# Supporting Information

# Post-synthetic conversion of 5-pivaloyloxymethyluridine present in a support-bound RNA oligomer into biologically relevant derivatives of 5-methyluridine

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## I. Synthesis of 5-isopentenylaminomethyluridine (5)

5-Pivaloyloxymethyluridine (**7**) (18 mg, 0.05 mmol, 1 equiv) [K. Bartosik and G. Leszczynska, *Tetrahedron Lett.* 2015, **56**, 6539] was dissolved in ethanol-water mixture (750 µL, 4/1 v/v) and tosylate salt of isopentenylamine (77 mg, 0.30 mmol, 6 equiv) and $Et_3N$ (145 µL, 1.05 mmol, 3.5 equiv in relation to the tosylate salt) were added. The reaction mixture was incubated in round-bottom flask sealed with rubber septum at 60 °C for 11 h. The mixture was then cooled to rt and concentrated under reduced pressure. The resulting material was dissolved in methanol and passed through the cation exchange resin (Dowex 50WX2-100, pyridinium form). Fraction containing the product **5** was concentrated under reduced pressure and purified by RP HPLC on C18 column (Hamilton PRP-1, 7.0 x 305 mm, 10 µm) with gradient of 10 mM TEAB (A) and acetonitrile (B) (flow: 3 mL/min; $R_t$ = 18.2 min). The mobile phase composition was as follows: start with 100% A, linear increase over 10 min to 10% B; linear increase over 20 min to 60% B; return to 100% A over 5 min. The appropriate fraction was concentrated under reduced pressure and lyophilized affording the product **5** in 81% yield (14 mg, 0.04 mmol). TLC $R_f$ = 0.58 (iPrOH/ammonia/$H_2O$ 8:1:1 v/v/v). NMR (δ [ppm], $D_2O$): $^1H$ (700 MHz) 1.72 (s, 3H), 1.81 (s, 3H), 3.57 (d, *J* = 7.7 Hz, 2H), 3.83 (s, 2H), 3.85 (dd, *J* = 4.2 Hz, *J* = 12.6 Hz, 1H), 3.96 (dd, *J* = 4.2 Hz, *J* = 12.6 Hz, 1H), 4.15-4.17 (m, 1H), 4.26 (t, *J* = 5.6 Hz, 1H), 4.35 (t, *J* = 4.2 Hz, 1H), 5.29-5.31 (m, 1H), 5.94 (d, *J* = 4.2 Hz, 1H), 7.90 (s, 1H); $^{13}C$ (176 Hz) 17.3, 25.0, 44.1, 44.2, 60.7, 69.3, 73.8, 83.9, 89.8, 106.9, 114.9, 140.6, 142.2, 157.4, 172.4. HRMS ($FAB^+$) calcd for $C_{15}H_{24}N_3O_6$ $[M + H]^+$ 342.1665, found 342.1671.

## II. Synthesis of 5-cyanomethyluridine (6)

5-Pivaloyloxymethyluridine (**7**) (18 mg, 0.05 mmol, 1 equiv) was treated with 0.1 M potassium cyanide in anhydrous ethanol (4 mL) and incubated in round-bottom flask sealed with rubber septum at 60 °C for 20 h. The reaction mixture was cooled to rt, neutralized with Dowex 50WX2-100 ($H^+$ form) and purified by RP-HPLC on C18 column (Hamilton PRP-1, 7.0 x 305 mm, 10µm) with gradient of water (A) and acetonitrile (B) (flow: 3 mL/min; $R_t$ = 14.3 min). The mobile phase composition was as follows: 100% A for 4 min; linear increase over 16 min to 10% B; linear increase over 10 min to 60% B; return to 100% A over 5 min. The appropriate fraction was concentrated under reduced pressure and lyophilized affording the product **6** in 80% yield (11 mg, 0.04 mmol). TLC $R_f$ = 0.36 ($CHCl_3$/MeOH 8:2 v/v). NMR (δ [ppm], DMSO-$d_6$): $^1H$ (700 MHz) 3.49 (s, 2H), 3.54-3.56 (m, 1H), 3.63-3.65

(m, 1H), 3.84 (q, *J* = 3.5 Hz, 1H), 3.96-3.97 (m, 1H), 4.02-4.04 (m, 1H), 5.06-5.09 (m, 2H), 5.39 (s, 1H), 5.78 (d, *J* = 5.6 Hz, 1H), 7.98 (s, 1H), 11.62 (s, 1H); $^{13}$C (176 Hz) 15.2, 60.9, 69.7, 73.4, 84.9, 87.8, 104.3, 118.0, 139.0, 150.4, 162.2. HRMS (EI) calcd for $C_{11}H_{13}N_3O_6$ 283.0804, found 283.0797.

## III. Synthesis of 5-pivaloyloxymethyluridine (7)

5-Pivaloyloxymethyluridine (**7**) and 5′-*O*-pivaloyl-5-pivaloyloxymethyluridine (**11**) were synthesized according to our previously reported procedure [K. Bartosik and G. Leszczynska, *Tetrahedron Lett.* 2015, **56**, 6539] excluding selective methods of the enzymatic or chemical 5′-depivaloylation of 5′-*O*-pivaloyl-5-pivaloyloxymethyluridine (**11**).

*Enzymatic 5′-depivaloylation of **11***

5′-*O*-Pivaloyl-5-pivaloyloxymethyluridine (**11**) (100 mg, 0.22 mmol) was dissolved in water-DMF mixture (10 mL, 9/1 v/v) and lipase from porcine pancreas (300 mg) was added. The reaction mixture was stirred at rt for 10 days. The solvents were removed, the solid residue was dissolved in water-ethyl acetate (10 mL, 3/7 v/v) and extracted with AcOEt (2 x 10 mL). The combained organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified on silica gel column with 4% MeOH in $CHCl_3$ to afford **7** in 75% yield (61 mg, 0.17 mmol). Spectral analysis of **7** showed very good agreement with previously published data [K. Bartosik and G. Leszczynska, *Tetrahedron Lett.* 2015, **56**, 6539].

*Chemical 5′-depivaloylation of **11***

5′-*O*-Pivaloyl-5-pivaloyloxymethyluridine (**11**) (100 mg, 0.22 mmol) was dissolved in 0.5 M methanolic NaOH (4.6 mL) and the mixture was stirred at rt for 1 h. The reaction mixture was cooled to 0 ºC and neutralized with 0.5 M aqueous HCl (4 mL) and extracted with ethyl acetate (3 x 10 mL). The combained organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product **7** was purified on a silica gel column with 6% MeOH in $CHCl_3$ as eluent to obtain a white foam in 60% yield (47 mg, 0.13 mmol).

