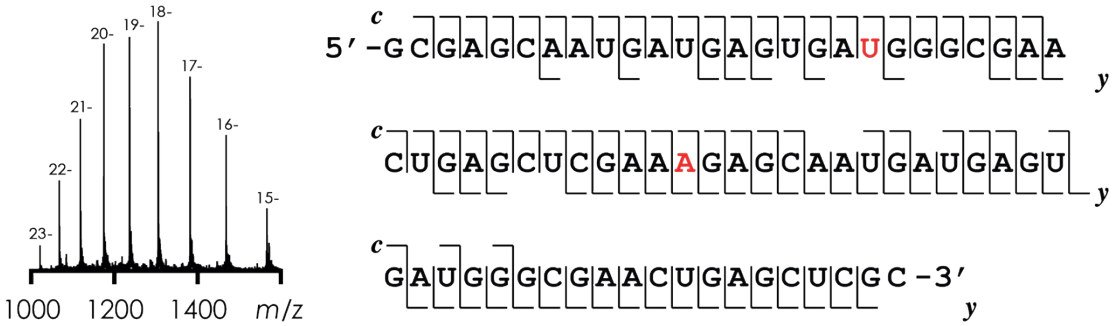
Topspin 3.5 pl6™ was used for data processing and for displaying the spectra.

Supporting Figure 1. CAD of $(M-15H)^{15-}$ ions of the synthetic 63 nt murine leukaemia virus (MLV) pseudoknot RNA: fragment ion map illustrating 90% sequence coverage with the modified residue 8-^{13}C-adenosine shown in red.

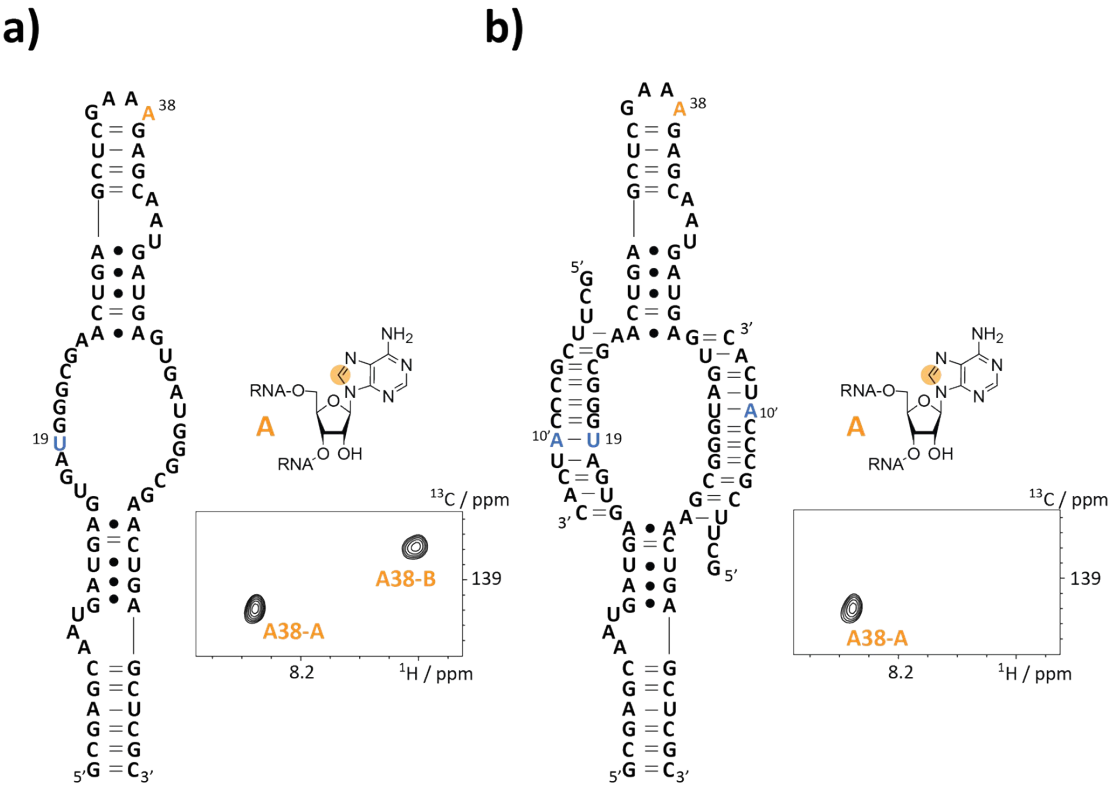
Supporting Figure 2. Anion-exchange (AIEX) chromatography of ssR26 RNA **(a)** and its 14 nt substrate RNA **(b)**. The chromatogram of the crude product after the deprotection steps and after AIEX-chromatographic purification (inset) are shown. Orange dot = ^{13}C, blue dot = ^{15}N.



