Enzymatic synthesis and reverse transcription of RNAs incorporating 2'-*O*-carbamoyl uridine triphosphate

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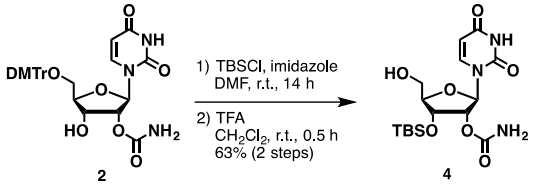
Contents

Experimental Section

General Remarks

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 500, 120 and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm), $CDCl_3$ (7.26 ppm) or DMSO-d_6 (2.49 ppm) for ^{1}H NMR spectra, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.7 ppm) for ^{13}C NMR spectra and 85% H_3PO_4 as an external standard for ^{31}P NMR spectra. Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd, and a minipump was conveniently used to attain sufficient pressure for rapid chromatographic separation. The synthesis of modified oligonucleotides was carried out by use of a DNA/RNA synthesizer 392 (Applied Biosystem). The unmodified oligonucleotides were purchased from Sigma-Aldrich Japan. DNase I, RNaseH and SuperScript III were purchased from Invitrogen. RNasin ribonuclease inhibitor was purchased from Promega. T7-RNA polymerase and EX Taq were purchased from Takara Bio.

Synthesis of 2′-*O*-carbamoyl-3′-*O*-(*tert*-butyldimethylsilyl)uridine (4)

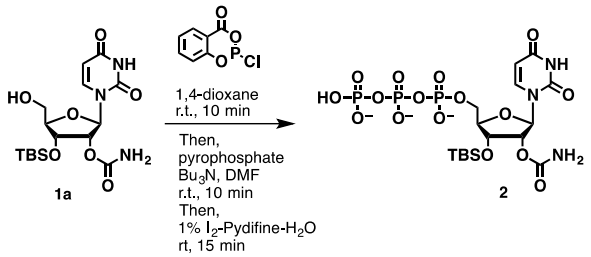


Compound **2** (2.12 g, 3.60 mmol) was rendered anhydrous by repeated coevaporation with pyridine, toluene, and CH_2Cl_2, and finally dissolved in anhydrous DMF (7.2 mL). TBSCl (0.81 g, 5.40 mmol) and imidazole (0.74 g, 10.8 mmol) was added to the solution, and the resulting mixture was stirred at room temperature for 14 h. The mixture was diluted with ethyl acetate (30 mL), and then washed three times with H_2O, sat $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. To the crude product, 4% trifluoroacetic acid in CH_2Cl_2 solution (36 mL) was added. After being stirred for 30 min, the reaction mixture was drop wisely added to the pyridine-methanol (1:1, v/v) solution (100 mL). After being stirred for 60 min, the resulting mixture was evaporated under reduced pressure. The residual precipitate was dissolved in CH_2Cl_2 (1 mL), and then hexane (10 mL) was added. The precipitate was collected by filtration to yield the product **4** (0.91 g, 63%)

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.39 (s, 1H), 7.89 (d, J = 8.1 Hz, 1H), 6.81 (s, 1H), 6.62 (s, 1H), 5.95 (d, J = 6.3 Hz, 1H), 5.69 (d, J = 8.1 Hz, 1H), 5.26 (t, J = 4.9 Hz, 1H), 5.04 (dd, J = 6.3, 5.1 Hz, 1H), 4.38 (dd, J = 5.1, 3.3 Hz, 1H), 3.87 (q, J = 3.3 Hz, 1H), 3.65 (dt, J = 12.0, 4.1 Hz, 1H), 3.54 (dt, J = 12.0, 4.1 Hz, 1H), 0.88 (s, 9H), 0.08 (s, 6H).

MS m/z calculated for $C_{16}H_{27}N_3NaO_7Si^+$ $[M+H]^+$: 424.1510, found: 424.1511.

Synthesis of 2′-*O*-carbamoyl-3′-*O*-(*tert*-butyldimethylsilyl)uridine 5′-triphosphate (5)

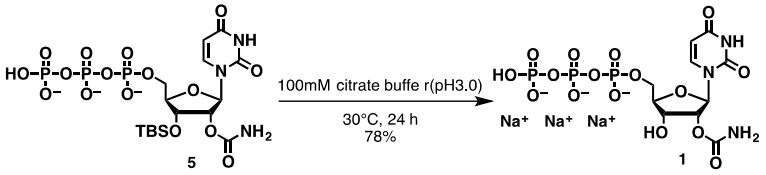


Compound **4** (181 mg, 0.45 mmol) was rendered anhydrous by repeated coevaporation with pyridine, and finally dissolved in anhydrous pyridine-1,4-dioxane (2:3, v/v, 1 mL). To the solution, 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (100 mg, 0.50 mmol) in 1,4-dioxane (500 μL) was added. After being stirred for 10 min, tributylamine (400 μL) and pyrophosphate tributylammonium salt (372 mg, 0.68 mmol) in DMF (1.3 mL) solution was added to the reaction mixture. After additional 10 min, 1% iodide pyridine-H_2O solution (98:2, v/v) was added. After being stirred for 15 min, the reaction mixture was diluted with H_2O (10 mL) and quenched with 5% $NaHSO_3$ solution (300 μL). After 30 min, the reaction mixture was concentrated under reduced pressure. The crude product was purified by reverse phase column chromatography (0.1 M triethylammonium acetate-MeCN, 70:30 to 50:50, v/v). The purified product was lyophilized and dissolved in MeOH (1 mL). To the solution, 0.6 M $NaClO_4$-acetone solution (10 mL) was added. The resulting mixture was centrifuged (7000 rpm, 4 min) and the supernatant liquid was removed. The precipitate was washed three times by acetone (10 mL) with centrifuge (7000 rpm, 4 min). The residual liquid was evaporated to yield the product **5** (182 mg, 61 %).