## IV. $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra of 5, 6 and 12-14

**Fig. S1.** $^{1}H$ NMR spectrum of **5** (700 MHz, $D_2O$)

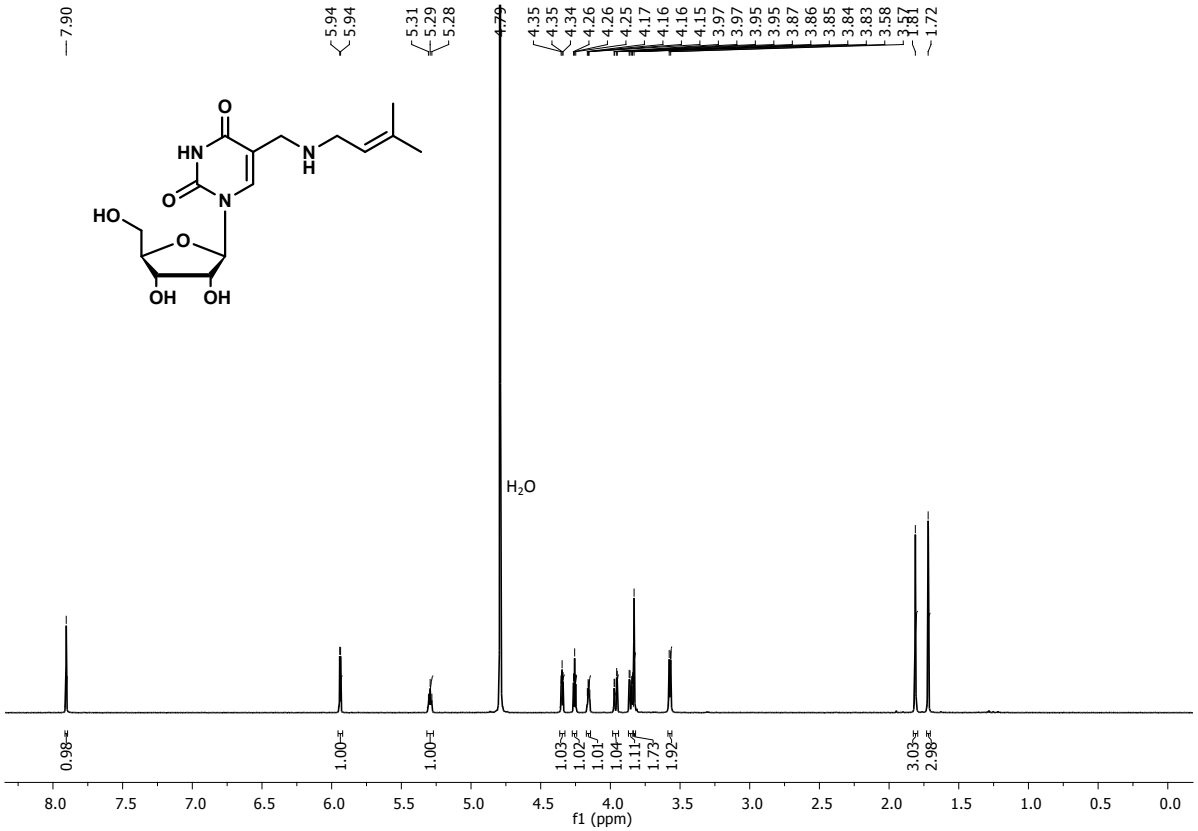



**Fig. S2.** $^{13}C$ NMR spectrum of **5** (176 MHz, $D_2O$)

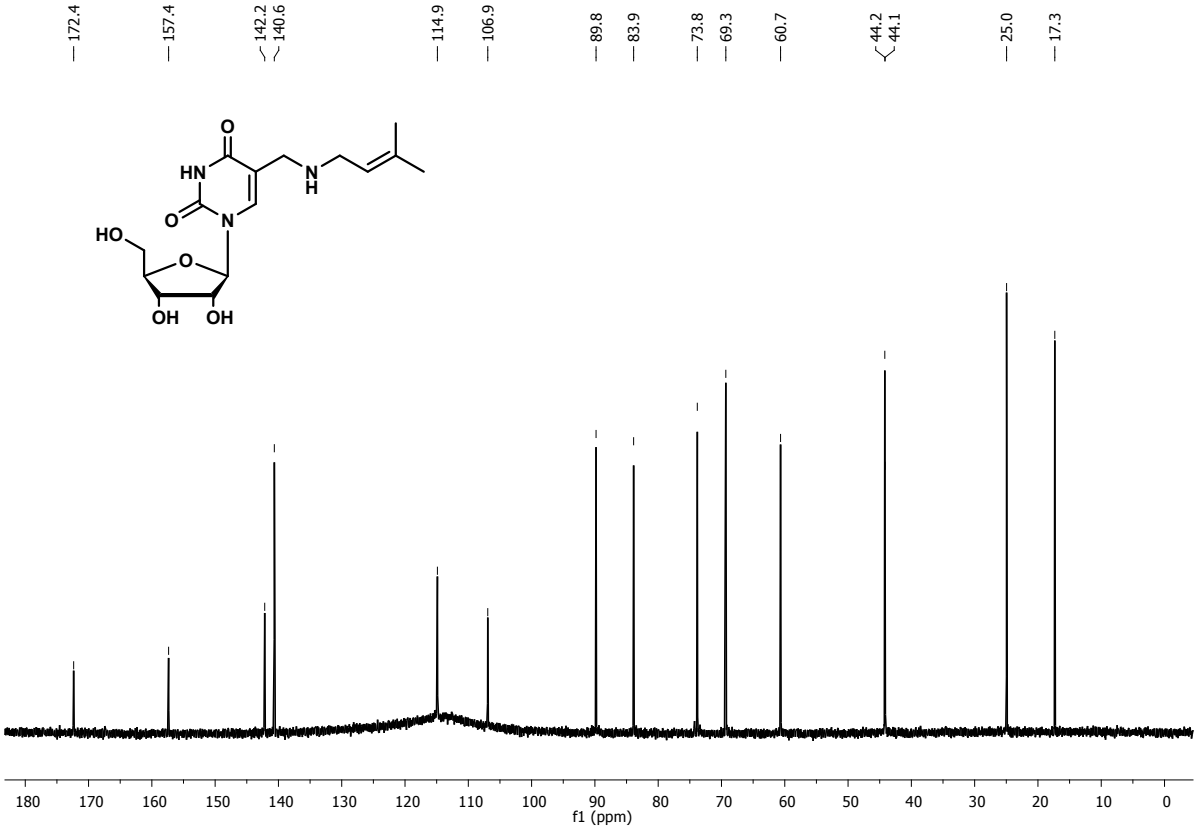


**Fig. S3.** $^{1}$H NMR spectrum of **6** (700 MHz, DMSO-$d_6$)

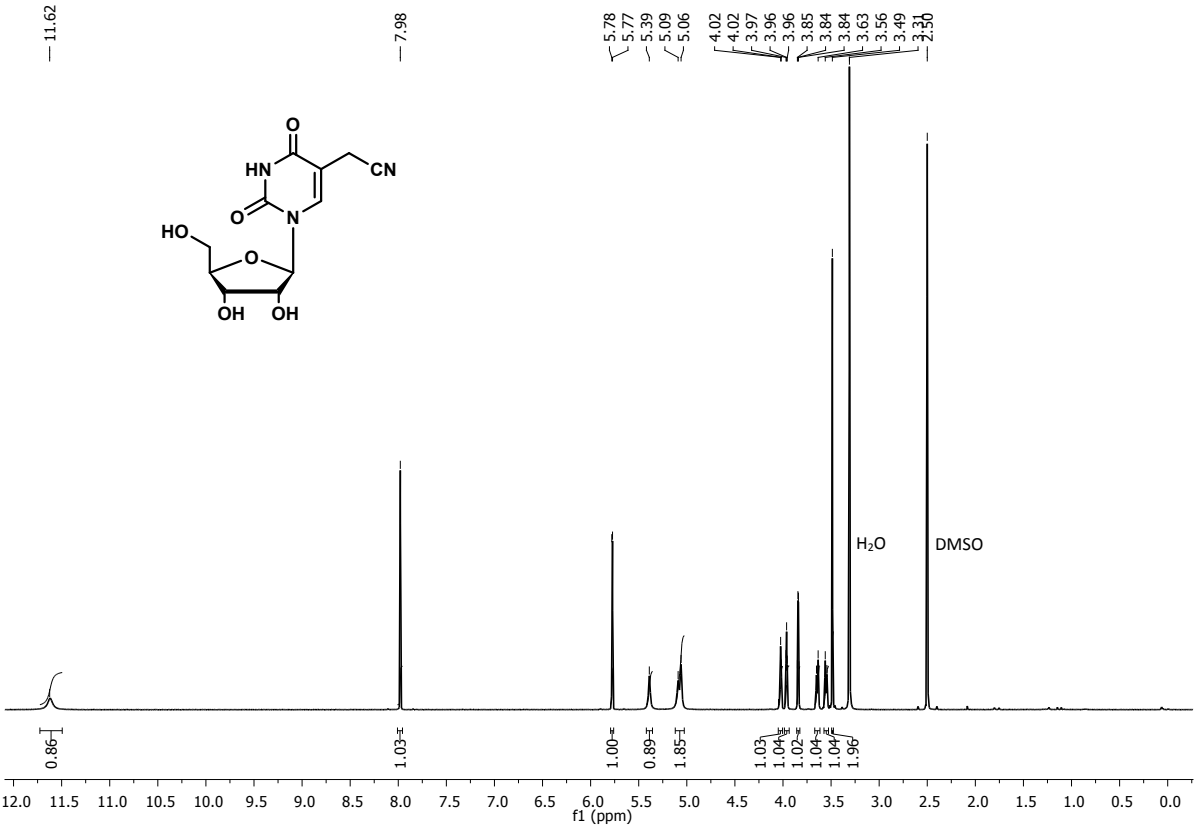



**Fig. S4.** $^{13}$C NMR spectrum of **6** (176 MHz, DMSO-$d_6$)

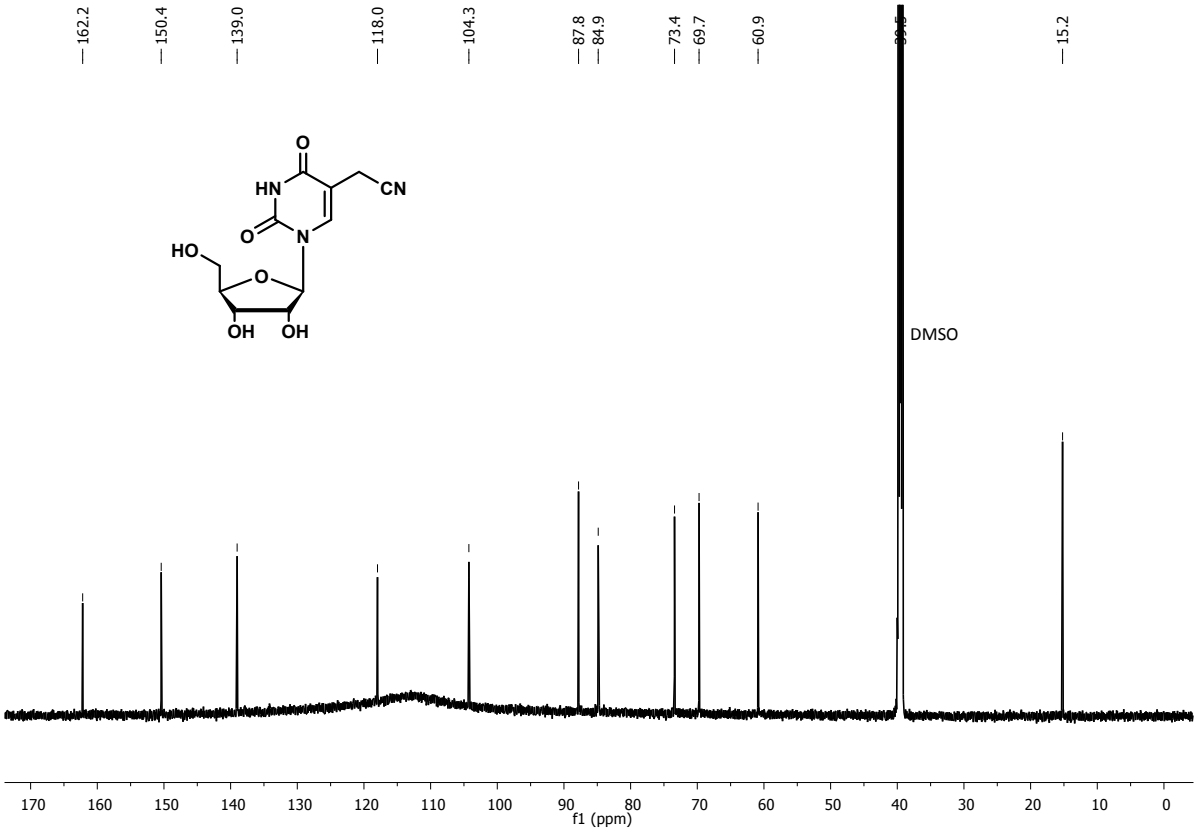


**Fig. S5.** $^{1}$H NMR spectrum of **12** (700 MHz, $CDCl_3$)

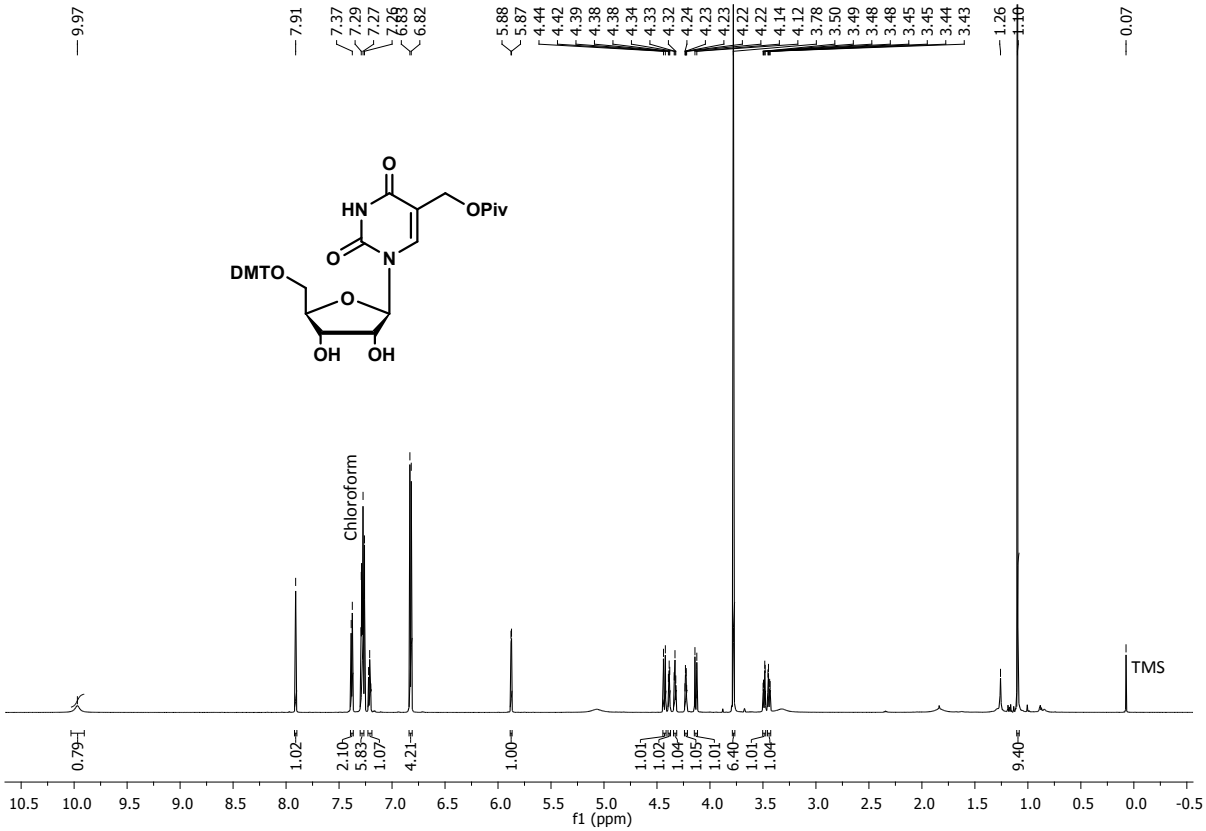



**Fig. S6.** $^{13}$C NMR spectrum of **12** (176 MHz, $CDCl_3$)

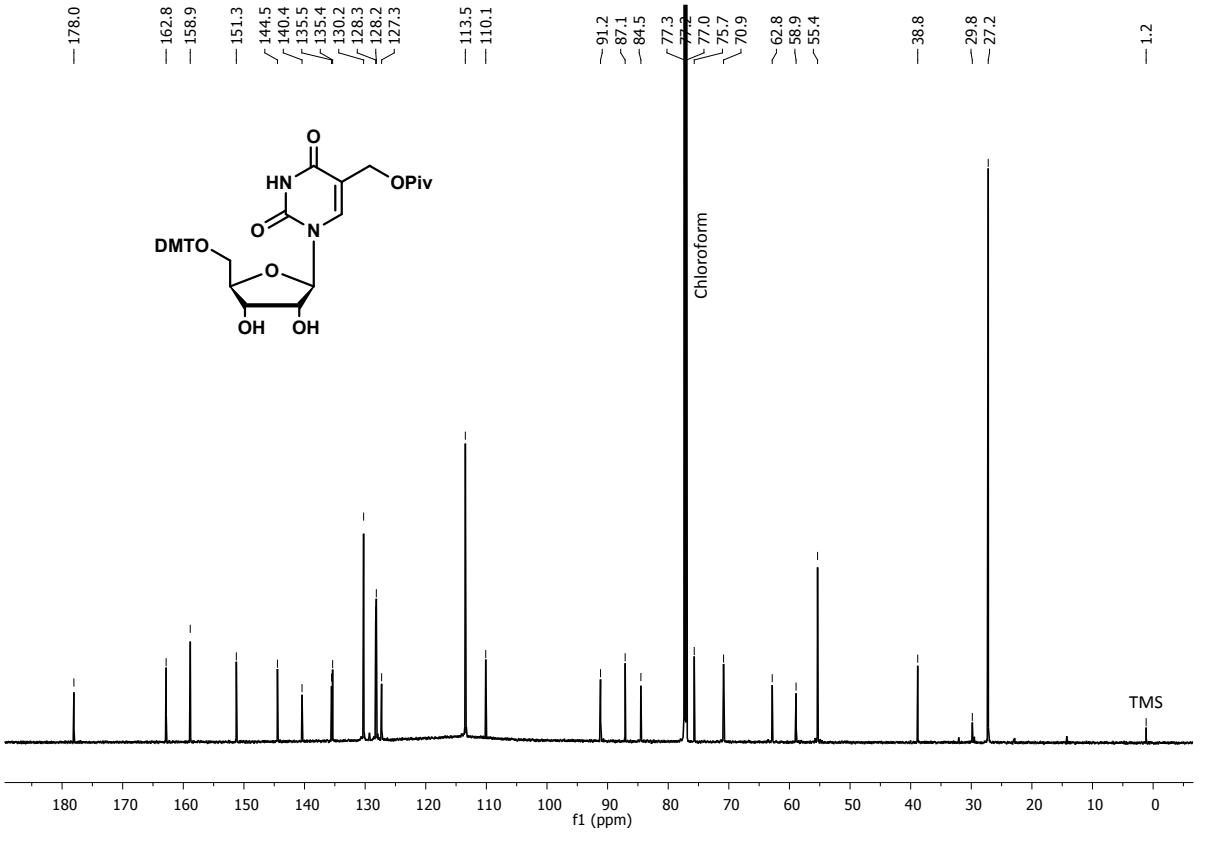


