Electronic Supporting Information

Efficient conversion of N^6-threonylcarbamoyladenosine (t^6A) into tRNA native hydantoin cyclic form (ct^6A) performed on nucleoside and oligoribonucleotide level

Michal Matuszewski, Katarzyna Debiec and Elzbieta Sochacka*

Institute of Organic Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924 Lodz, Poland
E-mail: elzbieta.sochacka@p.lodz.pl

Table of Contents

I. Pilot experiments of t^6A cyclization using active esters (*in situ* formed on N^6-threonylcarbamoyl residue)

General procedure

To a stirring solution of L-t^6A (50 mg, 0.12 mmol) in H_2O or DMF (0.5 mL) the appropriate condensing agent was added (Table 1, entry 1-5). The progress of each reaction was monitored by TLC, using elution system consisting of *n*-butanol/H_2O (85:15, v/v; R_f of t^6A=0.05; R_f of ct^6A=0.30). After consumption of the starting nucleoside, the reaction mixture was evaporated to dryness, except the reactions of entry 1-3, where before evaporation, the EDC bounded to the polymer support was filtered off and washed with H_2O. In each case, the residual oily material was dissolved in MeOH and the solution was mixed with a small amount of silica gel, evaporated, applied on a silica gel column and then eluted with *n*-butanol/H_2O system (0-2% of H_2O) to obtain ct^6A nucleoside.

Table S1. Reagents tested for cyclization of L-threonine in t^6A to L-ct^6A.

ENTRY	Reagent	Solvent	AMOUNT OF REAGENT	TIME [min]	YIELD
1	EDC-P	H_2O	5 equiv	300	40%
2	EDC-P/HOBt	H_2O	5 equiv/5 equiv	50	90%
3	EDC-P/ HOSu	DMF	5 equiv/5 equiv	90	90%
4	BOP/DMAP	DMF	5 equiv/5 equiv	120	60%
5	HATU/ HOAt	DMF	5 equiv/5 equiv	20	70%

II. L-ct^{6}A and D-*allo*-ct^{6}A using EDC-P with HOBt addition

Synthesis of cyclic L-N^6-threonylcarbamoyladenosine (L-ct^{6}A) using EDC-P/HOBt.

To L-N^6-threonylocarbamoyladenosine (L-t^{6}A) in carboxylic H^+ form (0.5 g, 1.21 mmol) dissolved in Milli-Q® water (50 mL) the polymer-bounded EDC was added (5 equiv., 4.33 g, 6.06 mmol, EDC-P with ~1.4 mmol/g loading). The obtained suspension has been vigorously stirred at ambient temperature for 5 min, and then HOBt (5 equiv., 0.82 g 6.06 mmol) was added and the stirring was continued at room temperature. The progress of the reaction was monitored by TLC using elution system consisting of *n*-butanol/H_2O (85:15, v/v). Complete conversion of the substrate (R_f=0.05) to one product (R_f=0.30) was observed after 1 h of reaction time. Next, the resin with condensing agent was filtered off from the reaction mixture, washed carefully with H_2O (3 x 50mL), MeOH (2 x 50 mL) and the collected solvents were removed under reduced pressure. The fluffy residue was dissolved in MeOH and the solution mixed with silica gel, evaporated and applied on silica gel column. Elution was carried out initially with *n*- butanol (100 mL) and 1% H_2O in *n*-butanol (100 mL) to remove HOBt (R_f =0.60 for HOBt in *n*-butanol/H_2O (85:15 v/v) system) and then using 2% H_2O in *n*-butanol (150 mL) to obtain 0.45 g (1.14 mmol) of L-ct^{6}A as a white solid (yield 90%).

Synthesis in a smaller scale (50 mg of L-t^{6}A) was monitored by analytical RP-HPLC and the complete conversion to L-ct^{6}A was observed after 1 h (ESI, Figure S1). The crude product was purified by preparative HPLC on Ascentis® C18 HPLC column (10 µm, 21.2 x 250 mm) with linear gradient of acetonitrile (B) in 0.1% AcOH in water (A) as follows: 0-30 min from 2% B to 30% B, 30-35 min from 30% B to 50% B, 35-37 min from 50% B to 2% B, 37-40 min 2% B with a flow rate of 7 ml/min. The fraction containing L-ct^{6}A (with retention time 10.11 min) was evaporated to dryness. The residue was lyophilized furnishing L-ct^{6}A as a white solid (48 mg, 0.121 mmol, 95%).

Synthesis of cyclic D-*allo*-N^6-threonylcarbamoyladenosine (D-*allo*-ct^{6}A) using EDC-P/HOBt.

The synthesis of D-*allo* isomer of cyclic N^6-threonylcarbamoyladenosine (D-*allo*-ct^{6}A) was performed according to the procedure described for the L isomer, starting from D-*allo*-N^6-threonylcarbamoyladenosine (D-*allo*-t^{6}A) carboxylic H^+ form (50 mg, 0.12 mmol). The progress of the reaction was monitored by TLC using *n*-butanol/H_2O (85:15, v/v) system as well as by HPLC (ESI Figure S2) and after 1 h of the reaction time complete consumption of the substrate occurred (TLC, R_f=0.04) to give one product (R_f=0.27). The crude product was purified by preparative HPLC (Ascentis® C18 HPLC Column, 10 µm, 21.2 x 250 mm) with linear gradient of acetonitrile (B) in 0.1% AcOH in water (A) as follows: 0-30 min from 2% B to 30% B, 30-35 min from 30% B to 50% B, 35-37 min from 50% B to 2% B, 37-40 min 2% B with a flow rate of 7 ml/min.; R_t=10.90 min of cyclic nucleoside) to obtain D-*allo*-ct^{6}A as a white solid (43 mg, 0.11 mmol, yield 92%).

III. HPLC monitoring of L-t^{6}A and D-*allo*-t^{6}A cyclization

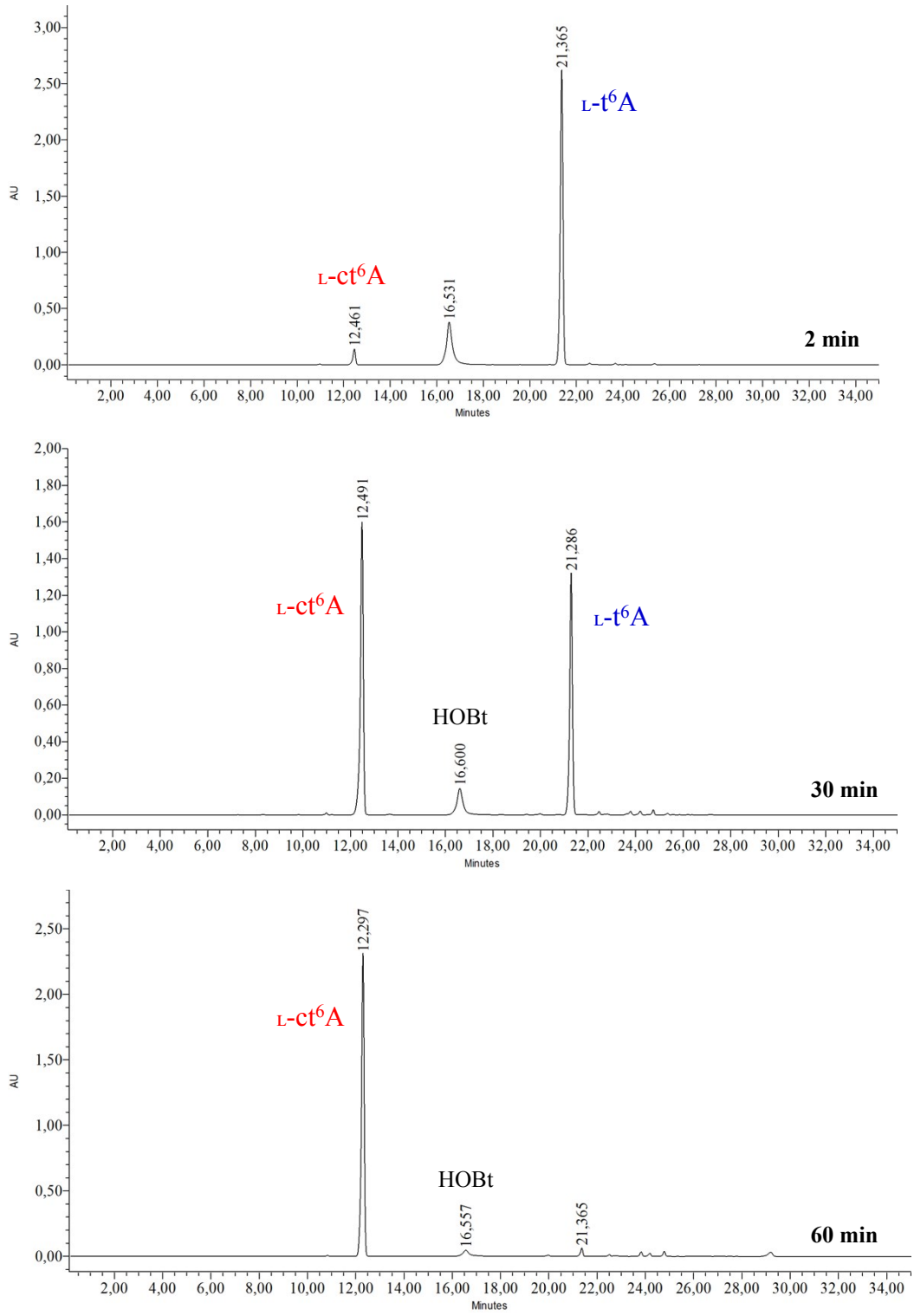


Figure S1. The HPLC monitoring of **L-t^{6}A** cyclization to **L-ct^{6}A** (Ascentis® C18 HPLC Column, 5 µm, 4.6 x 250 mm with linear gradient of buffer A (0.1% AcOH in H_2O) and buffer B (ACN) with a flow of 1 mL/min as follows: 0-15 min from 2%B to 8%B, 15-30 min from 8%B to 25%B 30-35 min 2%B).

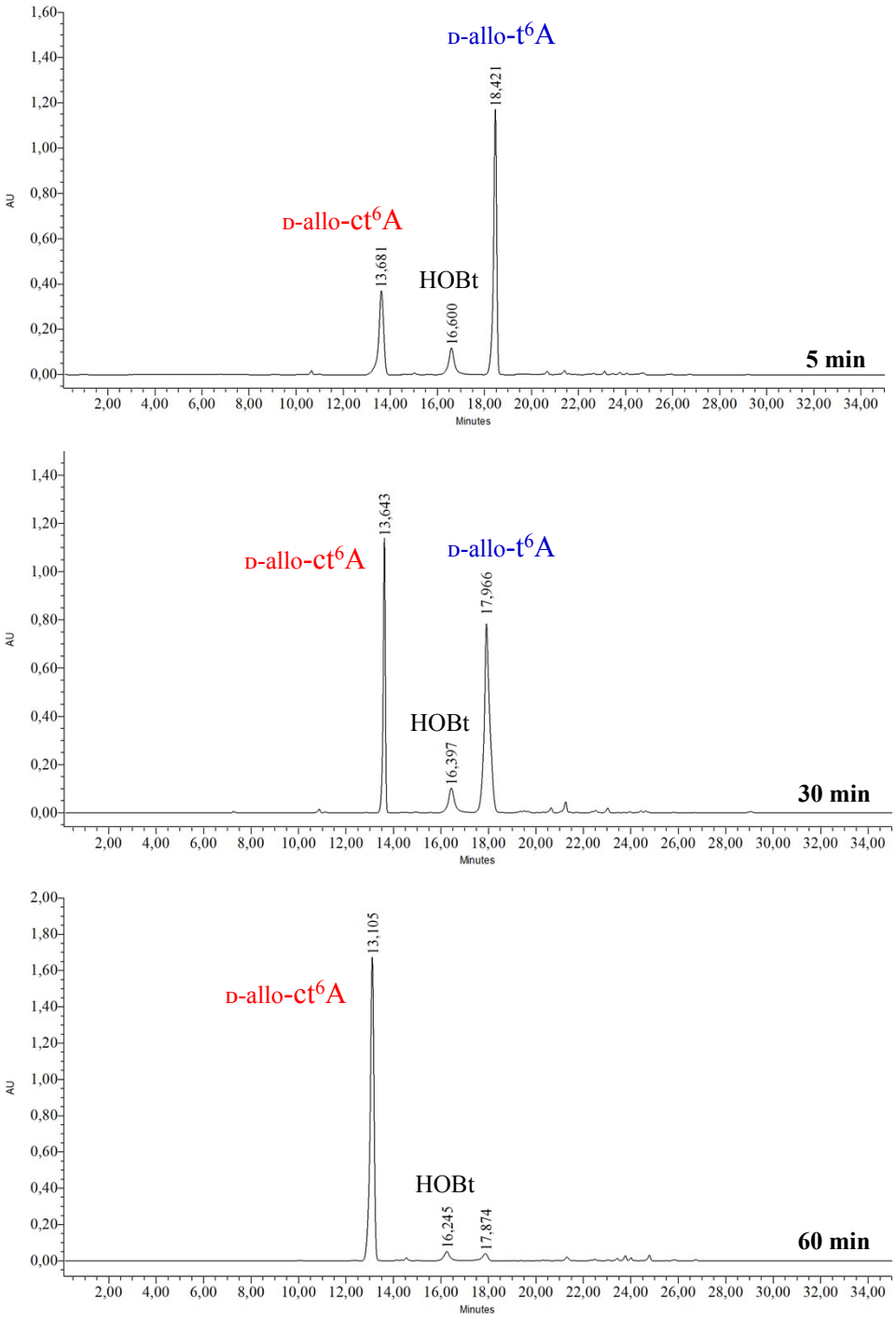


Figure S2. The HPLC monitoring of **D-*allo*-t^{6}A** cyclization to **D-*allo*-ct^{6}A** (Ascentis® C18 HPLC Column, 5 µm, 4.6 x 250 mm with linear gradient of buffer A (0.1% AcOH in H_2O) and buffer B (ACN) with a flow of 1 mL/min as follows: 0-15 min from 2%B to 8%B, 15-30 min from 8%B to 25%B 30-35 min 2%B).