^{1}H NMR (500 MHz, D_2O) δ 11.36 (br s, 1H), 7.86 (br s, 1H), 6.78 (br s, 1H), 6.70-6.41 (br s, 1H), 5.94 (br s, 1H), 5.67 (br s, 1H), 5.23 (br s, 1H), 5.02 (br s, 1H), 4.37 (br s, 1H), 3.86 (br s, 1H), 3.58-3.70 (m, 1H), 3.48-3.60 (m, 1H), 0.86 (s, 9H), 0.25-0.08 (br s, 6H). ^{31}P NMR (203 MHz, D_2O) δ -7.64, -10.44, -21.47.

MS m/z calculated for $C_{16}H_{29}N_3O_{16}P_3Si^-$ $[M-H]^-$: 640.0535, found: 640.0534.

Synthesis of 2′-*O*-carbamoyl-uridine 5′-triphosphate (1)



Compound **5** was dissolved in 100 mM citrate buffer (pH 3.0). After being stirred at 30 °C for 24 h, the reaction mixture was lyophilized. The residual mixture was dissolved in methanol (1 mL). To the solution, 0.6 M $NaClO_4$-acetone solution (10 mL) was added. The resulting mixture was centrifuged (7000 rpm, 4 min) and the supernatant liquid was removed. The precipitate was washed three times by acetone (10 mL) with centrifuge (7000 rpm, 4 min). The residual liquid was evaporated to yield the product **1** (139 mg, 78 %). The triphosphate was used immediately in the next enzymatic experiments to minimize the migration of the carbamoyl group.

^{1}H NMR (500 MHz, D_2O) δ 7.94 (d, J = 8.2 Hz, 1H), 6.09 (d, J = 4.1 Hz, 1H), 5.99 (d, J = 8.2 Hz, 1H), 5.23 (t, J = 4.9 Hz, 1H), 4.60 (t, J = 5.7 Hz, 1H), 4.24-4.39 (m, 3H). ^{31}P NMR (203 MHz, D_2O) δ -8.26, -10.00, -21.00.

MS m/z calculated for $C_{10}H_{13}N_3Na_2O_{16}P_3$- $[M-3H+2Na]^-$: 570.1215, found: 570.1221.

Single dNTP insertion using SuperScript III

The reaction mixtures (10 μL) containing 0.2 μM template RNA, 0.2 μM cDNA primer, Reverse Transcription buffer (2 μL), 50 mM DTT, 1 mM dNTP (dATP, dGTP, dCTP or dTTP) and 150 unit of SuperScript III (Invitrogen) were incubated at 42 °C for 30 min. After the reaction, the reaction mixtures were heated up to 75 °C for 10 min. Then, mixtures were placed in ice bath. To the mixture, 0.5 unit of RNaseH (Invitrogen) was added. The resulting mixture was incubated at 37 °C for 15 min. Then the reaction mixtures were heated up to 75 °C for 5 min. Quenching solution (95% formamide, 0.05% bromophenol blue, 10 μL) was added to the mixtures. The resulting solutions were heated up to 95 °C for 3 min. The solutions were immediately cooled down by ice bath. The resulting samples were separated by electrophoresis using 20% denaturing polyacrylamide gel containing 7 M urea and were fluorescently visualized by Fujifilm FLA-7000.

Template RNA (U_{cm}): 5′-r(GAGCUUGAAC **U_{cm}** CCGACCACACGCGUCCGAGA)

Template RNA (U): 5′-r(GAGCUUGAAC **U** CCGACCACACGCGUCCGAGA)

cDNA: 5′-FAM-d(TCTCGGACGCGTGTGGTCGG)

Primer extension using SuperScript III

Extension reactions were performed under the same conditions as described above except that the reaction were performed in the presence of four 1 mM dNTPs (dATP, dGTP, dCTP, and dTTP). The resulting mixture were incubated at 42 °C for 30 min, 1 h or 2 h. After the reaction, the reaction mixtures were heated up to 75 °C for 10 min. Then, mixtures were placed in ice bath. To the mixture, 0.5 unit of RNaseH (Invitrogen) was added. The resulting mixture was incubated at 37 °C for 15 min. Then the reaction mixtures were heated up to 75 °C for 5 min. Quenching solution (95% formamide, 0.05% bromophenol blue, 10 μL) was added to the mixtures. The resulting solutions were heated up to 95 °C for 3 min. The solutions were immediately cooled down by ice bath. The resulting samples were separated by electrophoresis using 20% denaturing polyacrylamide gel containing 7 M urea and were fluorescently visualized by Fujifilm FLA-7000.