**Fig. S7.** $^{1}$H NMR spectrum of **13** (700 MHz, $CDCl_3$)

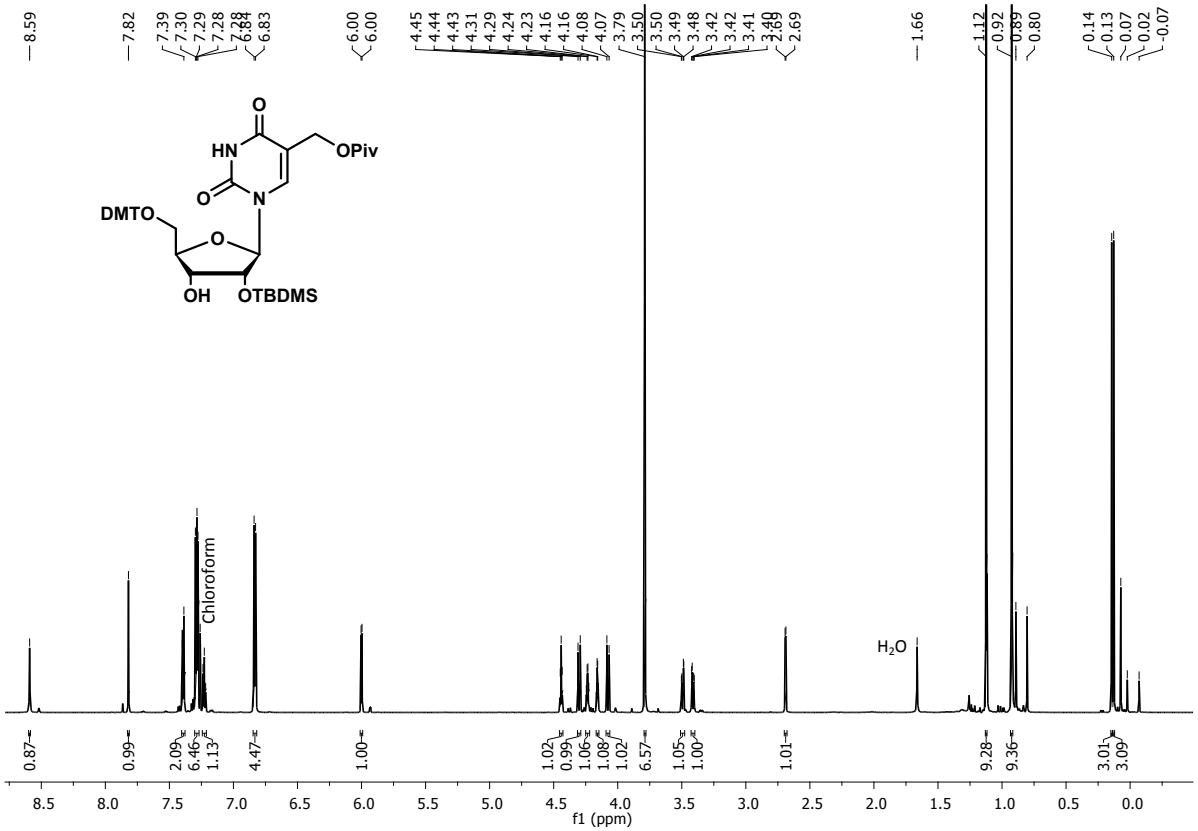



**Fig. S8.** COSY spectrum of **13** (700 MHz, $CDCl_3$)

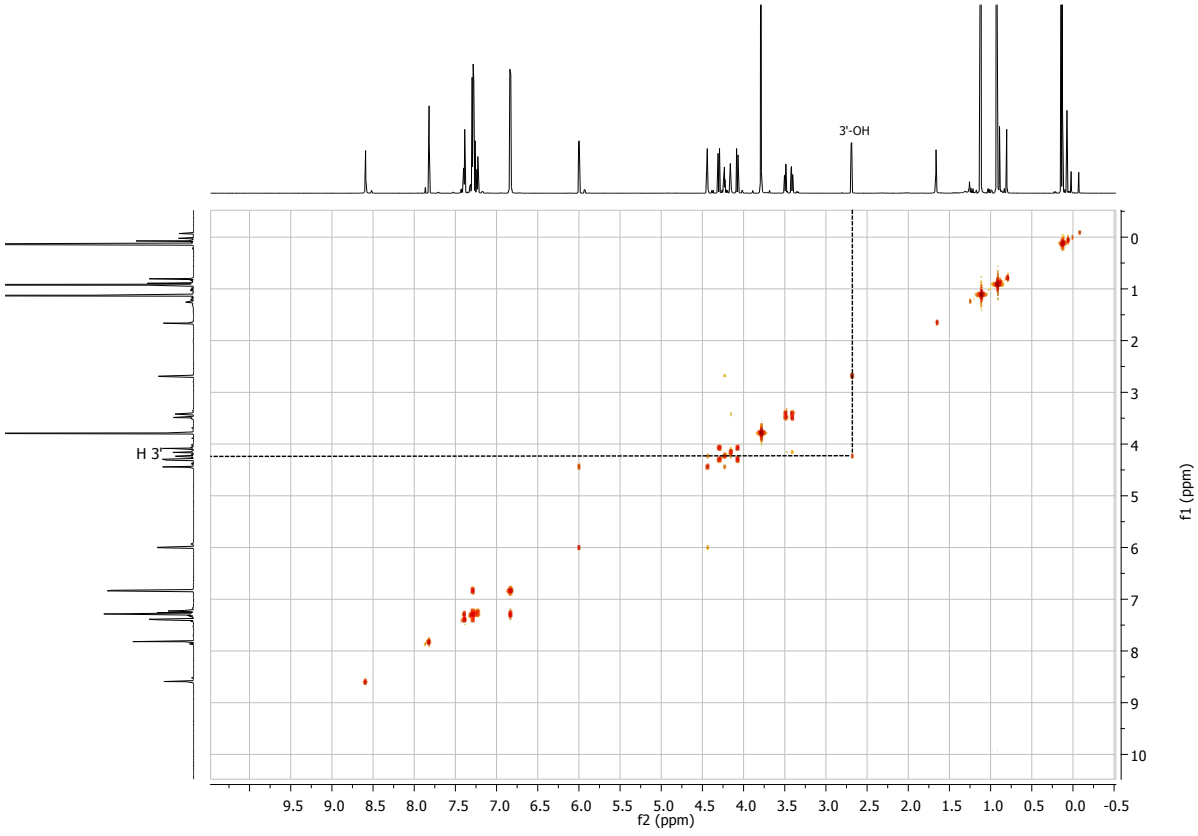


**Fig. S9.** $^{13}C$ NMR spectrum of **13** (176 MHz, $CDCl_3$)

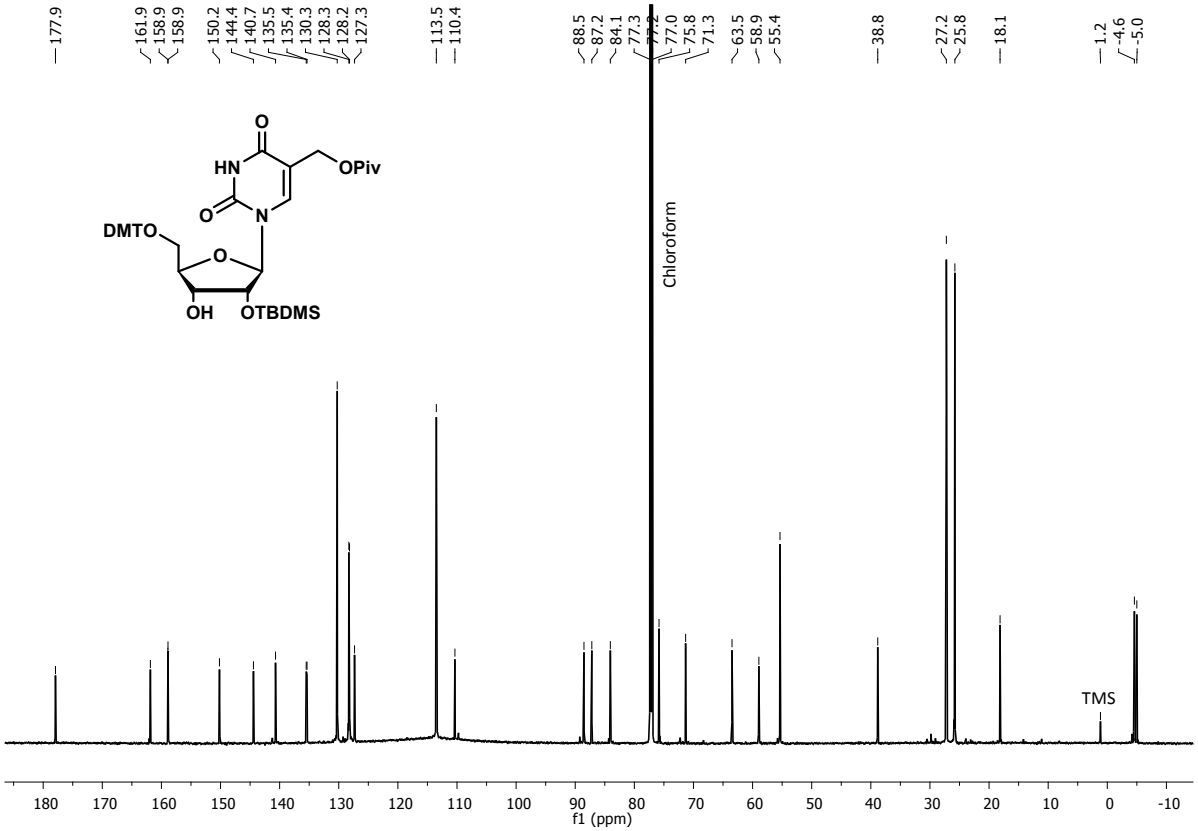



**Fig. S10.** $^{1}H$ NMR spectrum of **14** (700 MHz, $(CD_3)_2CO$)

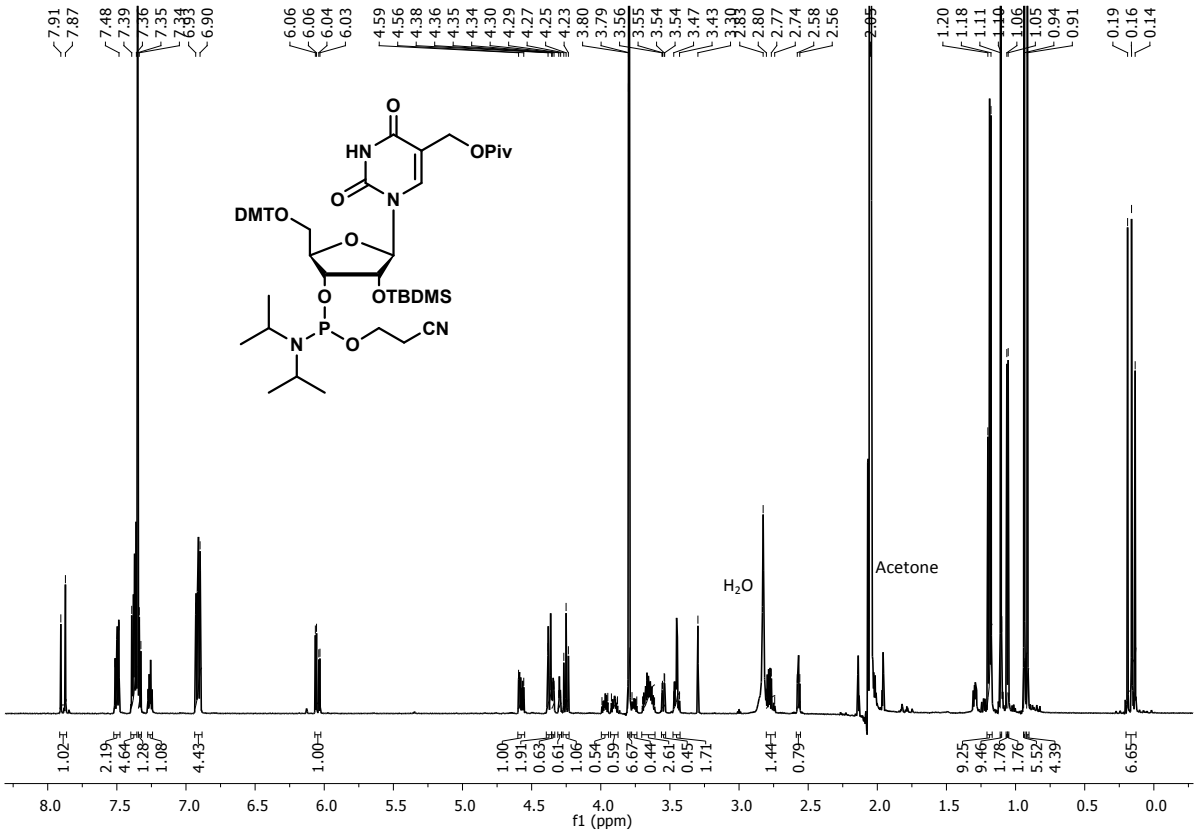
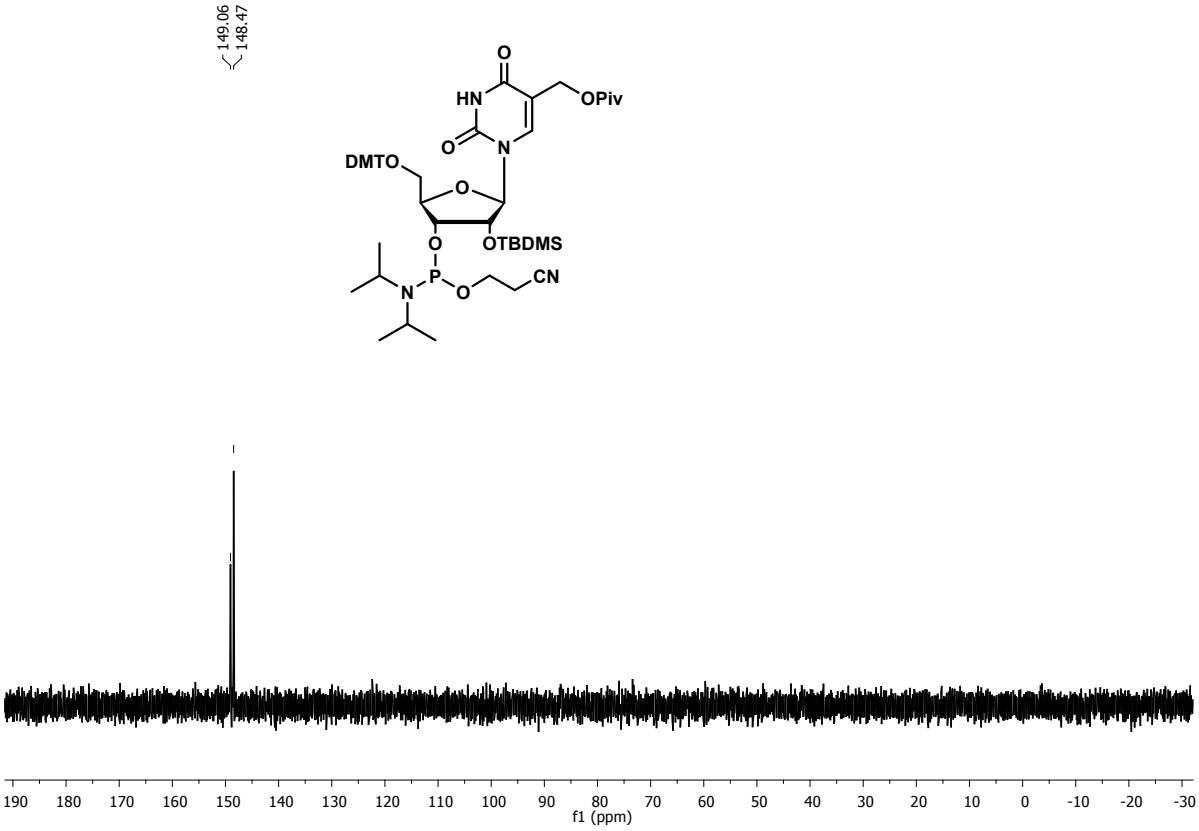


**Fig. S11.** $^{31}P$ NMR spectrum of **14** (101 MHz, $(CD_3)_2CO$)




## V. Deprotection and characterization of Pivom$^5$U-RNA (5-mer; 5'-GU(Pivom$^5$U)AC-3')

The "DMTr-off", CPG-linked Pivom$^5$U-RNA (0.1 μmol) was treated with $Et_3N$/acetonitrile mixture (136 μL, 1/1, v/v) for 20 