IV. RP-HPLC coinjection analysis of L-t^{6}A, L-ct^{6}A and D-*allo*-t^{6}A, D-*allo*-ct^{6}A

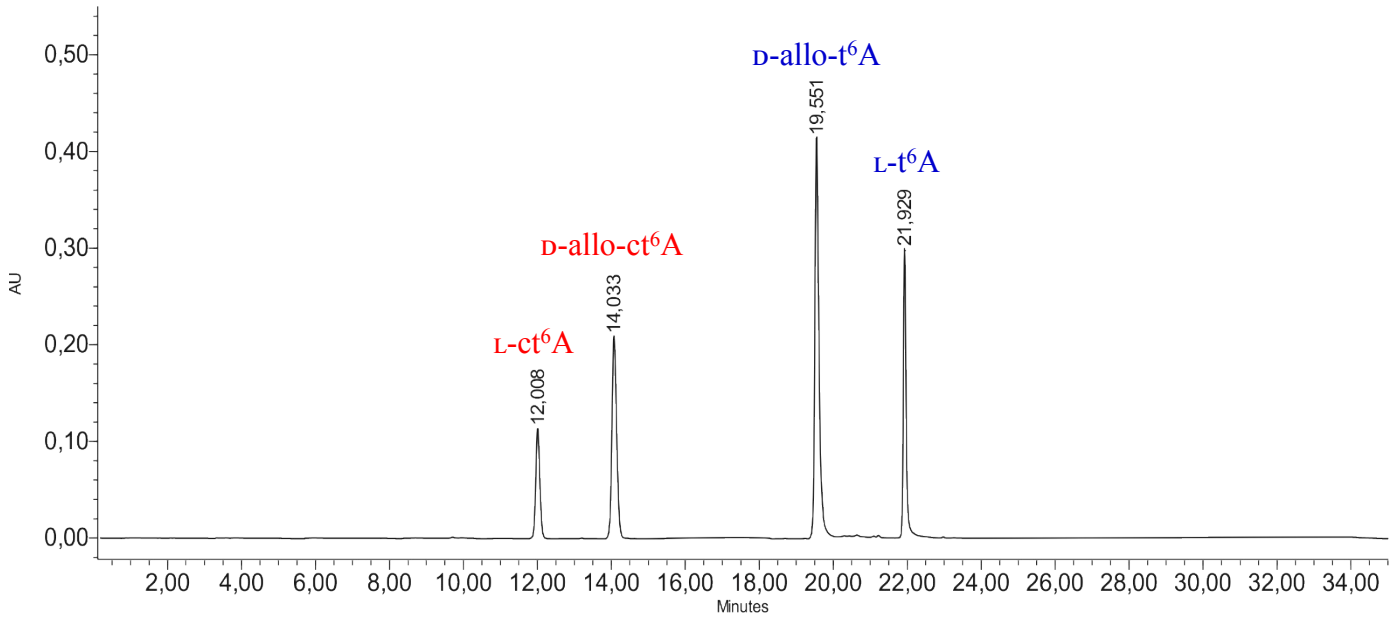


Figure S3. Reference HPLC chromatogram of the mixture of **L-ct^{6}A**, **D-*allo*-ct^{6}A**, **D-*allo*-t^{6}A**, **L-t^{6}A** nucleosides. RP-HPLC on C18 column (Kinetex C18 HPLC Column, 5 µm, 4.6 x 250 mm) with linear gradient of buffer A (0.1% AcOH in H_2O) and buffer B (ACN) with a flow of 1 mL/min as follows: 0-15 min from 2%B to 8%B, 15-30 min from 8%B to 25%B 30-40 min 2%B.

V. ^{1}H, and ^{13}C and spectra of L-t^{6}A, L-ct^{6}A and D-*allo*-t^{6}A, D-*allo*-ct^{6}A

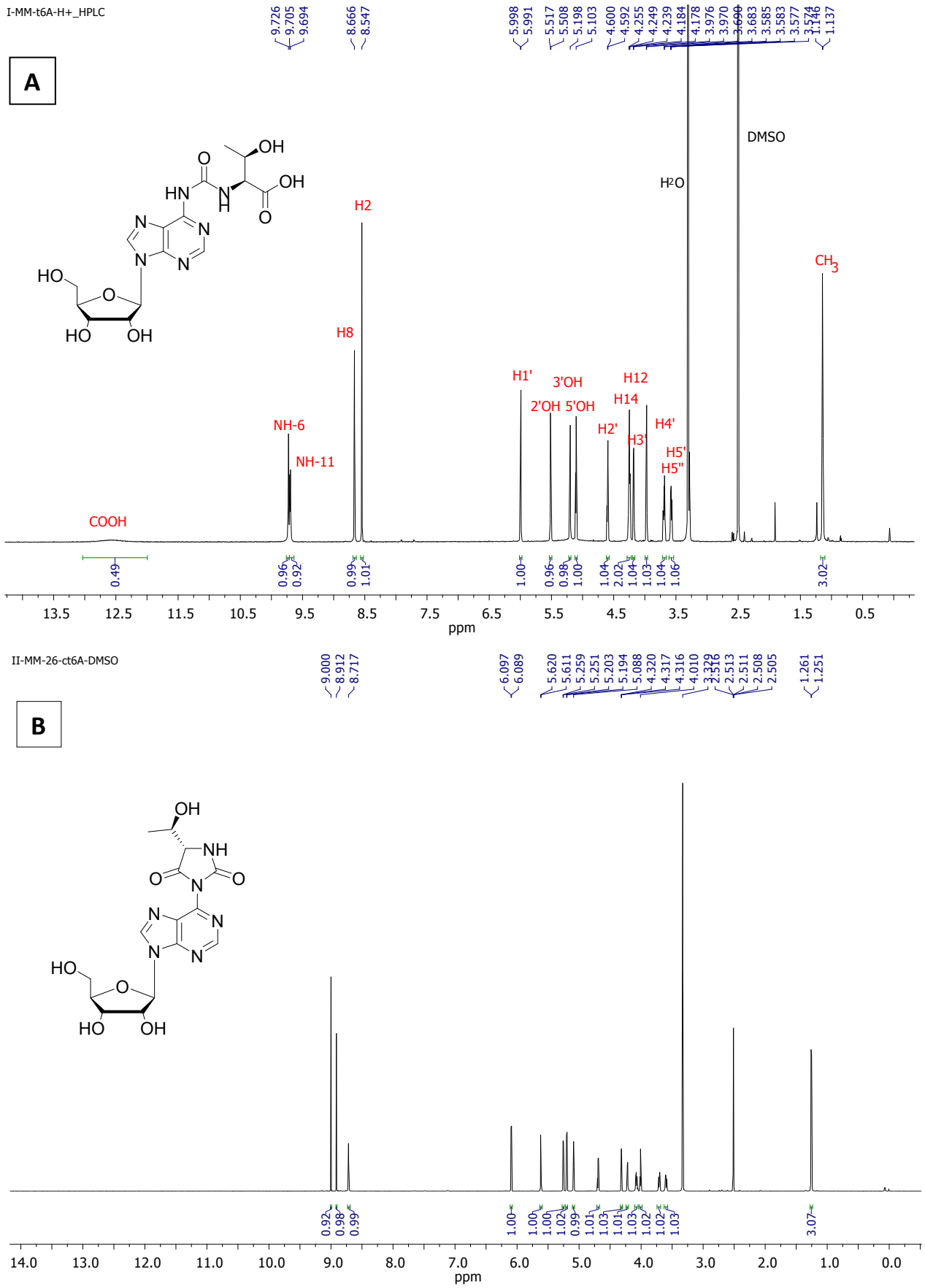
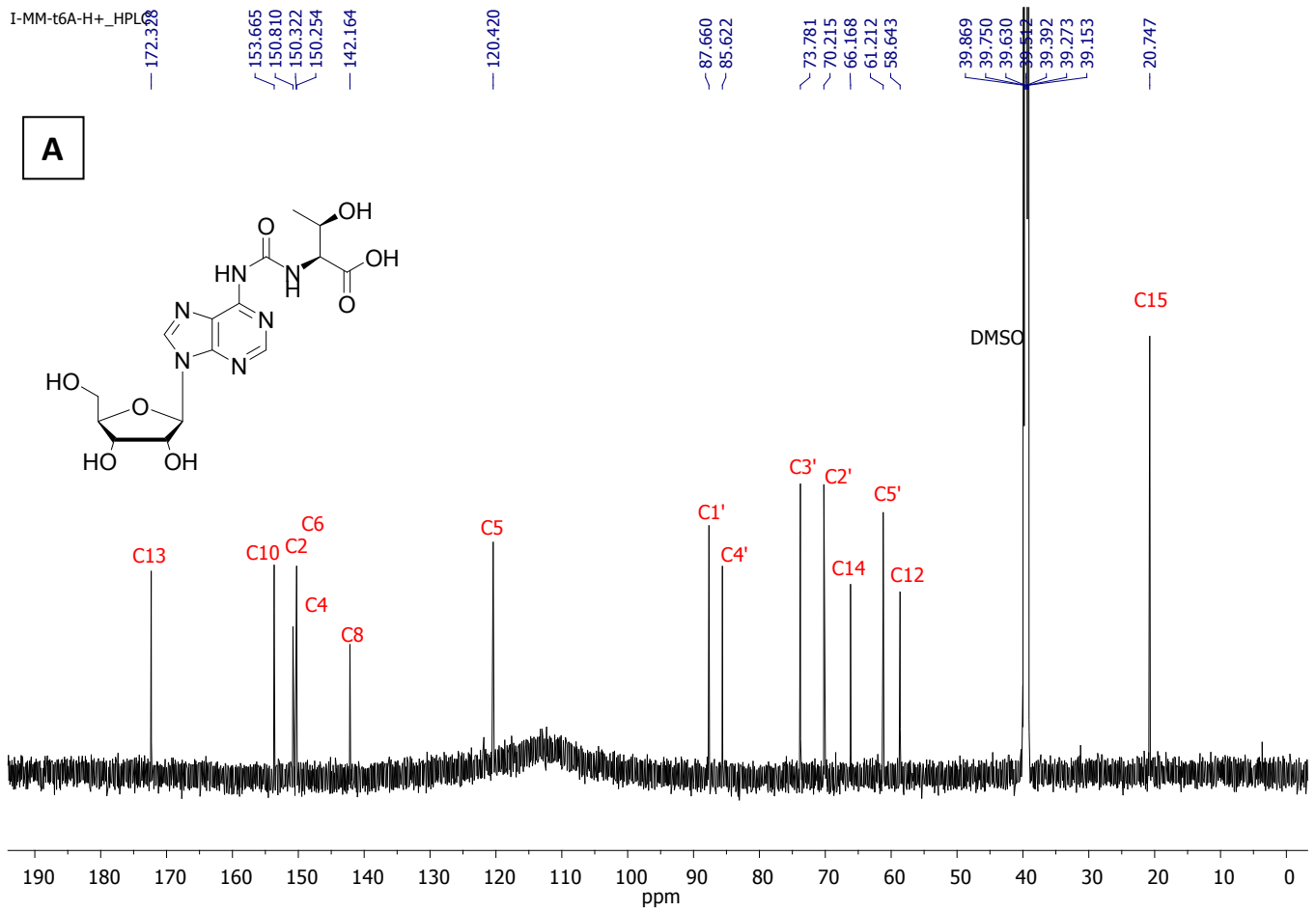


Figure S4. ^{1}H NMR (DMSO-d_6) spectrum of **L-t^{6}A** (A) and **L-ct^{6}A** (B).



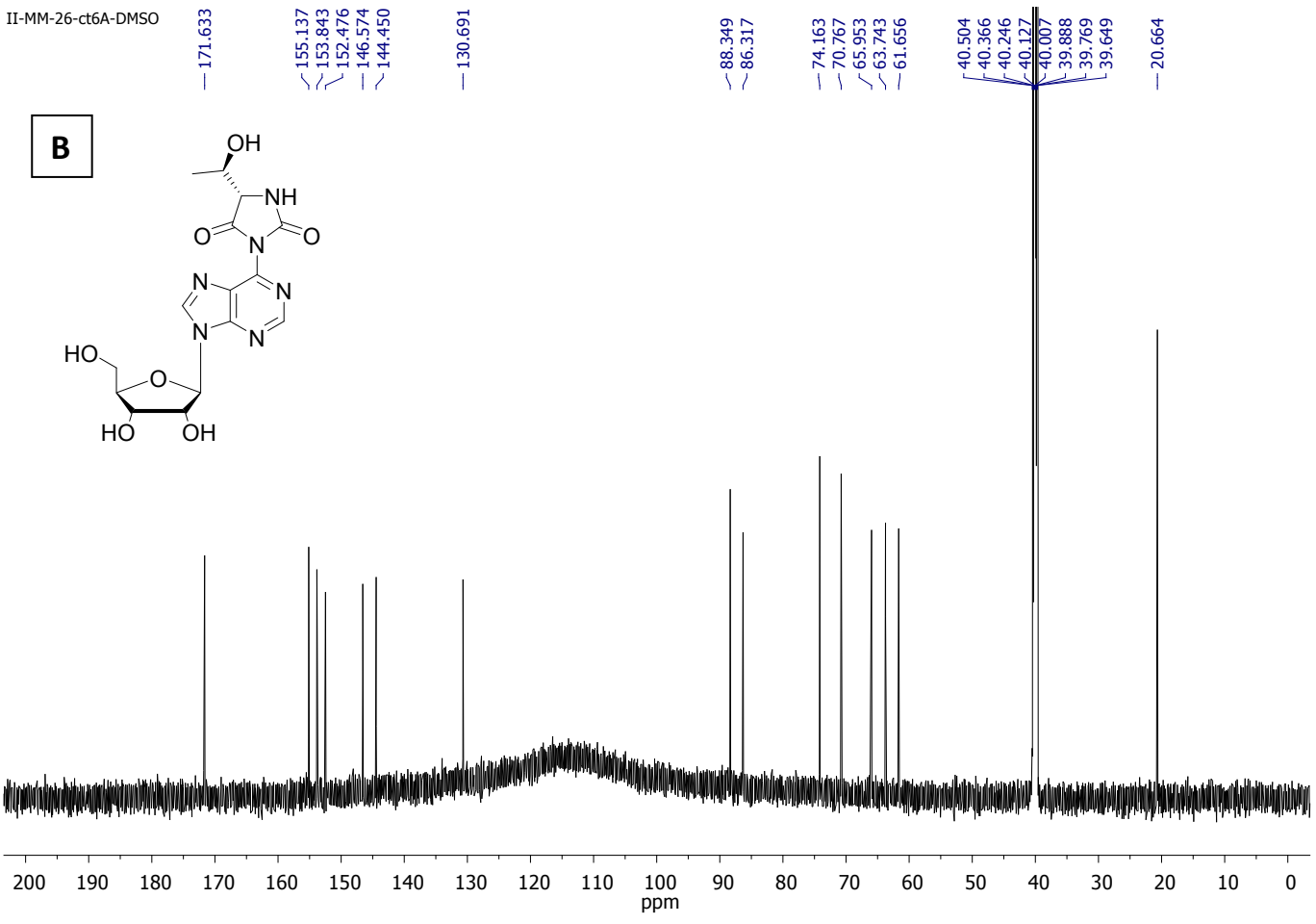


Figure S5. ^{13}C NMR (DMSO-d$_6$) spectrum of **L-t^{6}A** (A) and **L-ct^{6}A** (B).