T7 RNA polymerase transcription using U_{cm}TP

The reaction mixture (20 μL) containing 0.5 μM template dsDNA, T7 RNA polymerase buffer (40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine), 5 mM DTT, 0.4 mM ATP, 0.4 mM GTP, 0.32 mM CTP, 0.08 mM FAM-CTP, 0.4 mM U_{cm}TP and 100 unit of T7 RNA polymerase (Takara Bio) was incubated at 37°C for 4 h. After the reaction, the mixtures were heated up to 75 °C for 5 min. Then, mixtures were placed in ice bath. After addition of 5 unit of DNase I (Invitrogen), the mixture was incubated at room temperature for 15 min. Quenching solution (95% formamide, 0.05% bromophenol blue, 10 μL) was added to the mixtures. The resulting solutions were heated up to 95 °C for 3 min. The solutions were immediately cooled down by ice bath. The resulting samples were separated by electrophoresis using 20% denaturing polyacrylamide gel containing 7 M urea and were fluorescently visualized by Fujifilm FLA-7000.

Template DNA 1: 5′-TAATACGACTCACTATAGGGAGAAGAGTACTGTCTATGATCCACCGA-3′

Template DNA 2: 5′-TCGGTGGATCATAGACAGTACTCTTCTCCCTATAGTGAGTCGTATTA-3′

RNA product: 5′-pppGGGAGAAGAG**X**AC**X**G**X**C**X**A**X**GA**X**CCACCGA-3'

T7 RNA polymerase transcription using U_{cm}TP for MALDI-TOF-MS analysis

The reaction mixture (50 μL) containing 1 μM template dsDNA, T7 RNA polymerase buffer (40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine), 5 mM DTT, 1 mM ATP, 1 mM GTP, 1 mM CTP, 10 mM U_{cm}TP, 40 unit of RNasin (Promega) and 50 unit of T7 RNA polymerase (Takara Bio) was incubated at 37 °C for 6 h. After addition of 5 unit of DNase I (Invitrogen), the mixture was incubated at 37 °C for 1 h. Quenching solution (0.05% bromophenol blue, 10 M urea, 10 μL) was added to the mixtures. The resulting solutions were heated up to 95 °C for 1 min. The resulting samples were separated by electrophoresis using 20% denaturing polyacrylamide gel containing 7 M urea and were fluorescently visualized by Fujifilm FLA-7000.

Template DNA 3: 5′-TAATACGACTCACTATAGGGCCAATACAAAGA -3′

Template DNA 4: 5′-TCTTTGTATTGGCCCTATAGTGAGTCGTATTA-3′

Expected RNA product: pppGGGCCAA**X**ACAAAGA

Consecutive transcriprion, reverse transcription and PCR of Spinach aptamer sequence with U_{cm}TP

Preparation of template dsDNA

The reaction mixtures (50 μL) containing 0.5 nM template DNA, 1 μM Fw primer and 1 μM Rv primer, PCR reaction buffer (5 μL), 0.2 mM dNTPs, SYBR green (0.5 μL, 100x solution) and 1.25 unit of TaKaRa EX Taq (Takara Bio) were applied for PCR. The cycle conditions were 95 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min for 30 cycles.

Template DNA:

5′-

GGGAGACGCGACCGAAATGGTGAAGGACGGGTCCAGTGCTTCGGCACTGTTGAGTAGAGTGTGAGCTCCG

TAACTGGTCGCGTCAAACGCAAACGCAAA -3′

Fw Primer: 5′-GTATAATACGACTCACTATAGGGAGACGCGACCGAAA -3′

Rv Primer: 5′-TTTGCGTTTGCGTTTG -3′

T7 transcription

The reaction mixture (100 μL) containing template dsDNA (1 ng), T7 RNA polymerase buffer (40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine), 5 mM DTT, 40 unit of RNasin (Promega), 4 mM ATP, 4 mM GTP, 4 mM CTP, 8 mM U_{cm}TP and 125 unit of T7 RNA polymerase (Takara Bio) was incubated at 37 °C for 4 h. After the reaction, the mixtures were heated up to 75 °C for 5 min. Then, the mixtures were placed in ice bath. After addition of 10 unit of DNase I (Invitrogen), the mixture was incubated at room temperature for 15 min. To the solution of reaction mixture (5 μL), quenching solution (95% formamide, 0.05% bromophenol blue, 10 μL) was added and visualized as described above. The residual solution (97 μL) was desalted by using Zeba spin desalting column (7K MWCO) (Thermo Scientific). The RNA product was separated by following standard chloroform-phenol extraction (pH 5.2) and ethanol precipitation. The collected precipitate was dissolved in DEPC water (50 μL). The sequential dilutions were allowed preparing 10,000 times diluted solution.