min. The solution was removed and the support-bound RNA was washed with acetonitrile (3 x 100 μL), dried *in vacuo* for 30 min, and treated with 30% aq. $NH_3$ (150 μL) at rt for 3.5 h. The supernatant was removed and the support was washed with ethanol/water (3 x 150 μL, 1/1, v/v). The combined washings were evaporated on a Speed-Vac concentrator and the solid residue was treated with a solution of $Et_3N{\cdot}3HF$ in NMP (60 μL, 1/1, v/v) for 24 h at rt. The reaction was quenched by addition of 120 μL of ethoxytrimethylsilane and the crude RNA was precipitated using 300 μL of *tert*-butyl methyl ether. The fully deprotected RNA was purified by anion-exchange (IE) HPLC (Source 15Q 4.6/100PE) at constant flow rate of 1 mL/min. The column was eluted with a linear gradient 50 mM to 500 mM NaBr in 20 mM $Na_2HPO_4$-$NaH_2PO_4$ buffer solution pH 7.5, containing 50 μM EDTA and 10% ACN. Fractions containing the desired product (Pivom$^5$U-RNA, $R_t$ = 13.9 min., **Fig. S12**) and the product of post-synthetic conversion of Pivom$^5$U-RNA → nm$^5$U-RNA (nm$^5$U-RNA, $R_t$ = 7.3 min) were collected, concentrated, and desalted on a C-18

cartridge (Sep-Pak, Waters). The desalted Pivom$^5$U-RNA and nm$^5$U-RNA were lyophilized and analyzed by MALDI-TOF mass spectrometry (Pivom$^5$U-RNA: *m/z* calcd 1644, found 1642; **Fig. S13**; the spectral analyses of nm$^5$U-RNA was identical to that shown on page S11). The total yield of Pivom$^5$U-incorporation into RNA chain was calculated as 85%.