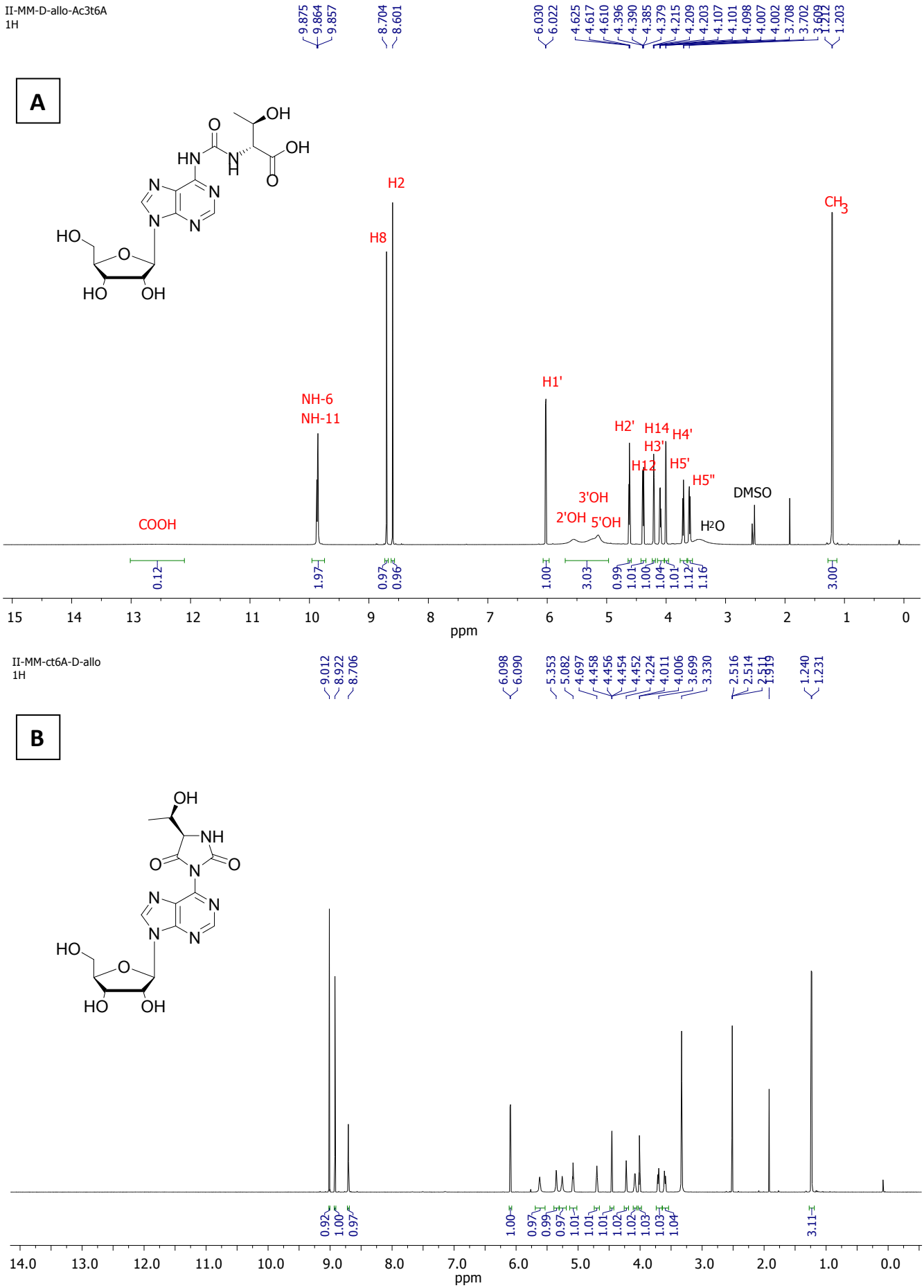


Figure S6. ^{1}H NMR (DMSO-d_6) spectrum of **D-*allo*-t^{6}A** (A) and **D-*allo* -ct^{6}A** (B).

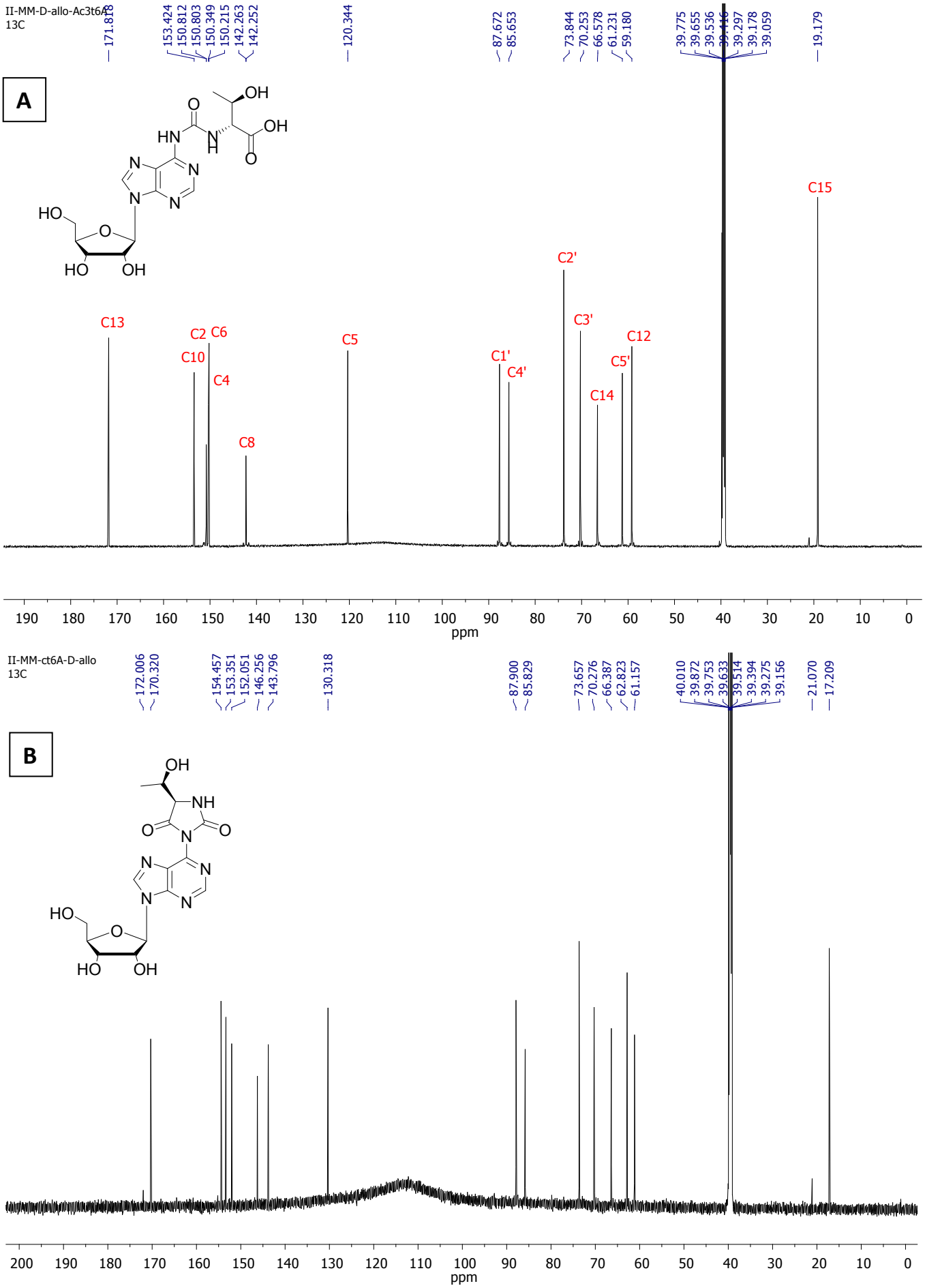


Figure S7. ^{13}C NMR (DMSO-d_6) spectrum of **D-*allo*-t^{6}A** (A) and **D-*allo*-ct^{6}A** (B).

Detailed data for Figure S4

L-t^{6}A - ^{1}H NMR: (700 MHz, DMSO-d6) δ: 12.56 (brs, 1H, COOH), 9.73 (s, 1H, NH-6), 9.70 (d, $^3J_{HH}$=4.9, NH-11), 8.67 (s, 1H, H-8), 8.55 (s, 1H, H-2), 6.00 (d, 1H, $^3J_{HH}$=4.9, H-1'), 5.52 (d, 1H, $^3J_{HH}$=6.3, 2'-OH), 5.20 (d, 1H, $^3J_{HH}$=4.9, 3'-OH), 5.10 (t, 1H, $^3J_{HH}$=5.6, OH-5'), 4.60 (dd, 1H, $^3J_{HH}$=5.6, $^3J_{HH}$=11.2, H-2'), 4.26-4.24 (m, 2H, H-12, H-14), 4.18 (dd, 1H, $^3J_{HH}$=4.2, $^3J_{HH}$=8.4, H-3'), 3.97 (dd, 1H, $^3J_{HH}$=3.5, $^3J_{HH}$=7.7, H-4'), 3.71-3.68 (m, 1H, H-5'), 3.59-3.56 (m, 1H, H-5"), 1.15 (d, 3H, $^3J_{HH}$=7.0, CH_3-15).

L-ct^{6}A - ^{1}H NMR: (700 MHz, **DMSO-d$_6$**) δ: 9.00 (s, 1H, H-2), 8.91 (s, 1H, H-8), 8.72 (brs, 1H, NH-11), 6.10 (d, $^3J_{HH}$=5.6, H-1'), 5.62 (d, 1H, $^3J_{HH}$=6.3, 2'-OH), 5.26 (d, 1H, $^3J_{HH}$=5.6, 3'-OH), 5.20 (d, 1H, $^3J_{HH}$=6.3, C14-OH), 5.09 (t, 1H, $^3J_{HH}$=5.6, 5'-OH), 4.69 (dd, 1H, $^3J_{HH}$=5.6, $^3J_{HH}$=10.5, H-2'), 4.32 (dd, 1H, $^3J_{HH}$=0.7 , $^3J_{HH}$=2.8, H-12), 4.22 (dd, 1H, $^3J_{HH}$=4.9 , $^3J_{HH}$=8.4, H-3'), 4.09-4.07 (m, 1H, H-14), 4.01 (dd, 1H, $^3J_{HH}$=3.5 , $^3J_{HH}$=7.7, H-4'), 3.72-3.69 (m, 1H, H-5'), 3.61-3.58 (m, 1H, H-5"), 1.26 (d, 3H, $^3J_{HH}$=7.0, CH_3-15).

Detailed data for Figure S5

L-t^{6}A - ^{13}C NMR (176.03 MHz, DMSO-d$_6$) δ: 172.33 (C-13), 153.67 (C-10), 150.81 (C-2), 150.32 (C-6), 150.25 (C-4), 142.17 (C-8), 120.42 (C-5), 87.66 (C-1'), 85.62 (C-4'), 73.78 (C-2'), 70.22 (C-3'), 66.17 (C-14), 61.21 (C-5'), 58.64 (C-12), 20.75 (C-15).

L-ct^{6}A - ^{13}C NMR (176.03 MHz, DMSO-d$_6$) δ: 171.63 (C-13), 155.14 (C-10), 153.84 (C-4), 152.48 (C-2), 146.57 (C-8), 144.45 (C-6), 130.69 (C-5), 88.35 (C-1'), 86.32 (C-4'), 74.16 (C-2'), 70.77 (C-3'), 65.95 (C-14), 63.74 (C-12), 61.66 (C-5'), 20.66 (C-15).

Detailed data for Figure S6

D-*allo*-t^{6}A - ^{1}H NMR: (700 MHz, DMSO-d6) δ: 12.56 (brs, 1H, COOH), 9.88-9.86 (m, 2H, NH-6, NH-11), 8.70 (s, 1H, H-8), 8.60 (s, 1H, H-2), 6.03 (d, 1H, $^3J_{HH}$=5.6, H-1'), 5.65-5.05 (m, 3H, 2'-OH, 3'-OH, 5'-OH), 4.63-4.31 (m, 1H, H-2'), 4.90 (dd, 1H, $^3J_{HH}$=4.2, $^3J_{HH}$=7.7, H-12), 4.22-4.20 (m, 1H, H-3'), 4.11-4.09 (m, 1H, H-14), 4.00 (dd, 1H, $^3J_{HH}$=4.2, $^3J_{HH}$=7.7, H-4'), 3.71 (dd, 1H, $^3J_{HH}$=3.5, $^3J_{HH}$=11.9, H-5'), 3.60 (dd, 1H, $^3J_{HH}$=4.2, $^3J_{HH}$=11.9, H-5''), 1.21 (d, 3H, $^3J_{HH}$=6.3, CH_3-15).