Reverse Transcription

A small amount of RNA product (2 μL of 1/10,000th diluted solution, which is 1/250,000th diluted product) was applied for SuperScript III mediated reverse transcription. The reaction mixtures (22 μL) containing transcripted RNA (2 μL), 10 μM cDNA primer, Reverse Transcription buffer (4 μL), 5 mM DTT, 0.25 mM dNTPs, 40 unit of RNasin (Promega) and 300 unit of SuperScript III (Invitrogen) were incubated at 45 °C for 4 h. After the reaction, the reaction mixtures were heated up to 90 °C for 5 min. Then, the mixtures were placed in ice bath. Then, the mixtures were placed in ice bath. To the mixture, 30 unit of RNaseH (Invitrogen) was added. The resulting mixture was incubated at 37 °C for 20 min. Then the reaction mixtures were heated up to 75 °C for 5 min. Then, mixtures were placed in ice bath. 0.5 μM FW primer and 0.5 μM RV primer, PCR reaction buffer (22 μL), 1 mM dNTPs, SYBR green (4 μL, 50x solution) and 5 unit of TaKaRa EX Taq (Takara Bio) were added and diluted with DEPC water up to 220 μL. The resulting reaction mixture was applied to PCR. The cycle conditions were 95 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min for 25 cycles.

Preparation of template dsDNA containing randomized sequence

The reaction mixtures (50 μL) containing 0.5 nM N=20 template, 1 μM FW primer and 1 μM RV primer, PCR reaction buffer (5 μL), 0.2 mM dNTPs, SYBR green (0.5 μL, 100x solution) and 1.25 unit of TaKaRa EX Taq (Takara) were applied for PCR. The cycle conditions were 90 °C for 1 min, 50 °C for 15 sec and 72 °C for 1 min for 14 cycles. The PCR products were purified by ethanol precipitation.

N=20 template:

5′-

GGGAGACGCGACCGAAATGGTGAAGGACGGGTCCAGTGCT**NNNNNNNNNNNNNNNNNNNN**GTGAGCTCC

GTAACTGGTCGCGTCAAACGCAAACGCAAA-3′

Fw Primer: 5′-GTATAATACGACTCACTATAGGGAGACGCGACCGAAA -3′

Rv Primer: 5′-TTTGCGTTTGCGTTTG -3′

T7 transcription

The reaction mixture (20 μL) containing the PCR products (1 μg), T7 RNA polymerase buffer (40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 20 mM spermidine), 5 mM DTT, 40 unit of RNasin (Promega), 4 mM ATP, 4 mM GTP, 4 mM CTP and 4 mM UTP or 8 mM $U_{cm}TP$ and 125 unit of T7 RNA polymerase (Takara) was incubated at 37 °C for 4 h. The mixtures were heated up to 75 °C for 5 min. Then, the mixtures were placed in ice bath. After addition of 10 unit of DNase I (Invitrogen), the mixture was incubated at 37 °C for 15 min. Quenching solution (95% formamide, 0.05% bromophenol blue, 20 μL) was added to the mixtures. The resulting solutions were heated up to 90 °C for 1 min. The resulting samples were analyzed by electrophoresis using 20% denaturing polyacrylamide gel containing 7 M urea and were fluorescently visualized by Fujifilm FLA-7000 using SYBR Gold dye.

Computational method

Molecular dynamic simulations were carried out using AMBER 12 program package.[23] the initial structure was obtained from reported NMR structure (PDB: 1QET). [24] The charge of non-canonical modified residues was obtained from previous report. The parm99, parmbsc0 and OL3 force fields were used for RNAs. The additional force field parameters were taken from GAFF.[25] The initial duplex was solvated in a periodic box with a 10 Å of water molecules, explicitly described by the TIP3P water model. Sodium and chloride ions were added to make 0.1 M NaCl solution. The minimization and equilibration (10.3 ns) were run accordingly to the protocol used in previous work.[15,26] The unrestrained extended simulations (25 ns) were performed with the Berendsen algorithm to maintain the temperature (300 K).[27] The 5000 steps of energy minimization were performed from the last snapshot of MD simulation. The obtained structures were used. During the MD simulations, hydrogen vibrations were removed using SHAKE bond constraints, allowing a longer time step of 2 fs. Long-range electrostatic interactions were treated using the Particle Mesh Ewald approach and a 10 Å cutoff.[28] All figures were produced by using Pymol.

Full citation of AMBER 12

D.A. Case, T.A. Darden, T.E. Cheatham, III, C.L. Simmerling, J. Wang, R.E. Duke, R. Luo, R.C. Walker, W. Zhang, K.M. Merz, B. Roberts, S. Hayik, A. Roitberg, G. Seabra, J. Swails, A.W. Götz, I. Kolossváry, K.F. Wong, F. Paesani, J. Vanicek, R.M. Wolf, J. Liu, X. Wu, S.R. Brozell, T. Steinbrecher, H. Gohlke, Q. Cai, X. Ye, J. Wang, M.-J. Hsieh, G. Cui, D.R. Roe, D.H. Mathews, M.G. Seetin, R. Salomon-Ferrer, C. Sagui, V. Babin, T. Luchko, S. Gusarov, A. Kovalenko, and P.A. Kollman (2012), AMBER 12, University of California, San Francisco.

a) b) c)

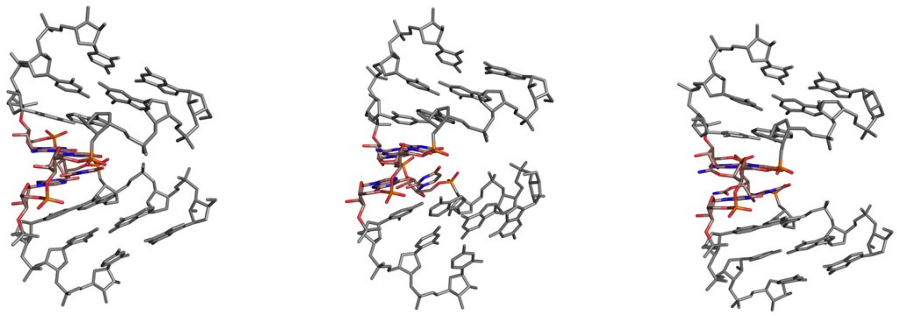


Fig S1 RNA duplex structure containing U-G mismatch base pair. a) NMR structure containing tandem G-U base pairs (PDB 1QET). b) The last snapshot of G-U base pair from 35 ns of MD simulation (initial structure is PDB 1QET). c) The last snapshot of G-U_{cm} base pair from 35 ns of MD simulation.