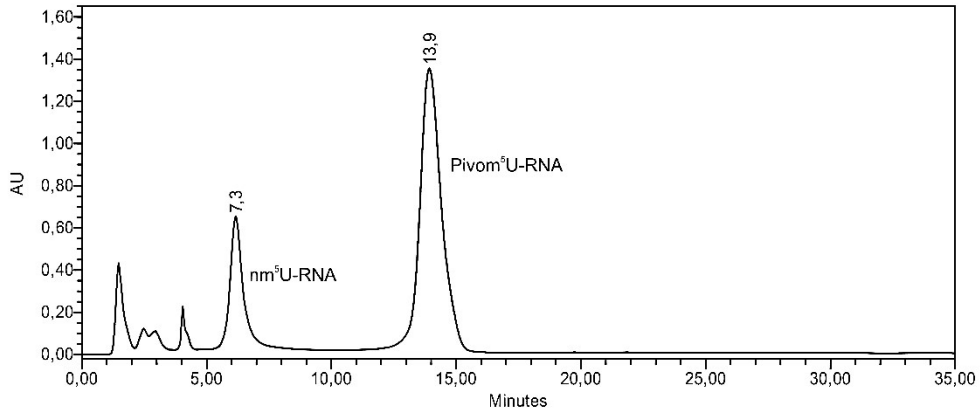



**Fig. S12.** IE-HPLC analysis of crude, deprotected Pivom$^5$U-RNA (5'-GU**Pivom$^5$U**AC-3').

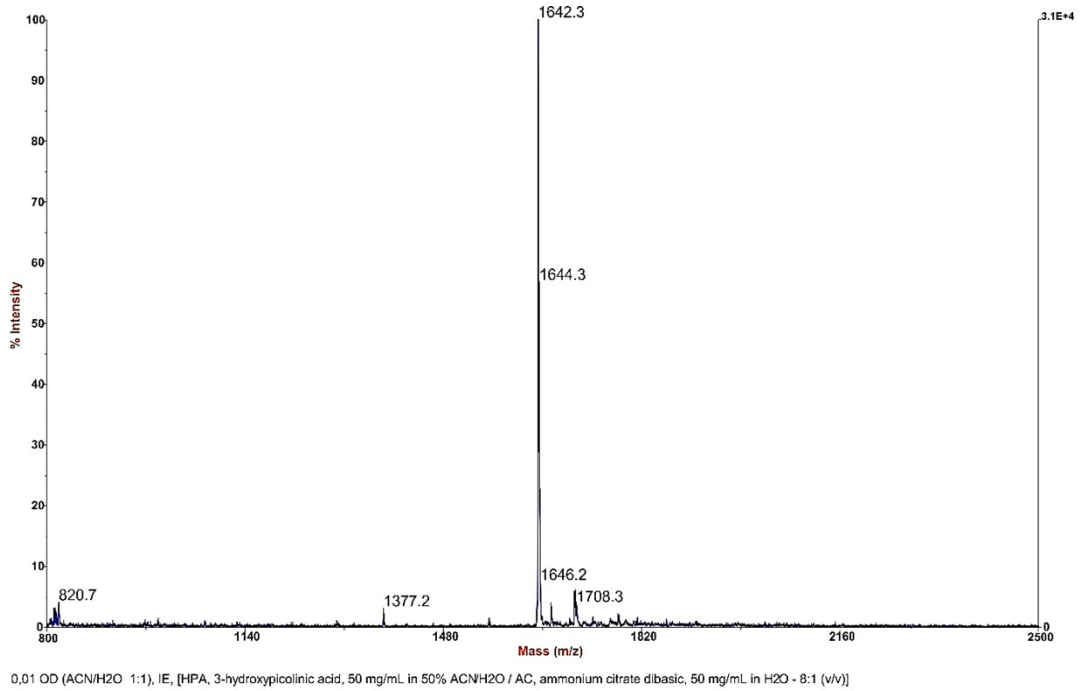



**Fig. S13.** The MALDI-TOF spectrum of 5'-GU**Pivom$^5$U**AC-3' (*m/z* calcd 1644, found 1642).

## VI. IE-HPLC analysis and spectral identification of xm$^5$U-RNA (5-mers)

### a) Post-synthetic transformation of Pivom$^5$U-RNA → nm$^5$U-RNA (Table 1, entry 1)

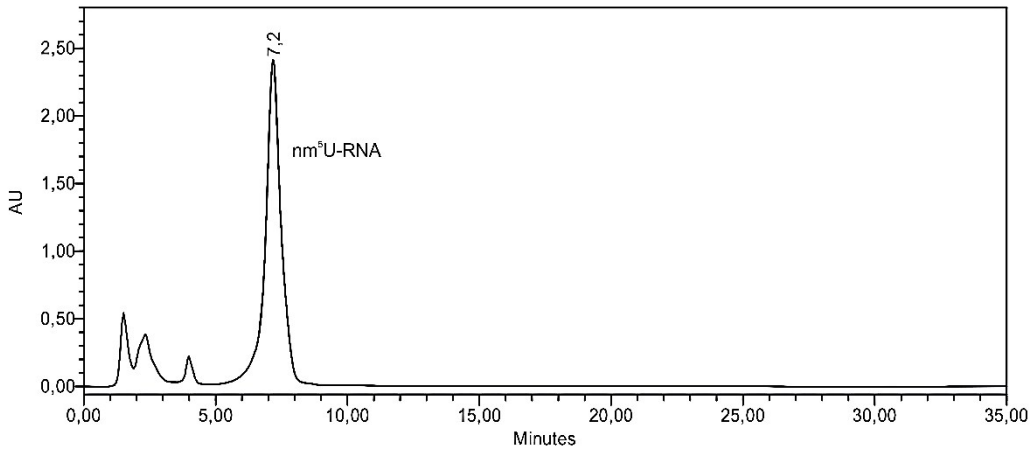



**Fig. S14.** IE-HPLC analysis of crude, deprotected nm$^5$U-RNA (5'-GU**nm$^5$U**AC-3').

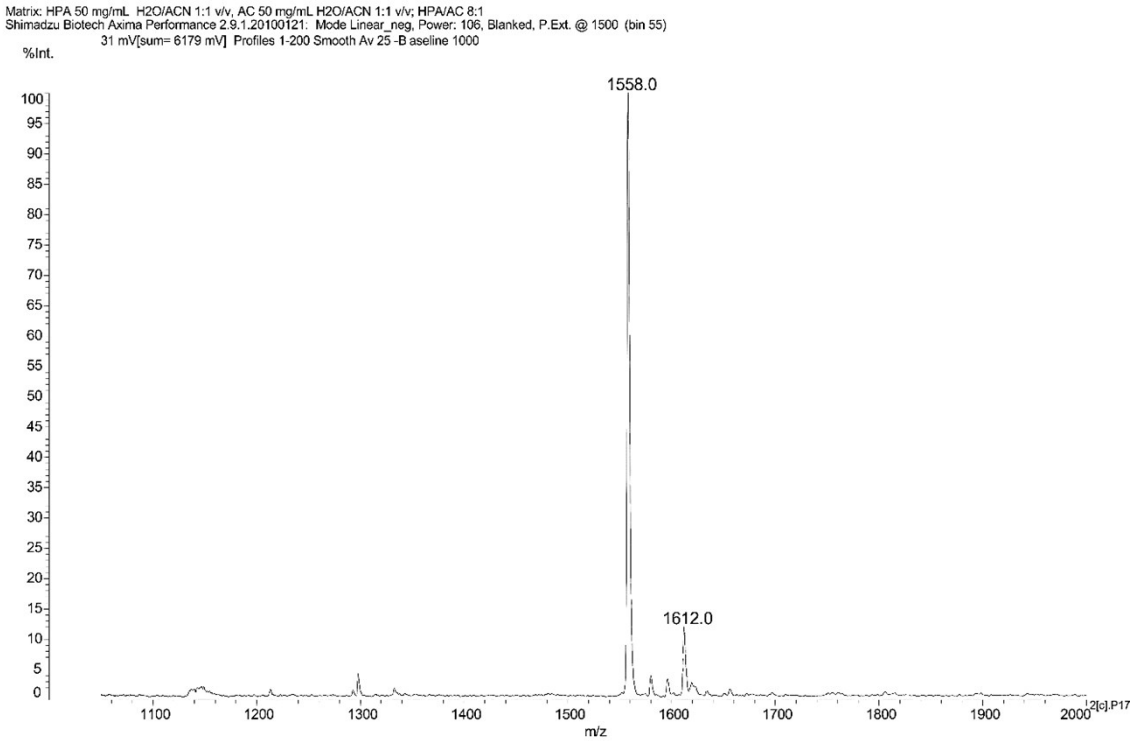



**Fig. S15.** The MALDI-TOF spectrum of 5'-GU**nm$^5$U**AC-3' (*m/z* calcd 1559, found 1558).

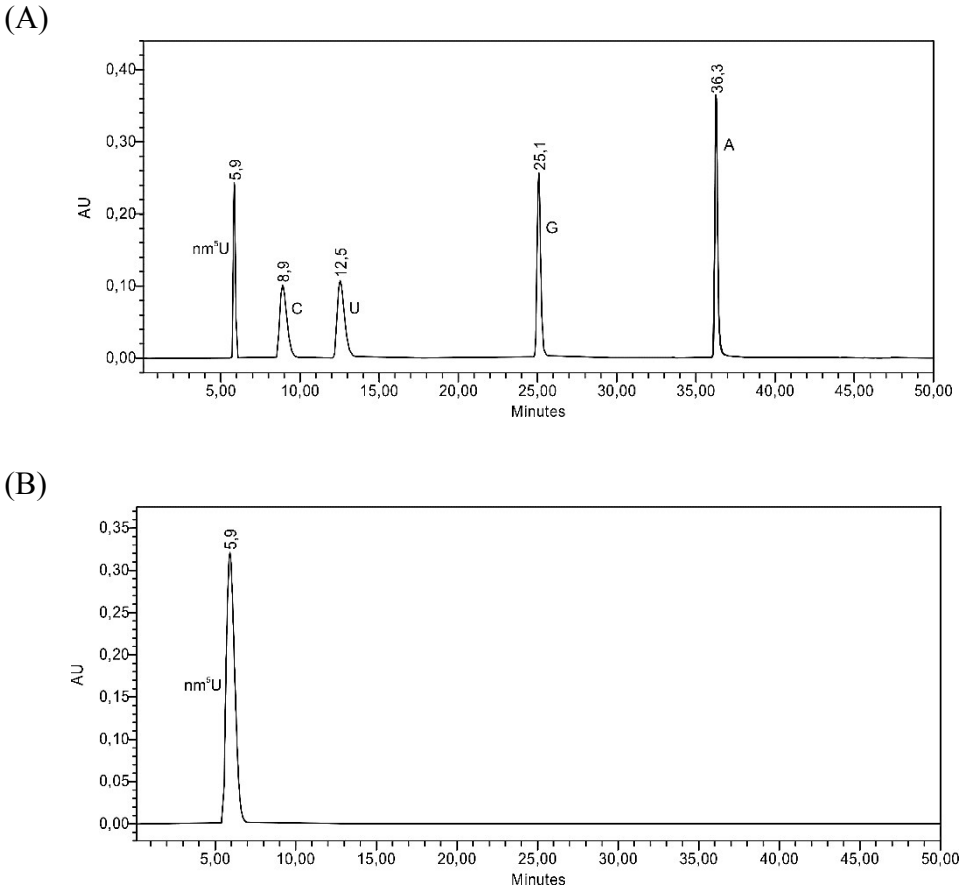



**Fig. S16.** RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of 5'-GU**nm$^5$U**AC-3'. (B) RP-HPLC of the reference of nm$^5$U nucleoside.