D-*allo*-ct^{6}A - ^{1}H NMR: (700 MHz, **DMSO-d$_6$**) δ: 9.01 (s, 1H, H-2), 8.92 (s, 1H, H-8), 8.71 (s, 1H, NH-11), 6.09 (d, $^3J_{HH}$=5.6, H-1'), 5.61-5.62 (m, 1H, 2'-OH), 5.34-5.35 (m, 1H, OH-3'), 5.25-5.26 (m, 1H, C14-OH), 5.08 (t, 1H, $^3J_{HH}$=4.9, OH-5'), 4.70-4.71 (m, 1H, H-2'), 4.46-4.45 (m, 1H, H-12), 4.22-4.23 (m, 1H, H-3'), 4.09-4.08 (m, 1H, H-14), 4.01 (dd, 1H, $^3J_{HH}$=4.2, $^3J_{HH}$=7.7, H-4'), 3.72-3.69 (m, 1H, H-5'), 3.61-3.58 (m, 1H, H-5"), 1.24 (d, 3H, $^3J_{HH}$=6.3, CH_3-15).

Detailed data for Figure S7

D-*allo*-t^{6}A - ^{13}C NMR (176.03 MHz, DMSO-d$_6$) δ: 171.82 (C-13), 153.42 (C-10), 150.81 (C-2), 150.80 (C-6), 150.35 (C-4), 142.26 (C-8), 120.34 (C-5), 87.67 (C-1'), 85.65 (C-4'), 73.84 (C-2'), 70.25 (C-3'), 66.58 (C-14), 61.23 (C-5'), 59.18 (C-12), 19.18 (C-15).

D-*allo*-ct^{6}A - ^{13}C NMR (176.03 MHz, DMSO-d$_6$) δ: 170.32 (C-13), 154.46 (C-10), 153.35 (C-4), 152.05 (C-2), 146.26 (C-8), 143.80 (C-6), 130.32 (C-5), 87.90 (C-1'), 85.83 (C-4'), 73.66 (C-2'), 70.28 (C-3'), 66.39 (C-14), 62.82 (C-12), 61.16 (C-5'), 17.21 (C-15).

VI. ^{31}P NMR and ESI MS spectra of L-t^{6}A, mnm^{5}s^{2}U and ψ phosphoramidities

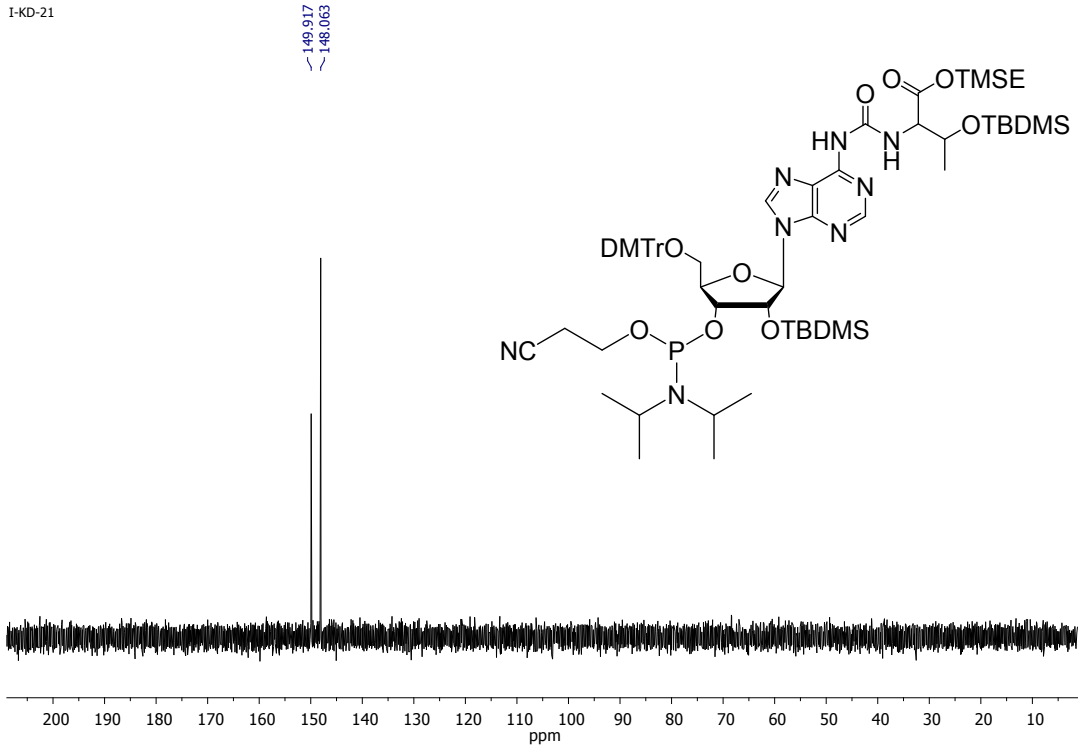


Figure S8. ^{31}P NMR (DMSO-d_6) spectrum of fully protected **L-t^{6}A phosphoramidite**.

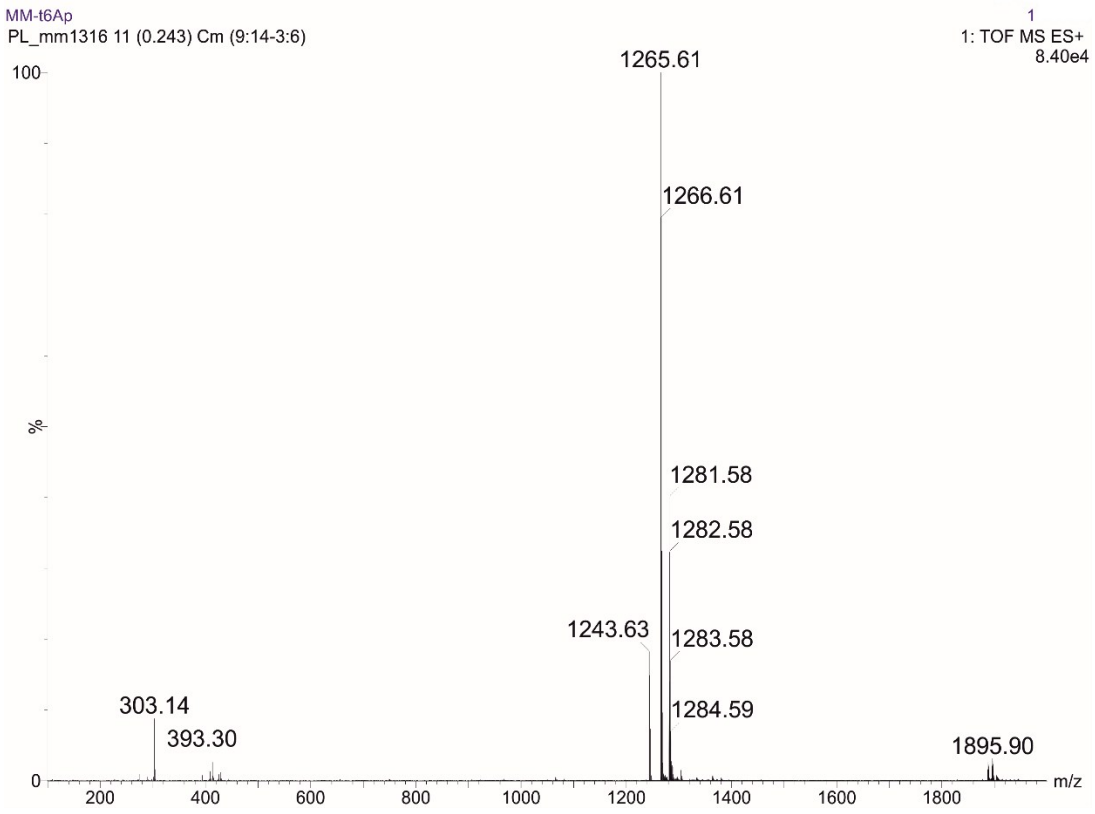


Figure S9. ESI$^+$ TOF MS spectrum of fully protected **L-t^{6}A phosphoramidite**; calculated monoisotopic mass is 1242.6166 [M]; measured *m/z* 1265.61 [M+Na]$^+$].

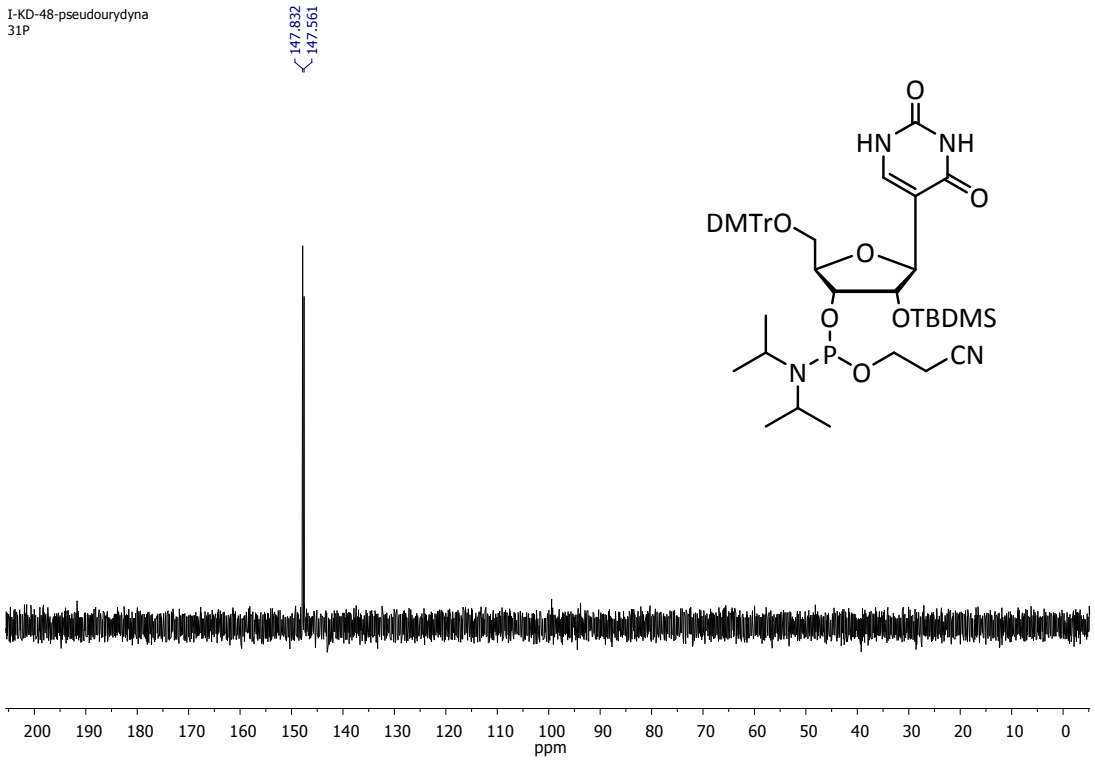


Figure S10. ^{31}P NMR (DMSO-d_6) spectrum of fully protected **ψ phosphoramidite**.

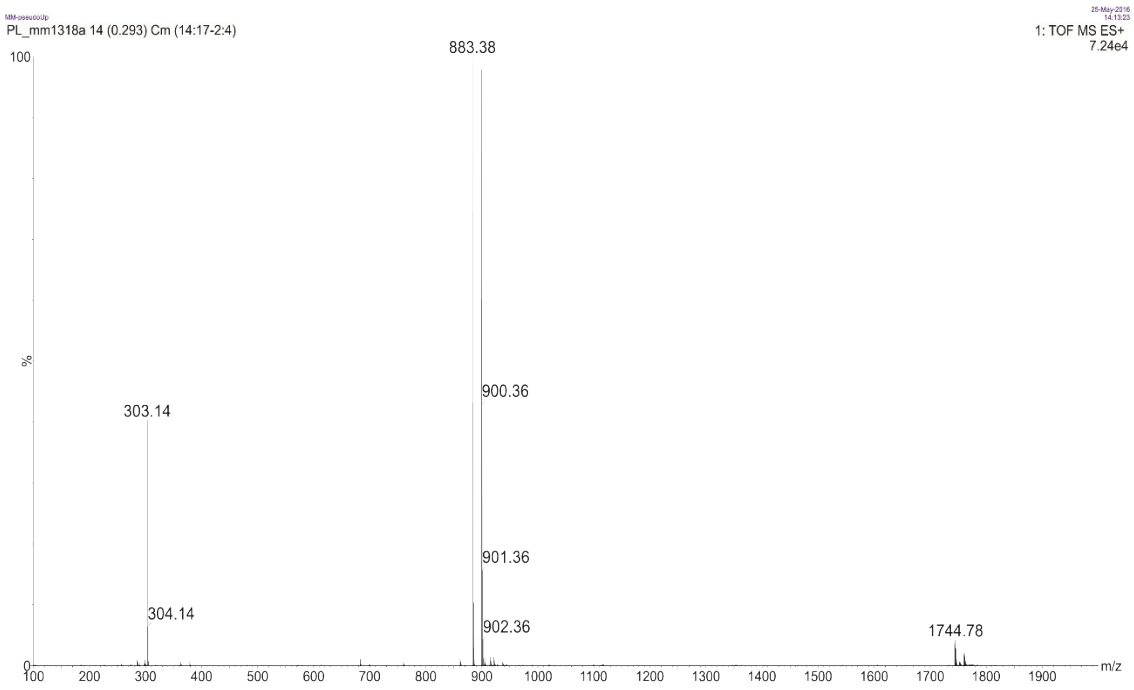


Figure S11. ESI$^+$TOF MS spectrum of fully protected **ψ phosphoramidite**; calculated monoisotopic mass is 860.3945 [M]; measured *m/z* is 883.38 $[M+Na]^+$.