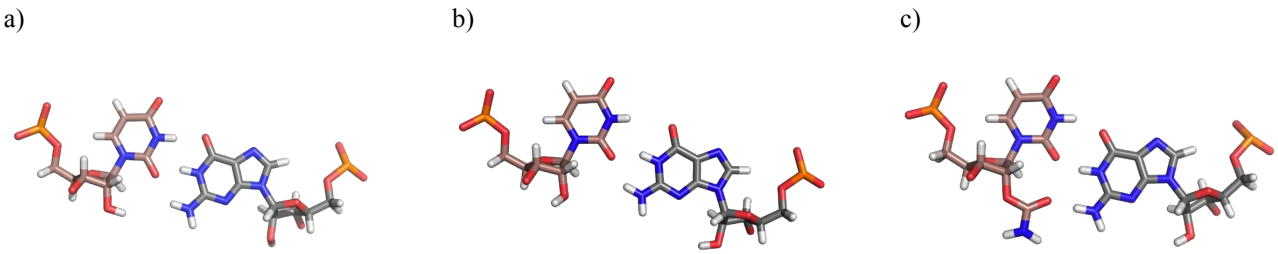


Fig S2 Structures of U-G mismatch base pair. a) NMR structure containing tandem G-U base pairs (PDB 1QET). b) The last snapshot of G-U base pair from 35 ns of MD simulation (initial structure is PDB 1QET). c) The last snapshot of G-U_{cm} base pair from 35 ns of MD simulation.

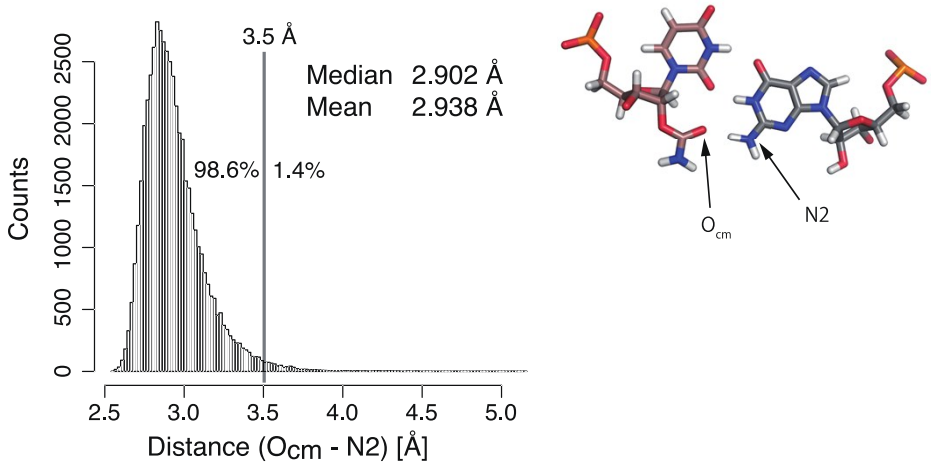


Fig S3 Population of hydrogen bonded state between the 2′-O-carbamoyl group and the 2-amino group.