**b) Post-synthetic transformation of Pivom$^5$U-RNA → mnm$^5$U-RNA (Table 1, entry 2)**

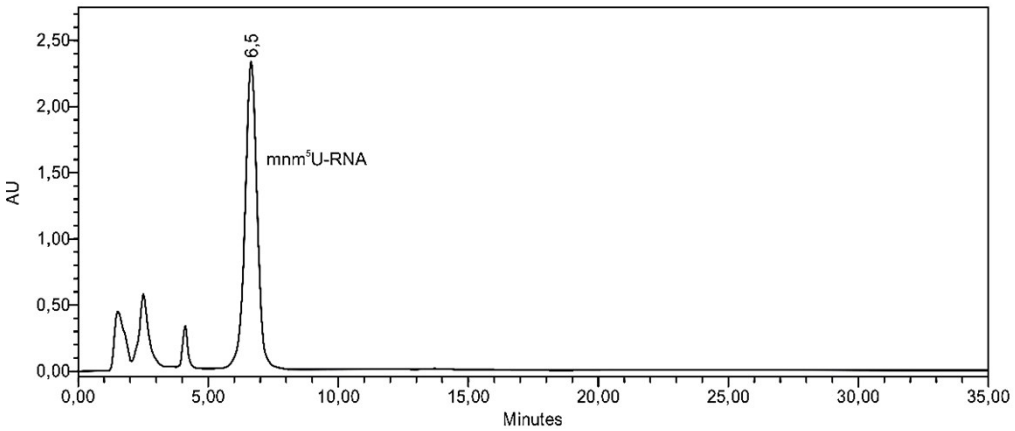



**Fig. S17.** IE-HPLC analysis of crude, deprotected 5'-GU**mnm$^5$U**AC-3'.

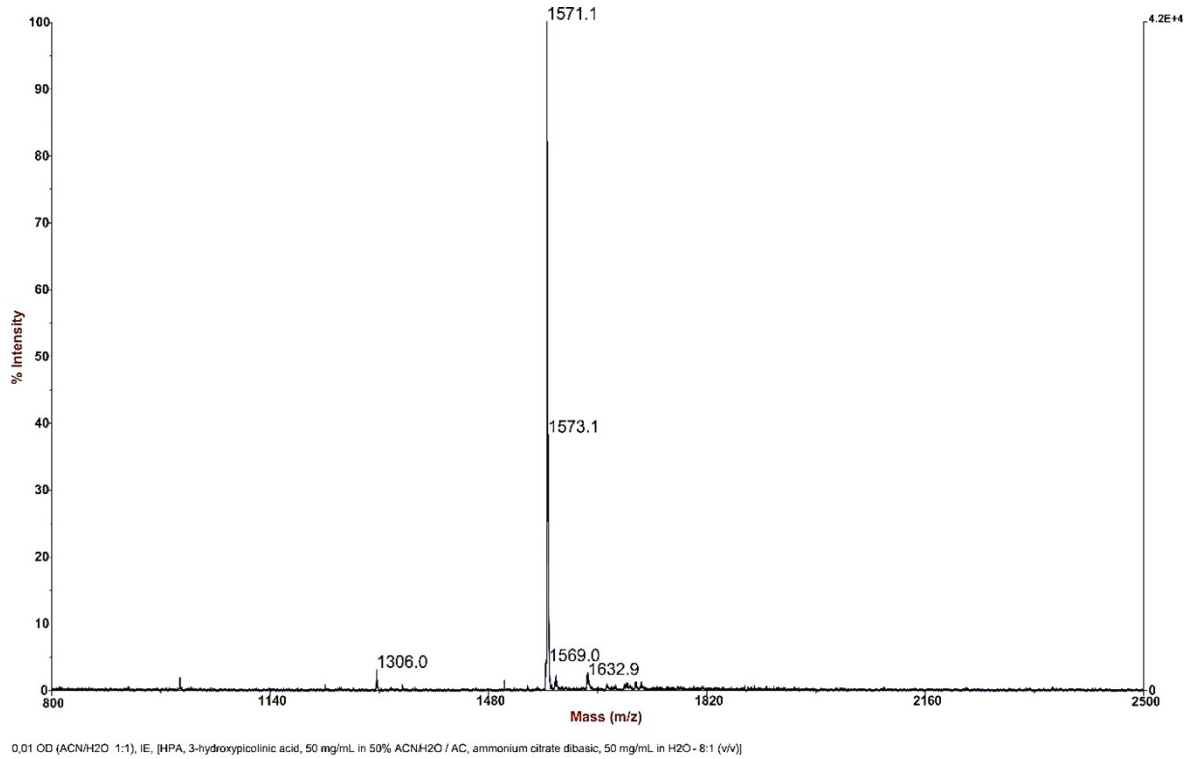



**Fig. S18.** The MALDI-TOF spectrum of 5'-GU**mnm$^5$U**AC-3' (*m/z* calcd 1573, found 1571).

**c) Post-synthetic transformation of Pivom$^5$U-RNA → cmnm$^5$U-RNA (Table 1, entry 3)**

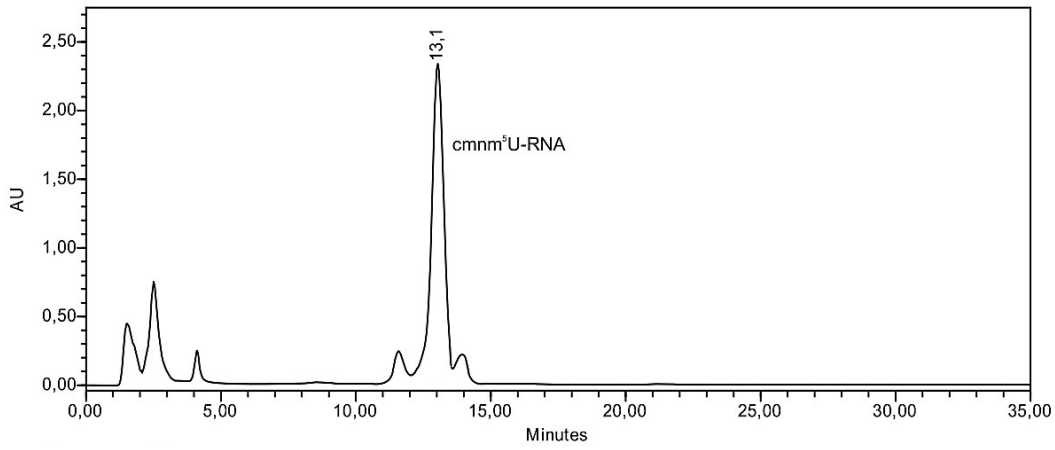



**Fig. S19.** IE-HPLC analysis of crude, deprotected 5'-GU**cmnm$^5$U**AC-3'.

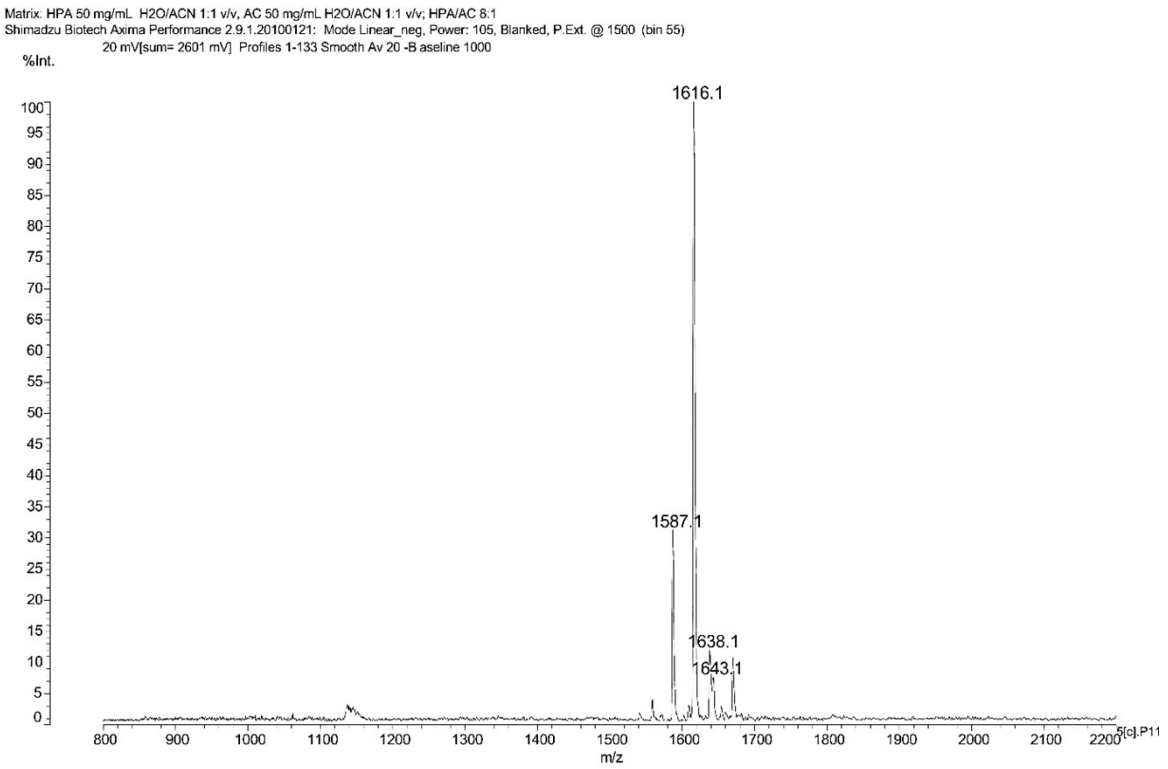
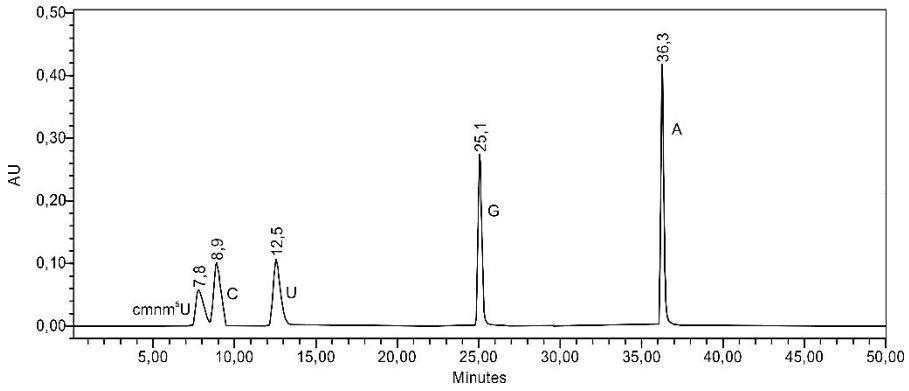



**Fig. S20.** The MALDI-TOF spectrum of 5'-GU**cmnm$^5$U**AC-3' (*m/z* calcd 1617, found 1616).

(A)




(B)

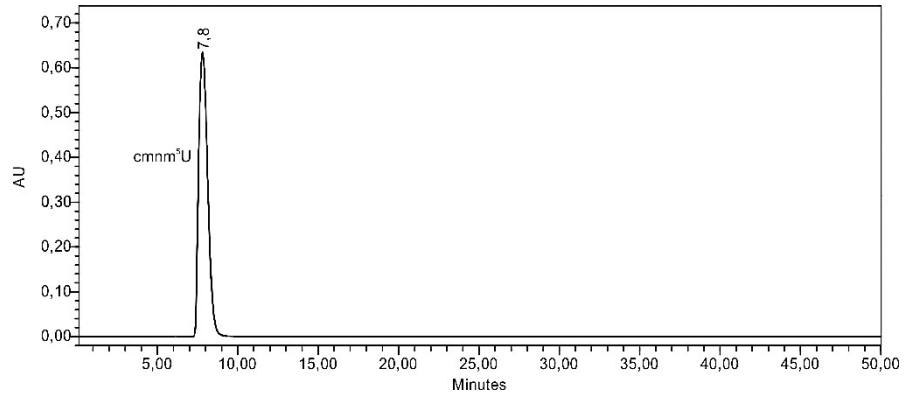



**Fig. S21.** RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of cmnm$^5$U-RNA. (B) RP-HPLC of the reference of cmnm$^5$U nucleoside.