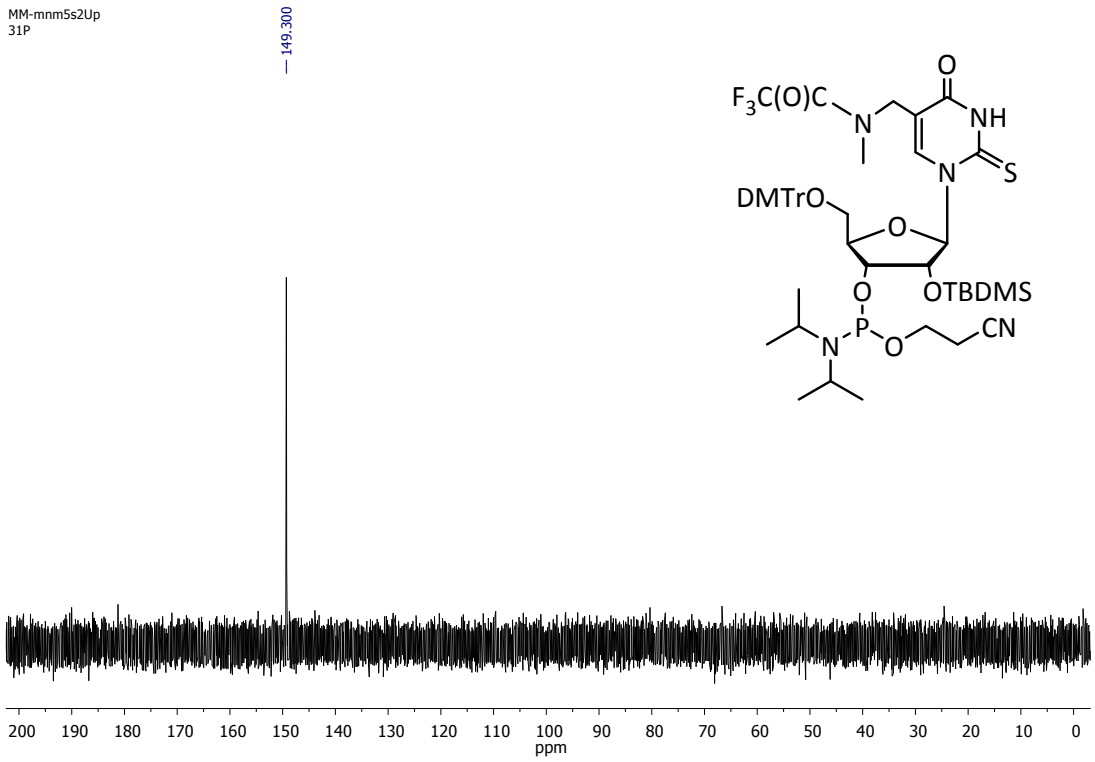


Figure S12. ^{31}P NMR (DMSO-d_6) spectrum of fully protected **mnm^{5}s^{2}U phosphoramidite**.

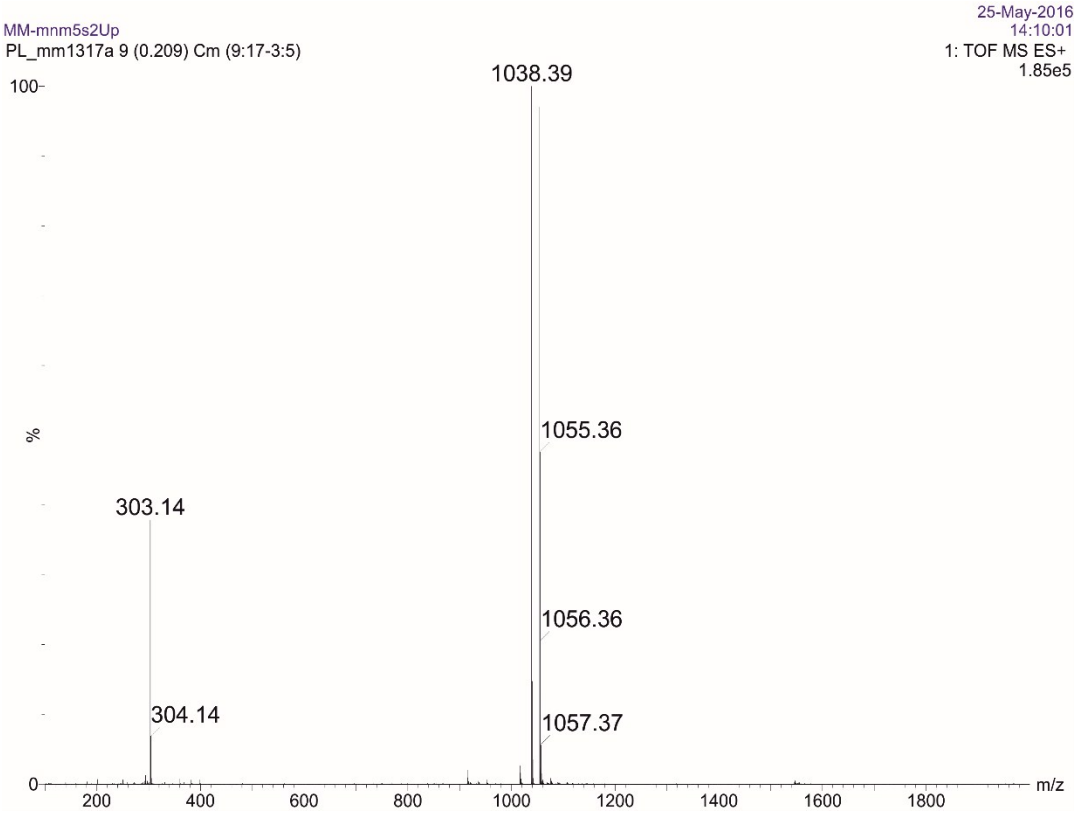


Figure S13. ESI$^+$ TOF MS spectrum of fully protected **mnm^{5}s^{2}U phosphoramidite**; the calculated monoisotopic mass is 1015.4503 [M]; measured *m/z* is 1038.39 [M+Na]$^+$.

Chemical synthesis of precursor oligoribonucleotides modified with t^6A (I and II)

5'-ACGGGCUCAU**t^6A**ACCCGU-3' (t^6A-ASL of *S. pombe* $tRNA_i^{Met}$), **I**

5'-GUUGACU**mnm^5s^2U**UUU**t^6A**A**Ψ**CAAC-3' (t^6A-ASL of *E. coli* $tRNA^{Lys}$), **II**

t^6A-containing precursor oligomers (**I**, **II**) were synthesized manually on 2.5 μmol scale using typical rU-succinyl-CPG (Proligo) support. Commercially available monomeric units A, C, U and G protected with DMTr and TBDMS on the 5'- and 2'-hydroxy functions, respectively, and with 4-*tert*-butylphenoxyacetyl (tac) (Proligo) on exocyclic amine function of A, C, and G were used as 0.1 M solutions in anhydrous acetonitrile. Canonical A, U, C and G amidities were coupled once in 8 molar excess for 8 min in the presence of Activator 42 (0.25 M solution of 5-(3,5-bis(trifluoromethyl)phenyl)-1*H*-tetrazole in ACN), while modified units were coupled twice, each time using 8 molar excess of amidite and 20 min coupling time. Capping was performed with tac anhydride (Fast protection Cap A : Cap B 1:1.1 v/v) for 2 min. A 0.02 M iodine solution in THF-H_2O-pyridine (90.54:9.05:0.41 v/v/v; 8 equiv.) was used as an oxidizing agent for 2 min for each oxidation step. For oligomer **II** (t^6A-ASL of *E. coli* $tRNA^{Lys}$), after incorporation of mnm^5s^2U unit, the oxidizing agent was changed for 0.25 M solution of *t*BuOOH in toluene and the oxidation step was carried out for 1.5 min. After the last coupling, the DMTr group was removed and the support was washed with acetonitrile, dried with argon and transferred to a screw cap glass vial.

Deprotection of t^6A-modified oligoribonucleotides.

To each of support-linked precursor oligoribonucleotides **I** and **II**, TEA/ACN mixture (3.3 mL, 1:1 v/v) was added. The suspension was stirred for 20 min in screw cap glass vial, and then the volatile components were removed on Speed-Vac. Next, the resin was washed with ACN (3 x 2.5 mL), dried *in vacuo* for 30 min. and the t^6A-RNA oligonucleotides (**I**, **II**) were deprotected and cleaved from the solid support by treatment with 8 M ethanolic ammonia (4.5 mL) for 16 h at 37 °C. The supernatant was removed and the support was washed with ethanol (3 x 4 mL). The combined solutions were evaporated on a Speed-Vac concentrator and the solid residue was treated with 1M TBAF in THF (1.8 mL) for 24 h at rt. The reaction was quenched by addition of phosphate buffer (0.2 M, 5mL, pH 7) and desalted on Sephadex column (G-25) using 20% $EtOH_{aq}$ as eluent. Each of the fully deprotected modified RNA oligomer was purified by anion-exchange (AEX) HPLC (Source 15Q 4.6/100PE) at constant flow rate of 1 mL/min. The column was eluted with linear gradient 50 mM to 600 mM NaBr in 20 mM Na_2HPO_4-NaH_2PO_4 buffer solution pH 7.5, containing 50 μM EDTA and 10% ACN. For precursor oligomer **I** (t^6A-ASL of *S. pombe* $tRNA_i^{Met}$) fractions at R_t = 34.198 min, while for oligomer **II** (t^6A-ASL of *E. coli* $tRNA^{Lys}$) at R_t = 42.154 min were collected and shortly evaporated on Speed-Vac concentrator to remove acetonitrile. The solutions containing oligonucleotides were diluted with 100 mM AcONa (up to ~ 6 mL) and loaded slowly onto Sep-Pak C18 cartridge (Waters) equilibrated with 100 mM AcONa (pH 6.5, 10 mL). The sample was desalted by slowly passing H_2O miliQ (4 x 6 mL) through the packing bed. Next, the cartridge was flushed with elution solution (MeOH/ACN/H_2O, 4:4:3, v/v) and collected elutes containing the desalted oligonucleotides were evaporated to dryness and lyophilized to give 102 OD_{260} units of **I** and 85 OD_{260} units of **II**. The obtained products **I** and **II** were analyzed by MALDI-TOF mass spectrometry.

VII. Purification and characterization of I (ASL of *S. pombe* $tRNA^{i}$) and II (ASL of of *E. coli* $tRNA^{Lys}$)

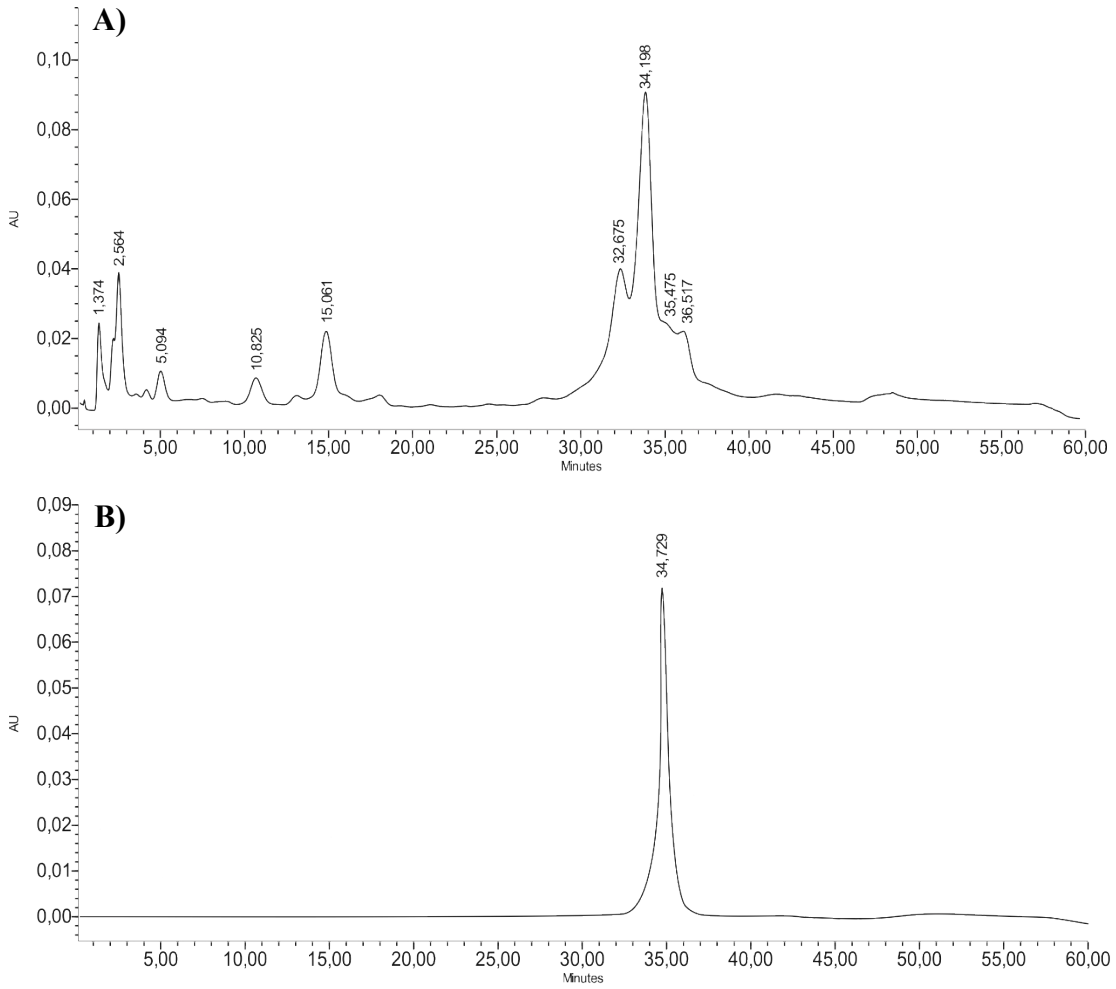


Figure S14. AEX-HPLC of crude **I** (t^6A-ASL of *S. pombe* $tRNA_i^{Met}$)(A); and analysis after purification (B).