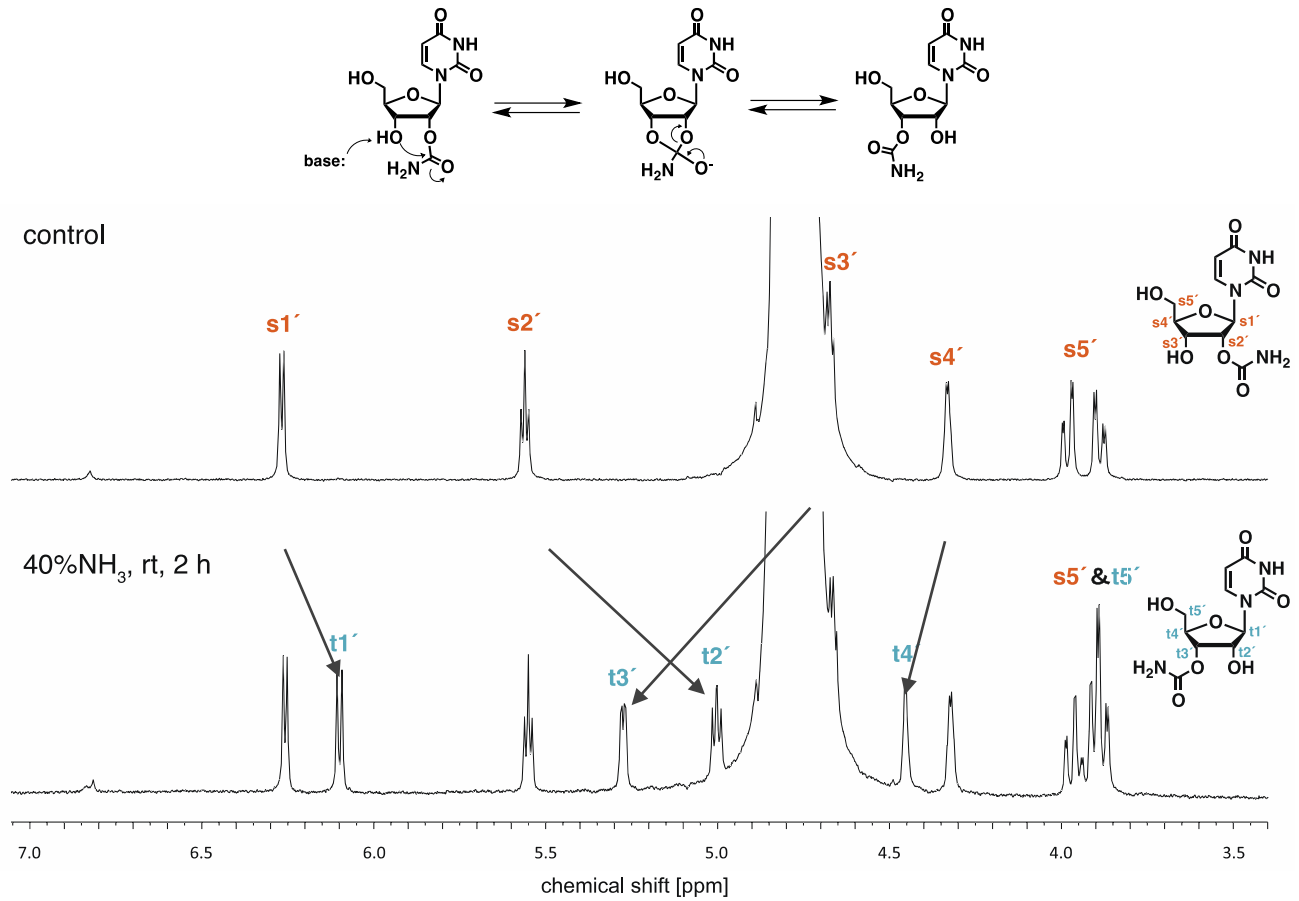
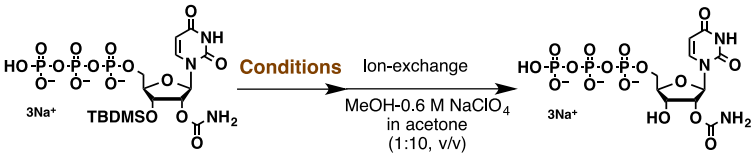


Fig S4 Migration of carbamoyl group to adjacent 3´-hydroxyl group under basic solution. The cleavage of carbamoyl group was not observed.

Table S1 Deprotection of TBS moiety in $U_{cm}TP$



Entry	Reagents	Solvent	Substrate concentration [M]	Temperature, Time	Yield
1	$Bu_4N^+ \cdot F^-$ 1.5 eq	THF	0.1	r.t., 1 h	≤5%
2	$Et_3N \cdot 3HF$ 1.8 eq Et_3N 3.5 eq	THF	0.1	r.t., 8 h	n.d.
3	8% AcOH	H_2O	0.1	30 °C, 60 h	54%
4	8% AcOH	H_2O	0.01	30 °C, 20 h	68%

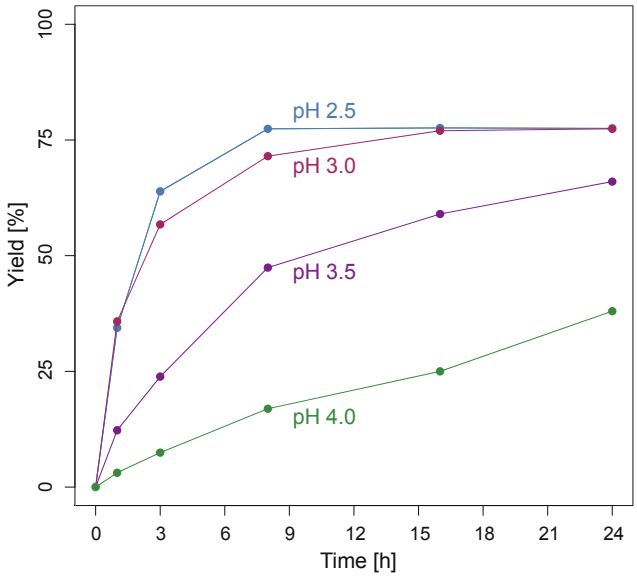


Fig S5 Deprotection of TBS moiety in $U_{cm}TP$ derivative under various pH buffer conditions. pH 2.5: 100 mM Glycine-HCl buffer, pH 3.0-4.0: 100 mM citric acid buffer.