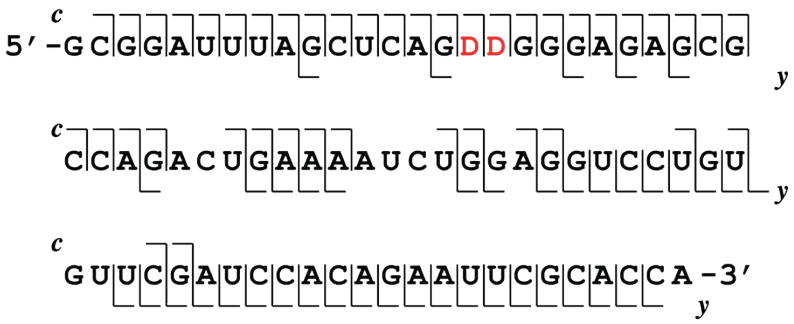
Supporting Figure 3. MS of the synthetic 72 nt box C/D guide ssR26 RNA, left: ESI mass spectrum (mol. wt. calc. 23509.25 Da, exp. 23509.24 Da), right: fragment ion map from CAD of $(M-18H)^{18-}$ ions illustrating 96% sequence coverage with the modified residues 3-^{15}N-uridine and 8-^{13}C-adenosine in red.



Supporting Figure 4. ^{1}H-^{13}C HSQC spectrum of ssR26 RNA with an 8-^{13}C-A38 label. **(a)** In the absence of substrate RNA the apo ssR26 RNA displays conformational heterogeneity. **(b)** After the addition of 2.2 eq. of substrate RNA only conformational state A is populated. Orange dot = ^{13}C.



Supporting Figure 5. CAD of (M-19H)$^{19-}$ ions of the synthetic 76 nt yeast tRNA^{Phe}: fragment ion map illustrating 88% sequence coverage with the modified 1,3-^{15}N-dihydrouridine residues in red.



1.3 µmol synthesis cycle on ABI 391 PCR mate

Supporting Table 1: Optimized cycle for solid phase synthesis of oligonucleotides with 2'-*O*-CEM protected phosphoramidites on an Applied Biosystems 391 automatic synthesizer and a 1.3 µmol synthesis scale. The coupling times for standard phosphoramidites (ACGU) were approx. 4 minutes and 6 minutes for modified amidites (X).