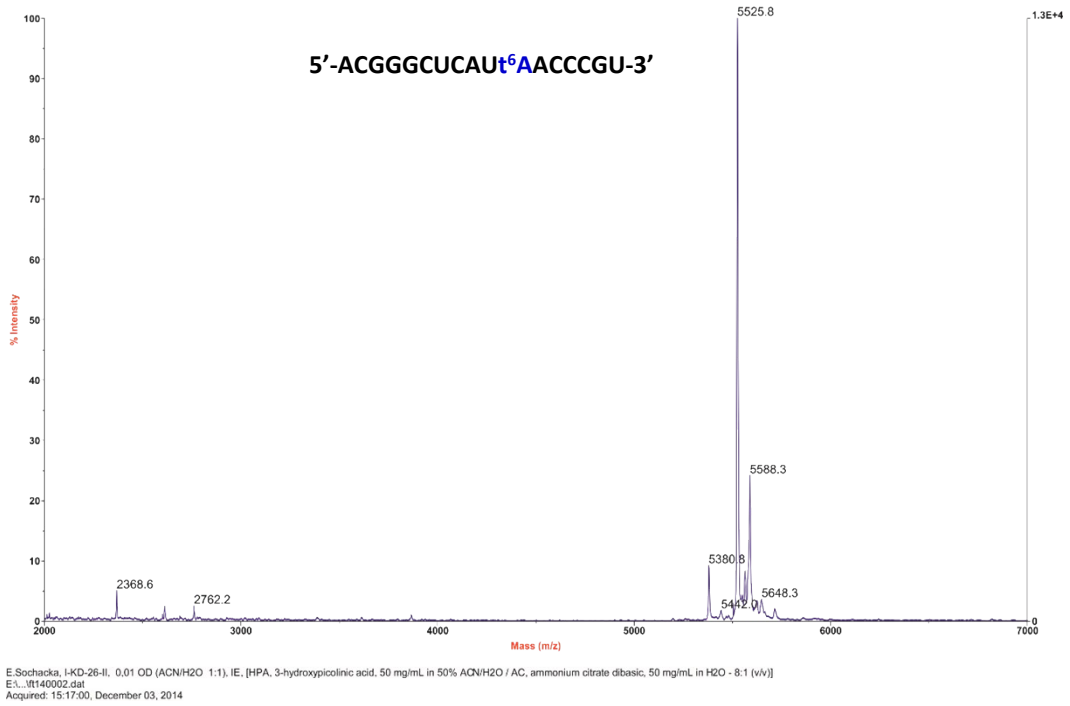


Figure S15. MALDI-TOF mass spectrum of **I**; calculated monoisotopic mass is 5530.2; measured *m/z* is 5525.8.

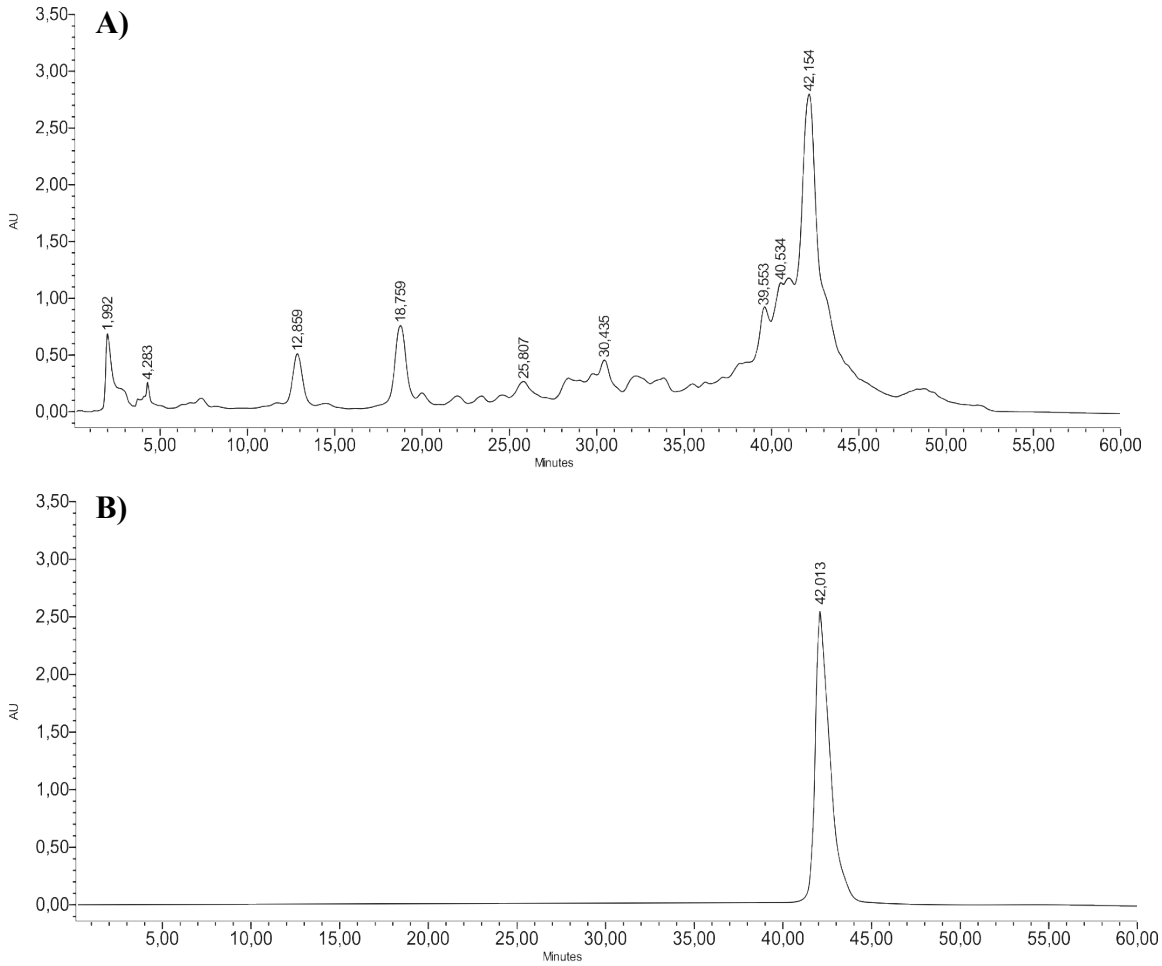


Figure S16. AEX-HPLC of crude **II** (t^6A-ASL of *E. coli* $tRNA^{Lys}$)(A) and analysis after purification (B).

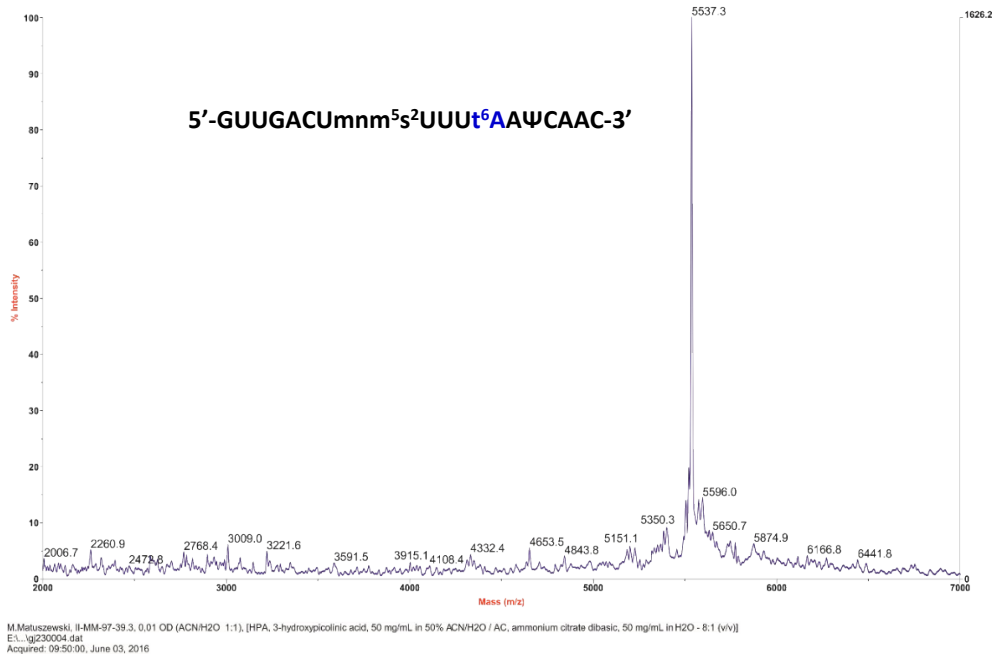


Figure S17. MALDI-TOF mass spectrum of **II**; calculated monoisotopic mass is 5538.4, measured *m/z* is 5537.3.

IX. **Post-synthetic transformation of I (ASL of *S.pombe* tRNA^{i}) to ct^{6}A containing oligomer cyclo-I (5'-ACGGGCUCAUt^{6}AACCCGU-3' (I) →5'-ACGGGCUCAUct^{6}AACCCGU-3'(cyclo-I))**.

To the lyophilized substrate **5'-ACGGGCUCAUt^{6}AACCCGU-3'** (**I**) (2 OD, n= $1 \cdot 10^{-5}$ mmole, m=0.08 mg) solution of EDC·HCl (5 equiv, n=$5 \cdot 10^{-5}$ mmole, m=0,0096 mg, V=2.8 µL from stock solution containing 3.5 mg (EDC·HCl) in 1mL H_2O) and solution of HOBt of (5 equiv, n=$5 \cdot 10^{-5}$ mmole, m=0.075 mg, V=3.2 µL from stock solution containing 2.2 mg (HOBt) in 1mL DMF). The resultant solution was incubated at 25 ºC. After 1 hour HPLC analysis of reaction mixture revealed one main product (R_t=11.813 min) and the crude oligonucleotide was purified by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1 M CH_3COONH_4, pH 6.0) and buffer B (40% ACN in 0.1 M CH_3COONH_4, pH 6.0) with a flow 1 mL/min as follows: 0-20 min from 2%B to 25%B, 20-25 min 2%B. The fraction (R_t=11.813 min) with cyclic oligonucleotide was shortly evaporated on Speed-Vac concentrator to remove acetonitrile and the concentrated solution was diluted with 100 mM AcONa (pH 6.0, up to 3 mL) and applied slowly on Sep-Pak C18 column (Waters) equilibrated previously with 100 mM AcONa (pH 6.0, 3 x 5 mL). Next, the cartridge was flushed with 100 mM AcONa (pH 6.0, 5 mL) and the sample of oligonucleotide retained on Sep-Pak was desalted by slowly passing of H_2O miliQ (4 x 6 mL) through the packing bed. Desalted oligonucleotide was washed out from the Sep-Pak with elution solution (MeOH/ACN/H_2O, 4:4:3, v/v) and the collected elute was evaporated to dryness to afford 1.64 OD_{260} units of **5'-ACGGGCUCAUct^{6}AACCCGU-3'** (**cyclo-I**), (82 % yield). Obtained (**cyclo-I**) oligonucleotide was analyzed by MALDI-TOF mass spectrometry (*m/z* calcd 5512, found 5509) and its homogeneity was confirmed by RP-HPLC on C18 column (Figure 18C; Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1 M CH_3COONH_4, pH 6.0) and buffer B (40% ACN in 0.1 M CH_3COONH_4, pH 6.0) with a flow 1 mL/min as follows: 0-20 min from 2%B to 25%B, 20-25 min 2%B.

HPLC monitoring: **5'-ACGGGCUCAUt^{6}AACCCGU-3' (I) → 5'-ACGGGCUCAUct^{6}AACCCGU-3' (cyclo-I)**.

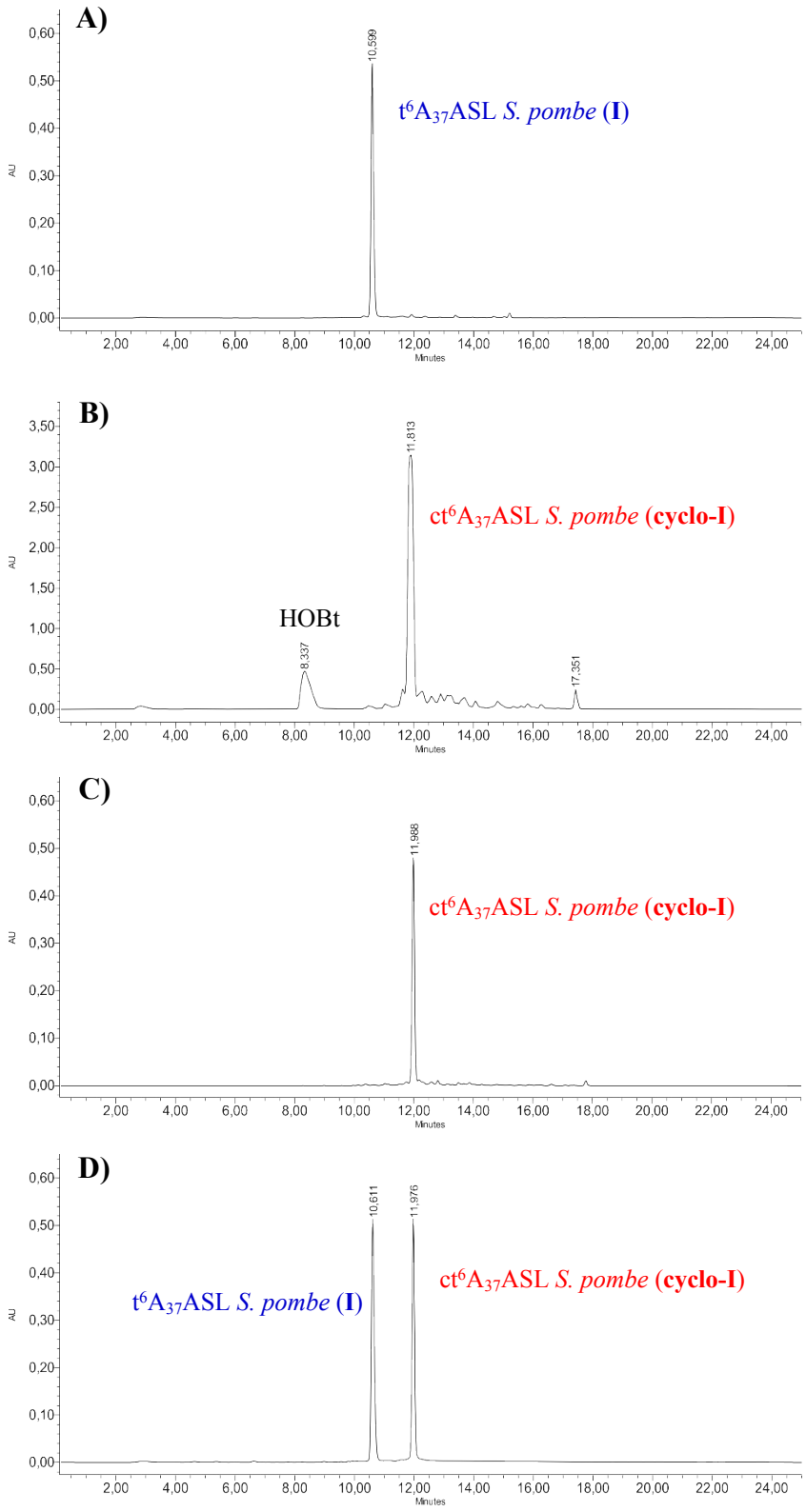


Figure S18. RP-HPLC of **I** (A); reaction mixture after 1h, containing crude **cyclo-I** (B)**;** purified **cyclo-I** (C); HPLC coinjection analysis of **I** and **cyclo-I** (D).