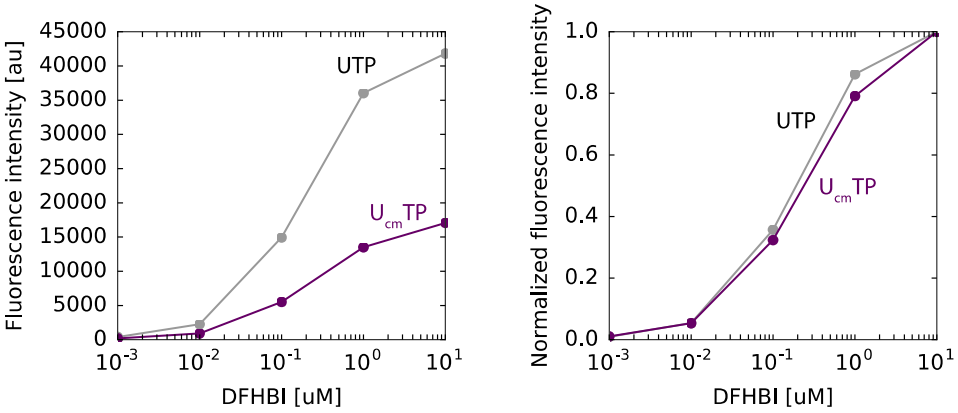


Fig S6 Dependence of fluorescence on DFHBI concentration. The sequence of RNA is Spinach sequence. While the RNA product transcribed with $U_{cm}TP$ showed lower fluorescence intensity than that transcribed with UTP in the presence of DFHBI, they showed comparable binding affinities to DFHBI.

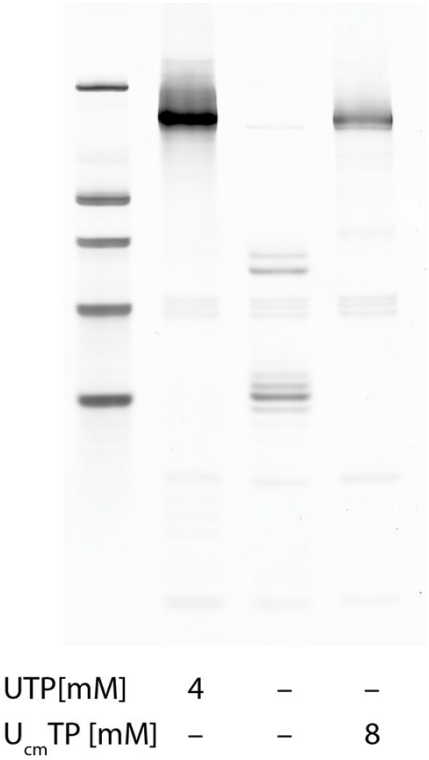
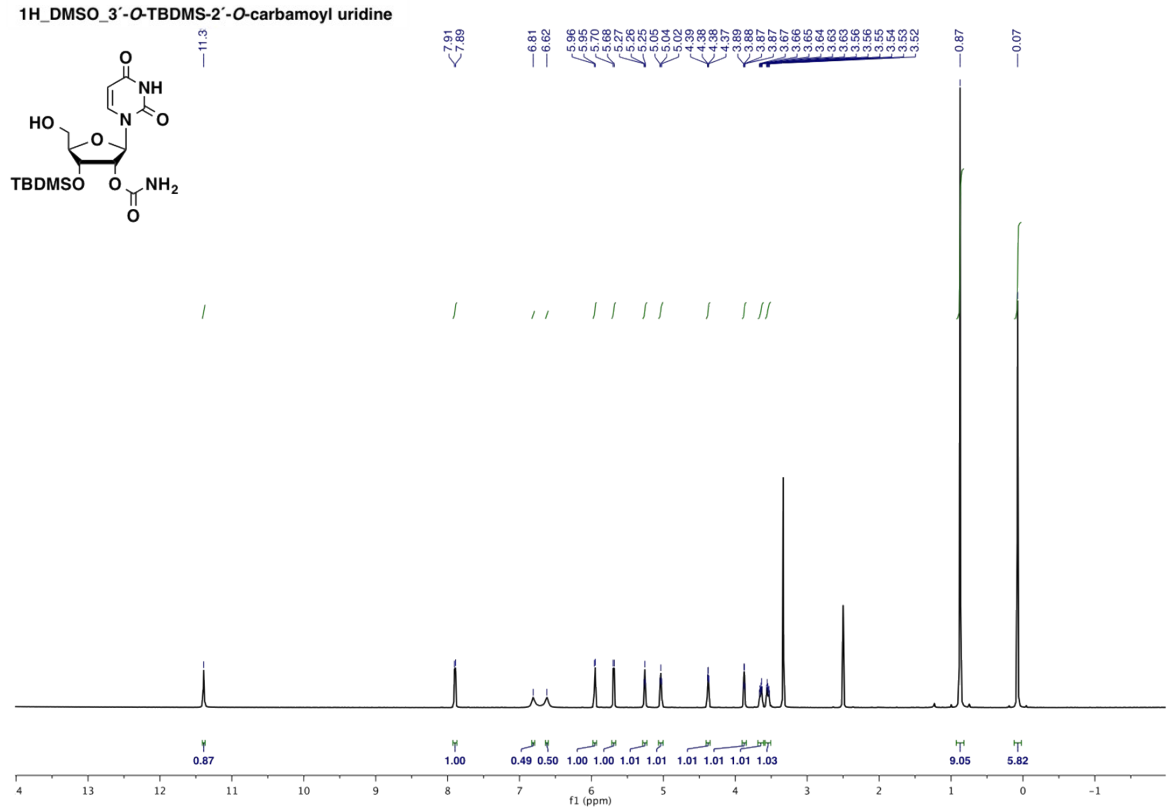
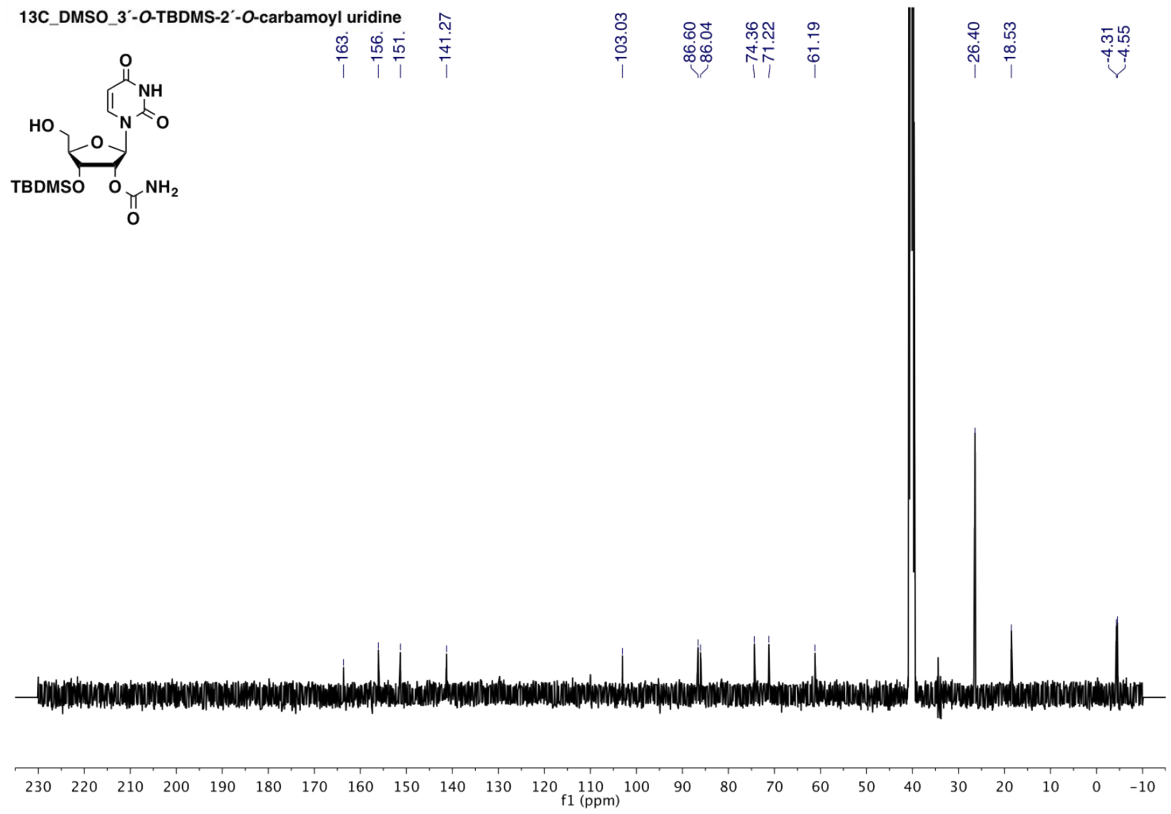