step	fxn	comment	time / s	amidite
1	10	#18 to waste	3	AGCUX
2	9	#18 to column	45	AGCUX
3	2	reverse flush	20	AGCUX
4	1	block flush	4	AGCUX
5	28	phos prep	3	AGCUX
6	61	tet to waste	2	AGCUX
7	19	b+tet to column	**3**	AGCUX
8	90	tet to column	2	AGCUX
9	19	b+tet to column	**2**	AGCUX
10	10	#18 to waste	4	AGCUX
11	1	block flush	3	AGCUX
12	61	tet to waste	2	AGCUX
13	19	b+tet to column	**3**	AGCUX
14	90	tet to column	2	AGCUX
15	19	b+tet to column	**2**	AGCUX
16	4	wait	**130**	AGCUX
17	4	wait	**200**	X
18	109	flush through col	1	AGCUX
19	4	wait	**30**	AGCUX
20	4	wait	**35**	X
21	2	reverse flush	1	AGCUX
22	4	wait	**25**	AGCUX
23	4	wait	**35**	X
24	109	flush through col	1	AGCUX
25	4	wait	**25**	AGCUX
26	4	wait	**25**	X
27	2	reverse flush	1	AGCUX
28	4	wait	**25**	AGCUX
29	4	wait	**25**	X
30	1	block flush	3	AGCUX
31	16	cap prep	3	AGCUX
32	10	#18 to waste	3	AGCUX
33	9	#18 to column	22	AGCUX
34	2	reverse flush	5	AGCUX
35	1	block flush	3	AGCUX
36	22	cap to column	15	AGCUX
37	4	wait	10	AGCUX

38	10	#18 to waste	3	AGCUX
39	2	reverse flush	6	AGCUX
40	1	block flush	3	AGCUX
41	81	#15 to waste	2	AGCUX
42	13	#15 to column	28	AGCUX
43	10	#18 to waste	3	AGCUX
44	1	block flush	3	AGCUX
45	4	wait	40	AGCUX
46	9	#18 to column	15	AGCUX
47	109	flush through col	6	AGCUX
48	9	#18 to column	15	AGCUX
49	109	flush through col	6	AGCUX
50	9	#18 to column	15	AGCUX
51	2	reverse flush	6	AGCUX
52	1	block flush	3	AGCUX
53	16	cap prep	3	AGCUX
54	10	#18 to waste	3	AGCUX
55	2	reverse flush	7	AGCUX
56	1	block flush	3	AGCUX
57	22	cap to column	12	AGCUX
58	4	wait	7	AGCUX
59	10	#18 to waste	3	AGCUX
60	1	block flush	3	AGCUX
61	9	#18 to column	15	AGCUX
62	2	reverse flush	15	AGCUX
63	1	block flush	3	AGCUX
64	9	#18 to column	20	AGCUX
65	2	reverse flush	12	AGCUX
66	1	block flush	3	AGCUX
67	9	#18 to column	25	AGCUX
68	2	reverse flush	4	AGCUX
69	1	block flush	3	AGCUX
70	33	cyc entry	1	AGCUX
71	10	#18 to waste	3	AGCUX
72	9	#18 to column	20	AGCUX
73	2	reverse flush	5	AGCUX
74	1	block flush	3	AGCUX
75	5	advance FC	1	AGCUX
76	6	waste port	1	AGCUX
77	82	#14 to waste	3	AGCUX
78	14	#14 to column	20	AGCUX
79	108	flush to trit	2	AGCUX
80	14	#14 to column	20	AGCUX
81	108	flush to trit	2	AGCUX
82	14	#14 to column	18	AGCUX

83	108	flush to trit	2	AGCUX
84	14	#14 to column	18	AGCUX
85	108	flush to trit	2	AGCUX
86	14	#14 to column	12	AGCUX
87	108	flush to trit	2	AGCUX
88	14	#14 to column	12	AGCUX
89	108	flush to trit	2	AGCUX
90	9	#18 to column	40	AGCUX
91	108	flush to trit	5	AGCUX
92	7	waste bottle	1	AGCUX
93	1	block flush	3	AGCUX
94	2	reverse flush	10	AGCUX
95	1	block flush	3	AGCUX
96	9	#18 to column	20	AGCUX
97	2	reverse flush	5	AGCUX
98	1	block flush	3	AGCUX
99	34	cyc end	1	AGCUX

7 References

1. M. A. Juen, C. H. Wunderlich, F. Nußbaumer, M. Tollinger, G. Kontaxis, R. Konrat, D. F. Hansen and C. Kreutz, *Angewandte Chemie International Edition*, 2016, **55**, 12008-12012.