Enzymatic digestion of 5'-ACGGGCUCAUct^{6}AACCCGU-3' (cyclo-I).

The nucleoside composition of oligoribonucleotide was confirmed by neutral one-step enzymatic digestion (*Nat. Chem Biol*. 2013, 9(2), 105-111) of the final 5'-ACGGGCUCAUct^{6}AACCCGU-3' (**cyclo-I**) with nuclease P_1 and alkaline phosphatase in 20 mM TEA·HCl (pH 7). The resulting mixture of nucleosides was analyzed by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1% AcOH in H_2O) and buffer B (ACN) with a flow 1 mL/min as follows: 0-15 min from 2%B to 8%B, 15-30 min from 8%B to 25%B 30-35 min 2%B. The retention time (R_t) of the ct^{6}A was compared to the R_t of the reference sample in a separate control experiment.

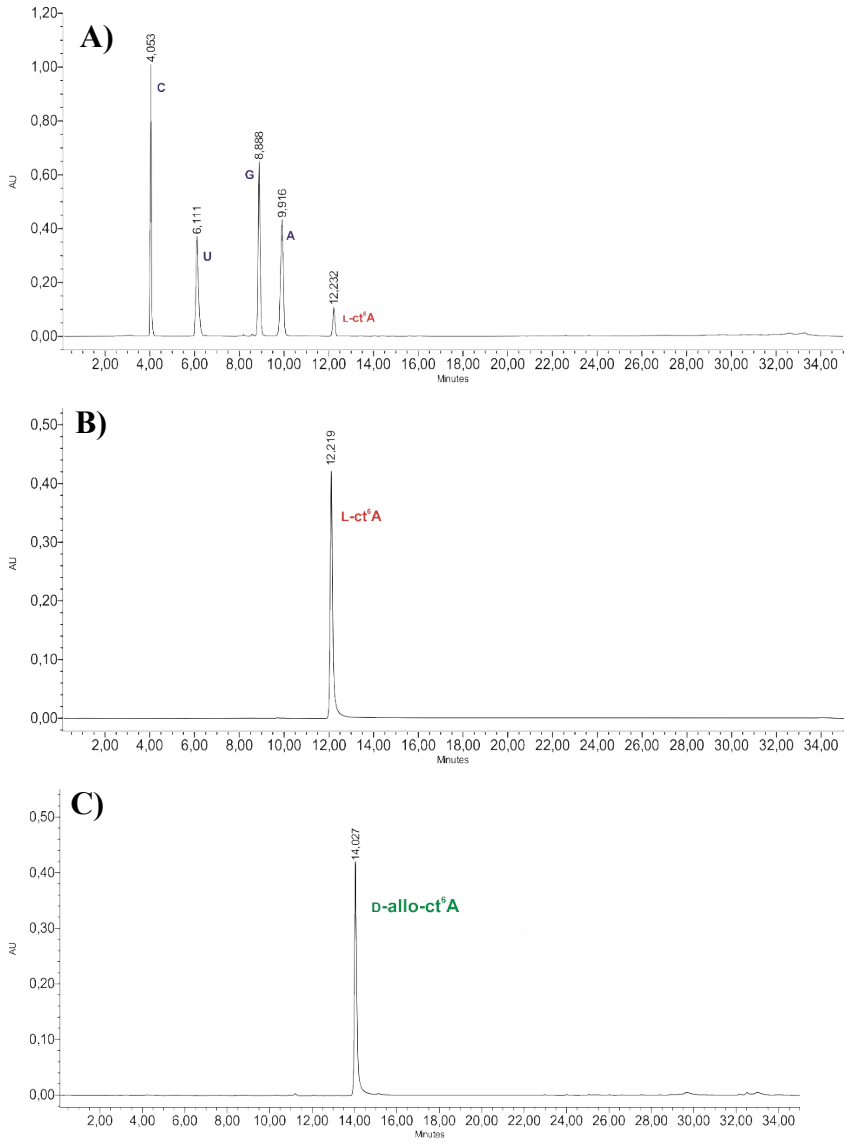


Figure S19. RNA enzymatic digestion of **cyclo–I** oligonucleotide. RP-HPLC analysis of nucleoside composition of ct^{6}A-ASL tRNA$^{Met}_i$ *S. pombe* (A); RP-HPLC of the references of **L-ct^{6}A** (B) and **D-*allo*-ct^{6}A** (C) nucleosides.

X. **Control of L-ct^{6}A stability under conditions used for NMR structural studies.**

Oligonucleotide **cyclo-I** (0.1 OD) was dissolved in 50 µL of 10 mM phosphate buffer (at pH 7.4, 6.5 and 5.5). and resultant solutions were incubated at 25 ºC. The stability of L-ct^{6}A was monitored by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1 M CH_3COONH_4, pH 6.0) and buffer B (40% ACN in 0.1 M CH_3COONH_4, pH 6.0) with a flow 1 mL/min as follows: 0-20 min from 2%B to 25%B, 20-25 min 2%B.

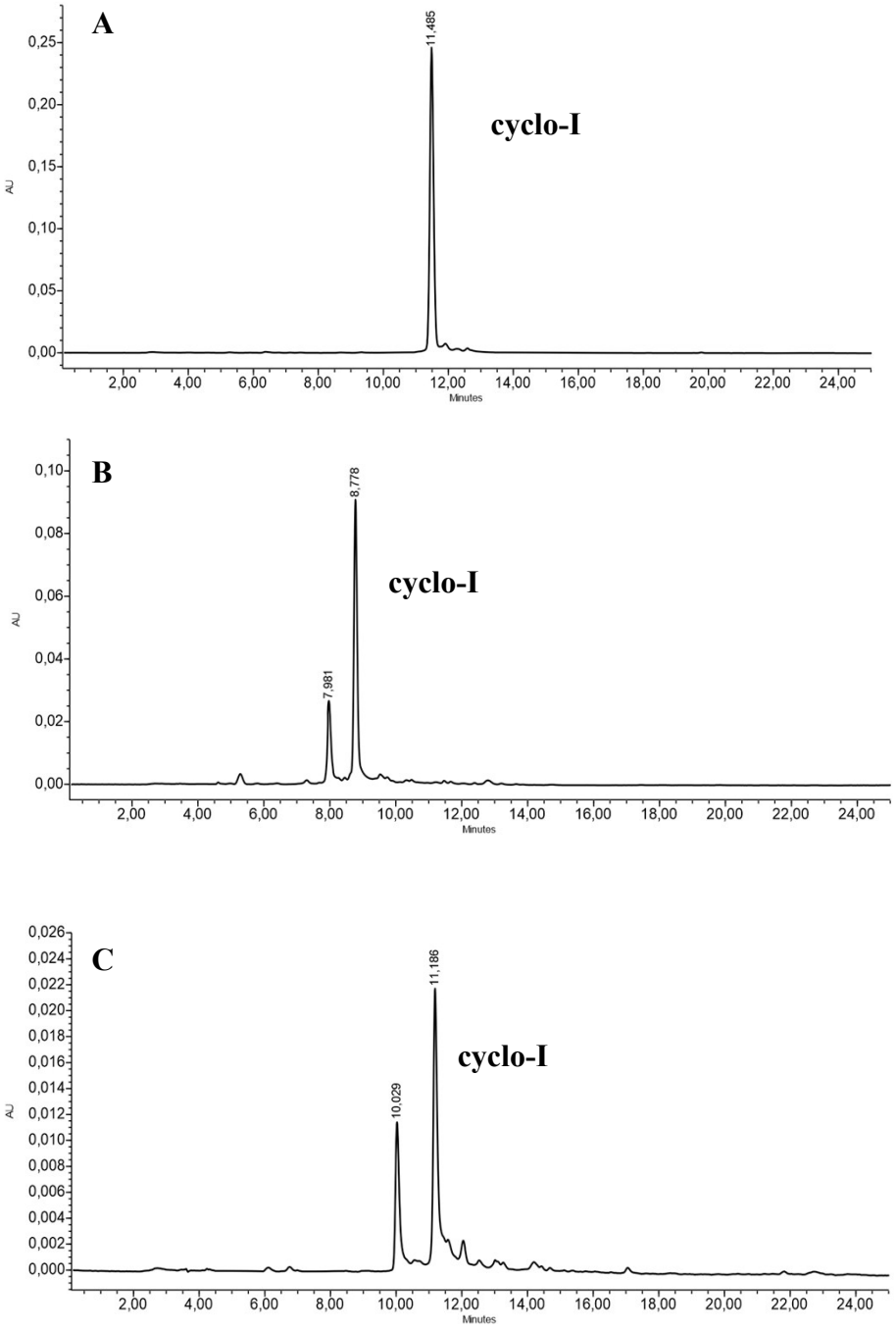


Figure S20. HPLC analysis of **cyclo-I** (A) stability at pH 7.4 after incubation for 96 (A) and 145 (B) hours at 25 ºC.

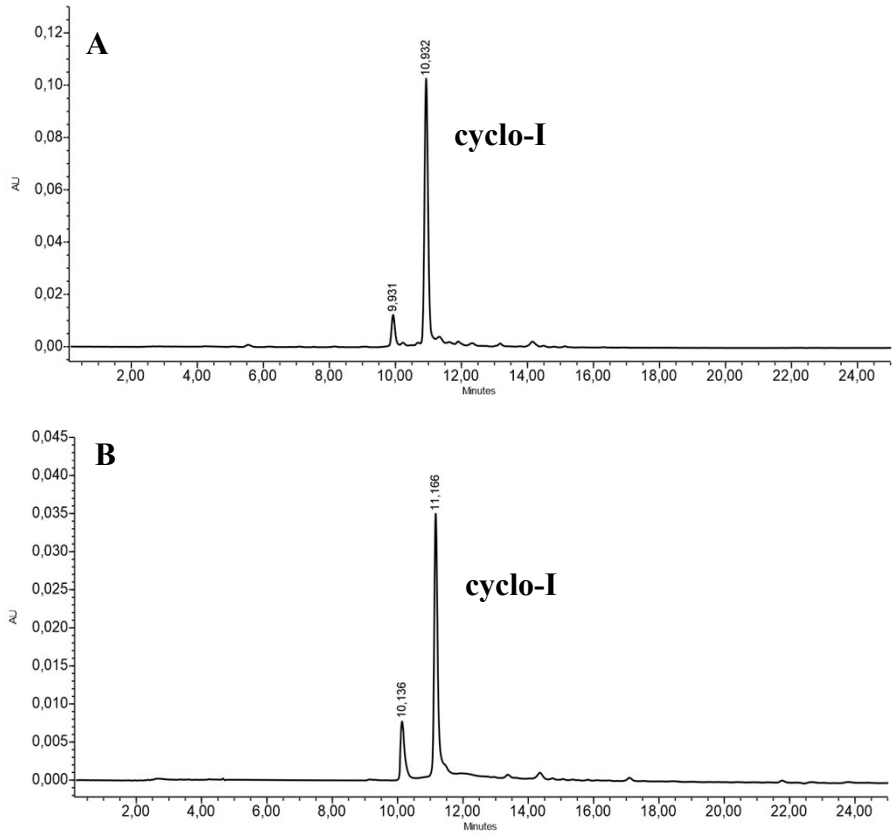


Figure S21. HPLC analysis of **cyclo-I** (A) stability at pH 6.5 after incubation for 96 (A) and 145 (B) hours at 25 ºC.

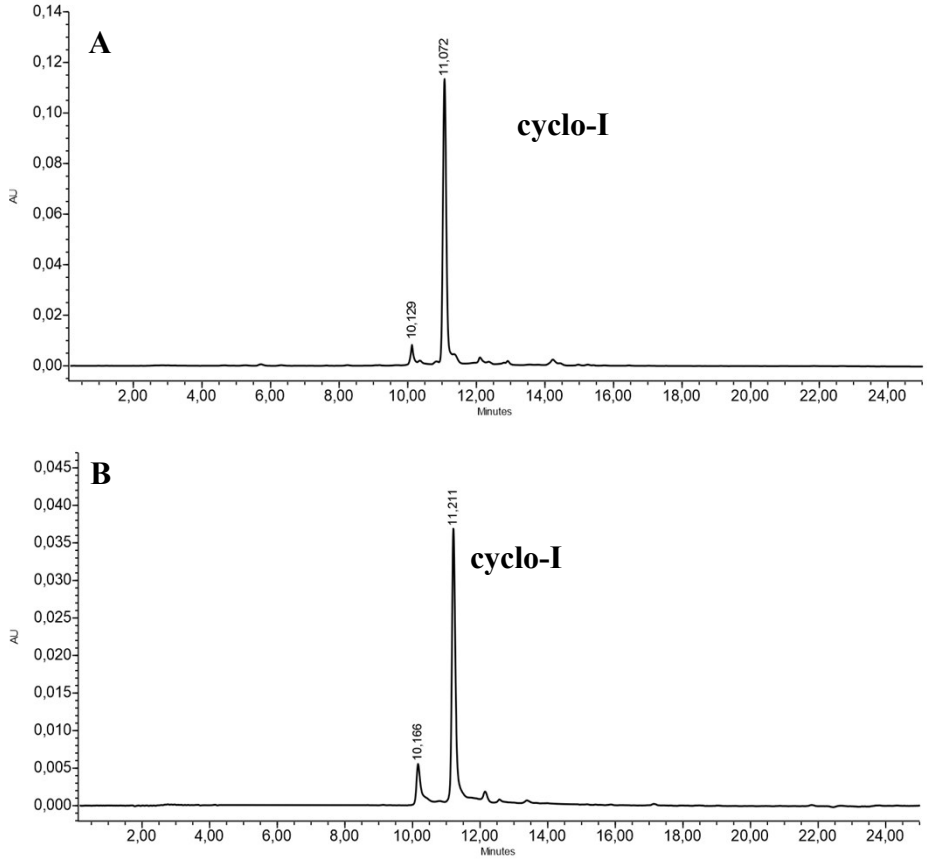


Figure S22. HPLC analysis of **cyclo-I** (A) stability at pH 5.5 after incubation for 96 (A) and 145 (B) hours at 25 ºC.