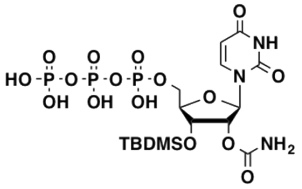
Fig S7 Gel electrophoresis of RNA products transcribed from randomized DNA template. Although gel-band intensity of the products transcribed with $U_{cm}TP$ was weaker than that with UTP, gel-band pattern of the RNA products containing Ucm was comparable with that containing UTP.

NMR spectra

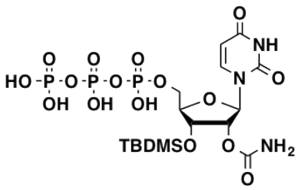

1H_DMSO_3´-O-TBDMS-2´-O-carbamoyl uridine
HO
TBDMSO
NH
NH2
11.3
7.91
7.89
6.81
6.62
0.87
0.07
0.87
1.00
0.49 0.50 1.00 1.00 1.01 1.01 1.01 1.01 1.01 1.03
9.05
5.82
f1 (ppm)


13C_DMSO_3´-O-TBDMS-2´-O-carbamoyl uridine
HO
TBDMSO
NH
NH2
163.
156.
151.
141.27
103.03
86.60
86.04
74.36
71.22
61.19
26.40
18.53
4.31
4.55
f1 (ppm)

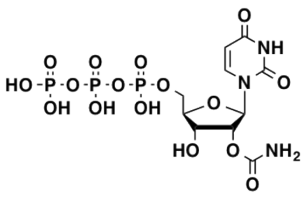
1H_D_2O_3′-*O*-TBDMS-2′-*O*-carbamoyl uridine triphosphate



31P_D_2O_3′-*O*-TBDMS-2′-*O*-carbamoyl uridine triphosphate



1H_D_2O_2′-*O*-carbamoyl uridine triphosphate



31P_D_2O_2′-*O*-carbamoyl uridine triphosphate