XI. Post-synthetic transformation of II (ASL of *E.coli* $tRNA^{Lys}$) to ct^6A oligomer cyclo-II

5'-GUUGACU**mnm^{5}s^2**UUU**t^{6}A**AΨCAAC-3' (**II**) → 5'-GUUGACU**mnm^{5}s^2**UUU**ct^{6}A**AΨCAAC-3' (**cyclo-II**).

To the lyophilized substrate 5'-GUUGACU**mnm^{5}s^2**UUU**t^{6}A**AΨCAAC-3' (**II**) (2 OD, n= $1\cdot10^{-5}$ mmole, m=0.08 mg) solution of EDC·HCl (5 equiv, n=$5\cdot10^{-5}$ mmole, m=0,0096 mg, V=2.8 µL from stock solution containing 3.5 mg (EDC·HCl) in 1mL miliQ H_2O) and solution of HOBt (5 equiv, n=$5\cdot10^{-5}$ mmole, m=0.075 mg, V=3.2 µL from stock solution containing 2.2 mg (HOBt) in 1mL DMF) were added. The resultant solution was incubated at 25 ºC. After 1 hour HPLC analysis of reaction mixture revealed one main product (R_t=13.021 min) and crude oligonucleotide was purified by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1 M CH_3COONH_4, pH 6.0) and buffer B (40% ACN in 0.1 M CH_3COONH_4, pH 6.0) with a flow 1 mL/min as follows: 0-20 min from 2%B to 25%B, 20-25 min 2%B. The fraction (R_t=13.021 min) with collected oligonucleotide was shortly evaporated on Speed-Vac concentrator to remove acetonitrile and the concentrated solution was diluted with 100 mM AcONa (pH 6.5, up to 3 mL) and applied on Sep-Pak C18 column (Waters) equilibrated with 100 mM AcONa (pH 6.0, 3 x 5 mL). The cartridge was flushed with 100 mM AcONa (pH 6.0, 5 mL) and the sample of oligonucleotide retained on Sep-Pak was desalted by slowly passing of H_2O miliQ (4 x 6 mL) through the packing bed. Desalted oligonucleotide was washed out with elution solution (MeOH/ACN/H_2O, 4:4:3, v/v) and the collected elute was evaporated to dryness to afford 1.6 OD_{260} units of 5'-GUUGACU**mnm^{5}s^2**UUU**ct^{6}A**AΨCAAC-3' **(cyclo-II)**, (80% yield). The obtained product was analyzed by MALDI-TOF mass spectrometry (*m/z* calcd 5520.1, found 5520.3) and its homogeneity was confirmed by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1 M CH_3COONH_4, pH 6.0) and buffer B (40% ACN in 0.1 M CH_3COONH_4, pH 6.0) with a flow 1 mL/min as follows: 0-20 min from 2%B to 25%B, 20-25 min 2%B.

HPLC monitoring:

5'-GUUGACU**mnm^{5}s^2**UUU**t^{6}A**AΨCAAC-3' (**II**) → 5'-GUUGACU**mnm^{5}s^2**UUU**ct^{6}A**AΨCAAC-3' (**cyclo-II**).

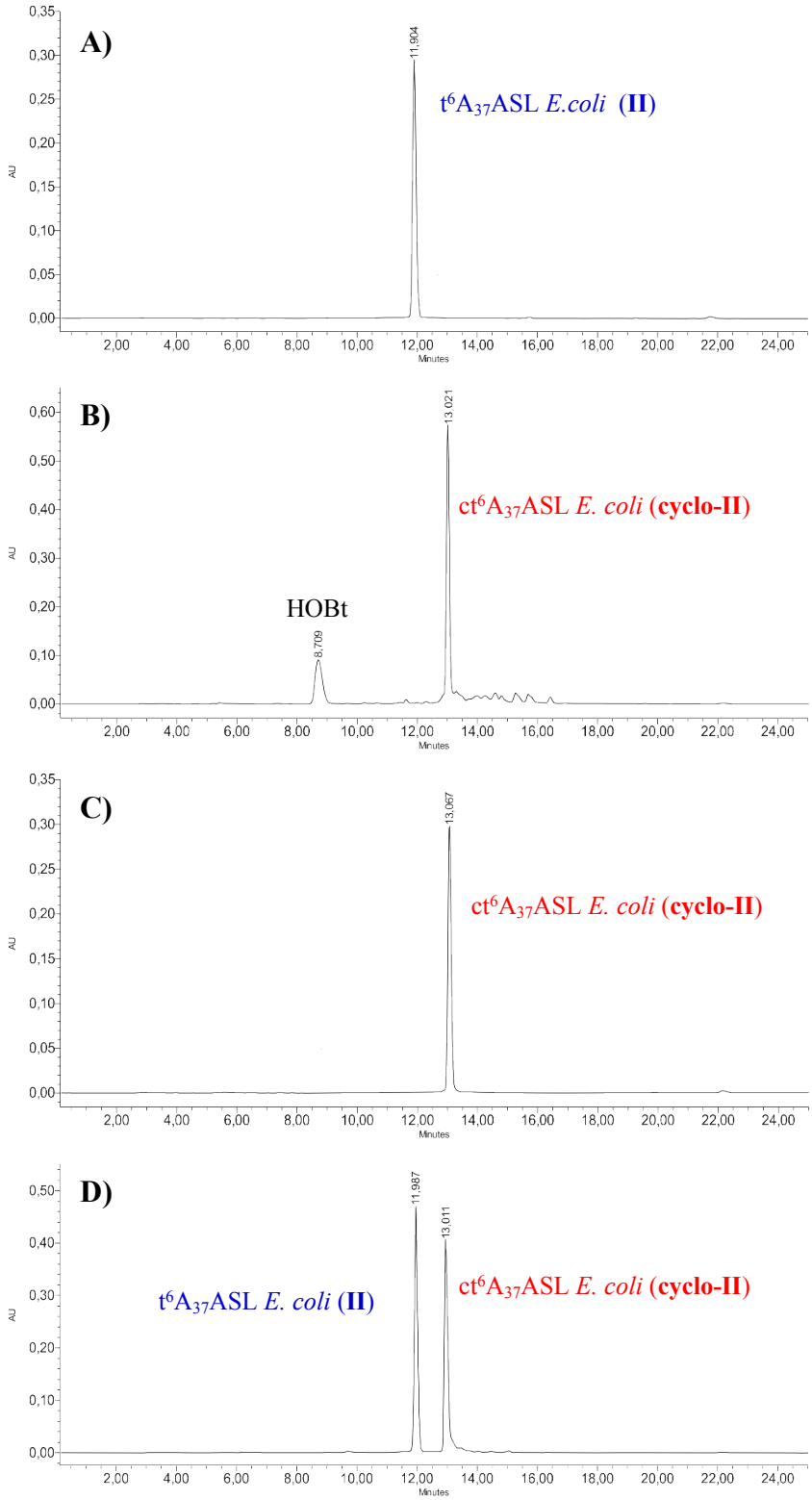
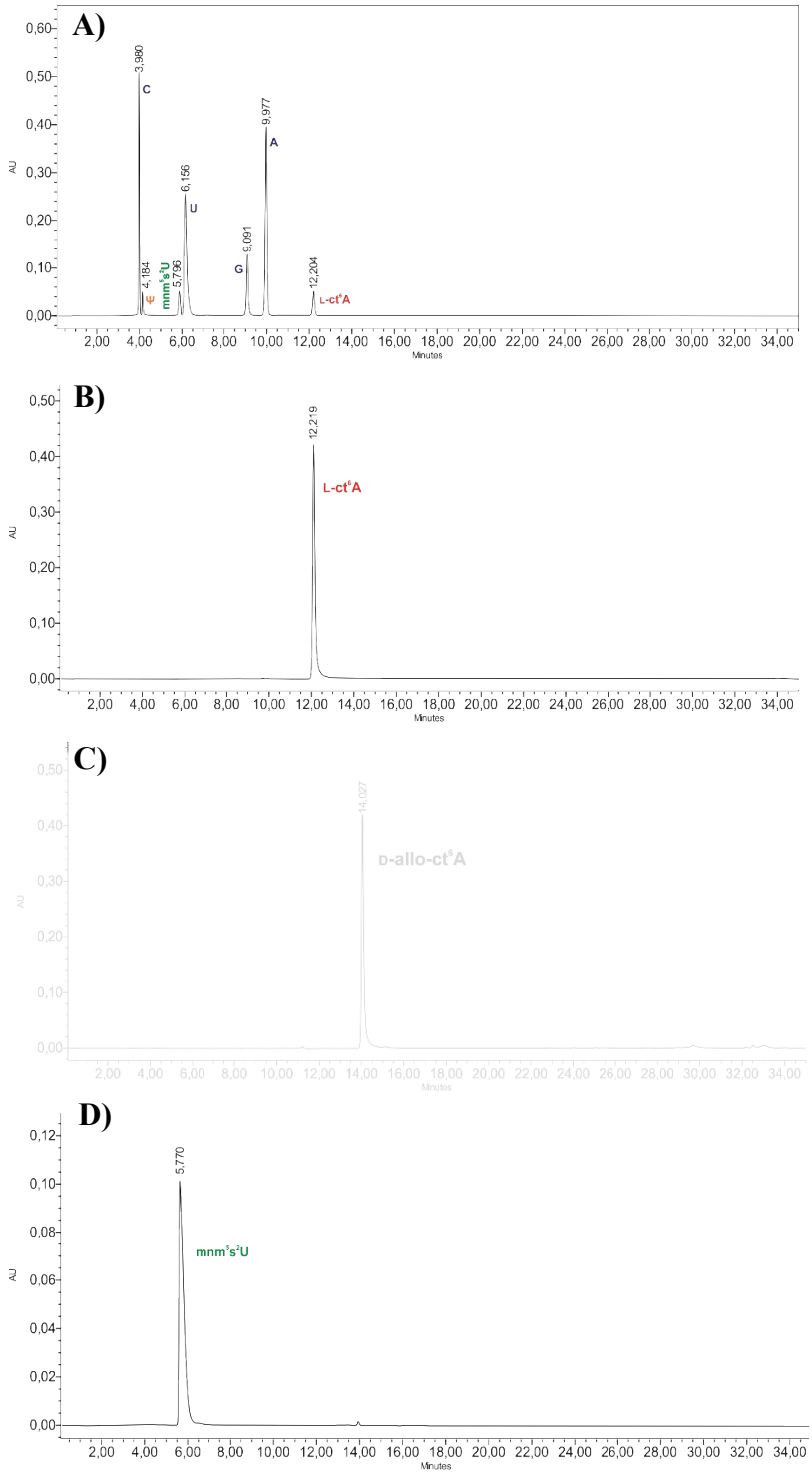


Figure S23. RP-HPLC analysis of **II** (A); reaction mixture after 1h, containing crude product **cyclo-II** (B)**;** purified **cyclo-II** (C); HPLC coinjection analysis of **II** and **cyclo-II** (D).

Enzymatic digestion of 5'-GUUGACU**mnm^{5}s^2**UUU**ct^{6}A**AΨCAAC-3' **(cyclo-II).**

The nucleoside composition of oligoribonucleotide was confirmed by neutral one-step enzymatic digestion (*Nat. Chem Biol*. 2013, 9(2), 105-111) of the final 5'-GUUGACU**mnm^{5}s^2**UUU**ct^{6}A**AΨCAAC-3' **(cyclo-II)** with nuclease P_1 and alkaline phosphatase in 20 mM TEA·HCl (pH 7). The resulting mixture of nucleosides was analyzed by RP-HPLC on C18 column (Kinetex, 4.6 x 250 mm, 5 µm) with linear gradient of buffer A (0.1% AcOH in H_2O) and buffer B (ACN) with a flow 1 mL/min as follows: 0-15 min from 2%B to 8%B, 15-30 min from 8%B to 25%B 30-35 min 2%B. The retention time (R_t) of the ct^{6}A was compared to the R_t of the reference sample in a separate control experiment.



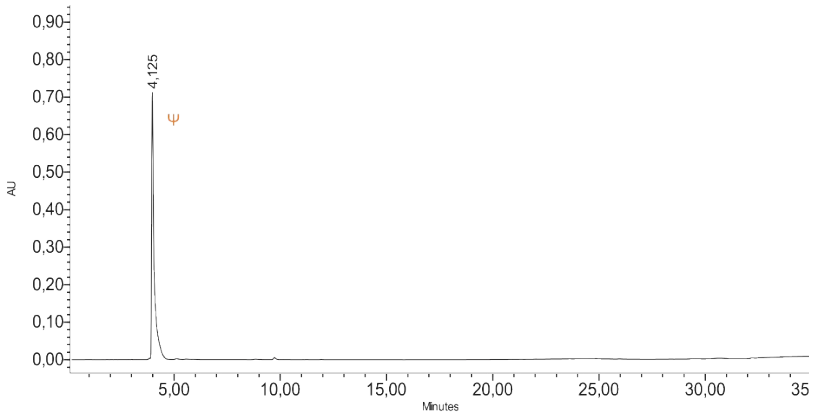


Figure S24. RNA enzymatic digestion of **cyclo-II** (**A**) RP-HPLC analysis of nucleoside composition of ct^{6}A-ASL of ***E. coli*** tRNA^{Lys}. RP-HPLC of the references of **L-ct^{6}A** nucleoside (B), **D-*allo*-ct^{6}A** nucleoside (**C**), 5-methylaminomethyl-2-thiouridine (**mnm^{5}s^{2}U**, D), pseudouridine (**Ψ**, E).