## d) Post-synthetic transformation of Pivom$^5$U-RNA → τm$^5$U-RNA (Table 1, entry 4)

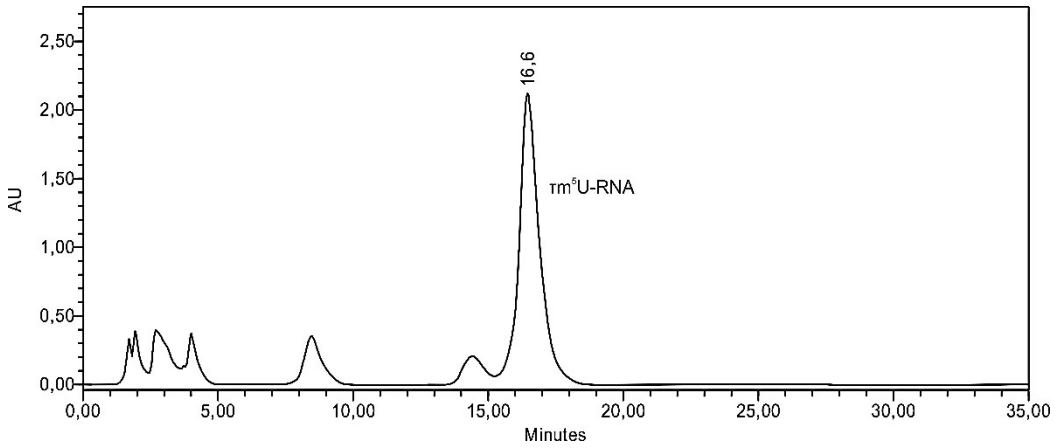



**Fig. S22.** IE-HPLC analysis of crude, deprotected 5'-GU**τm$^5$U**AC-3'.

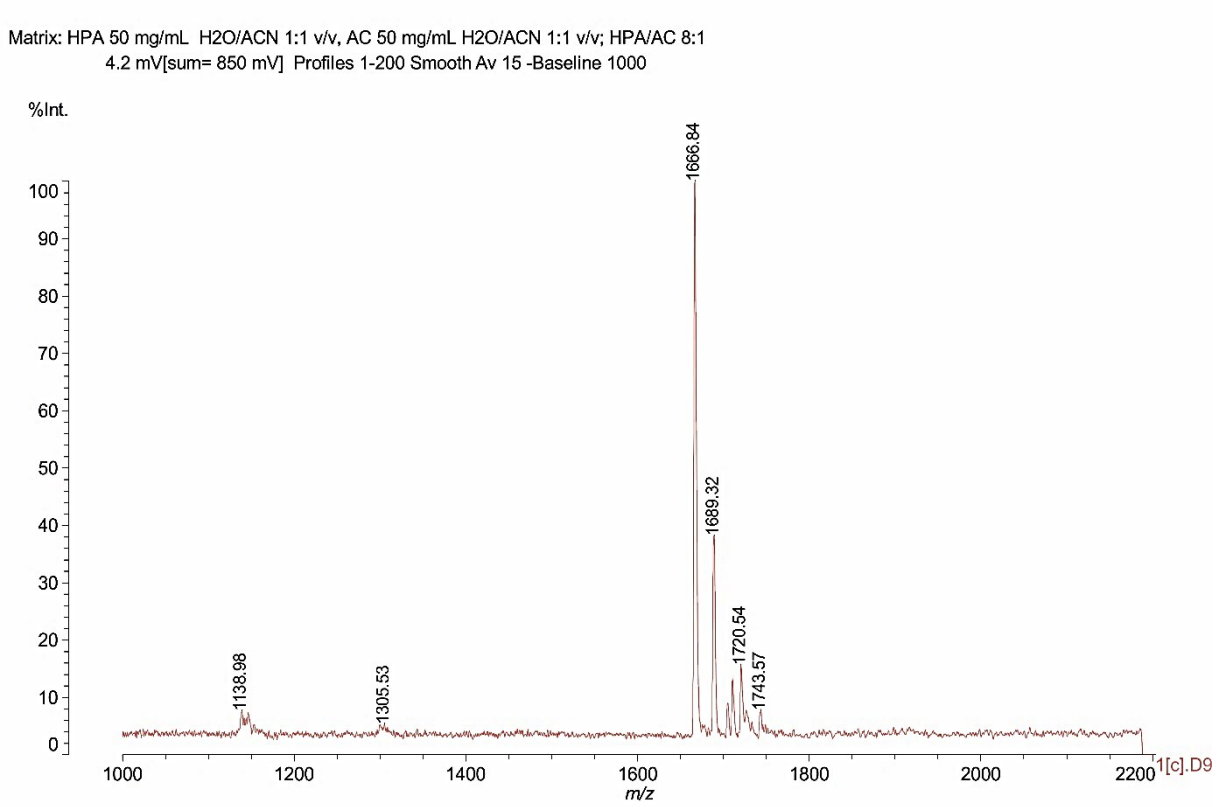
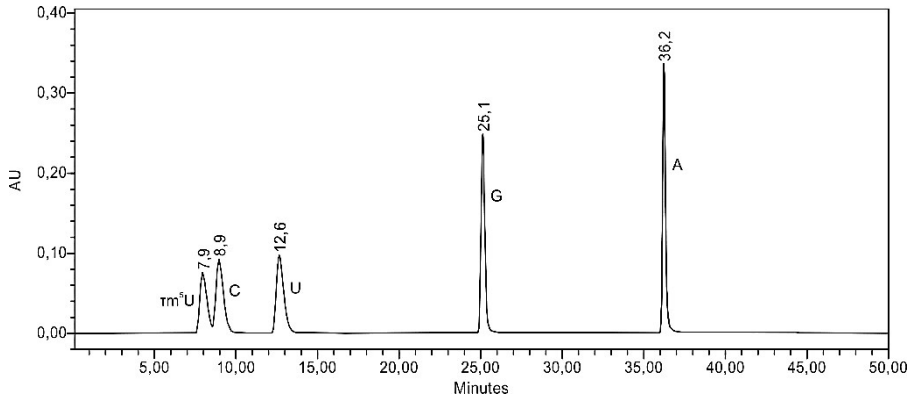



**Fig. S23.** The MALDI-TOF spectrum of 5'-GU**τm$^5$U**AC-3' (*m/z* calcd 1667, found 1667).

(A)




(B)

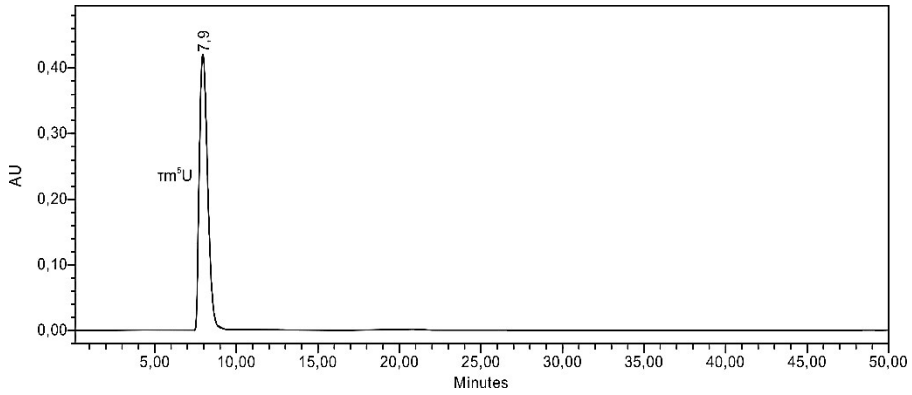



**Fig. S24.** RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of τm$^5$U-RNA. (B) RP-HPLC of the reference of τm$^5$U nucleoside.

**e) Post-synthetic transformation of Pivom$^5$U-RNA → inm$^5$U-RNA (Table 1, entry 5)**

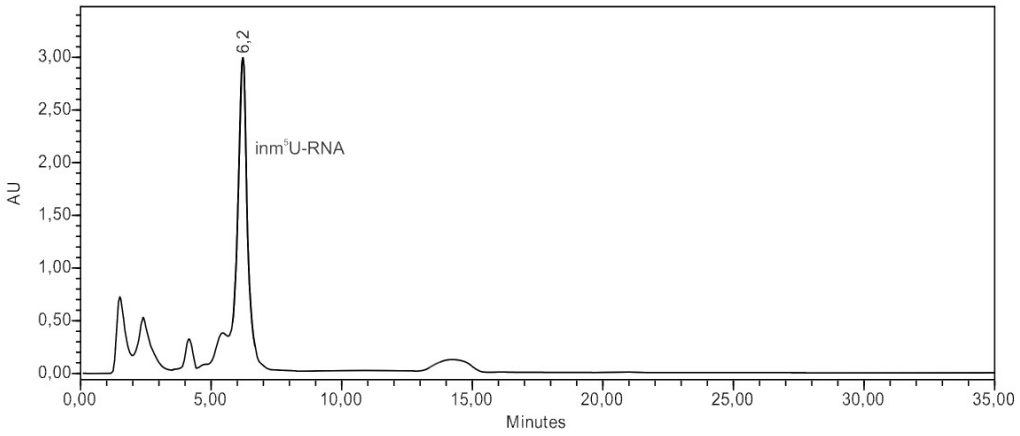



**Fig. S25.** IE-HPLC analysis of crude, deprotected 5'-GU**inm$^5$U**AC-3'

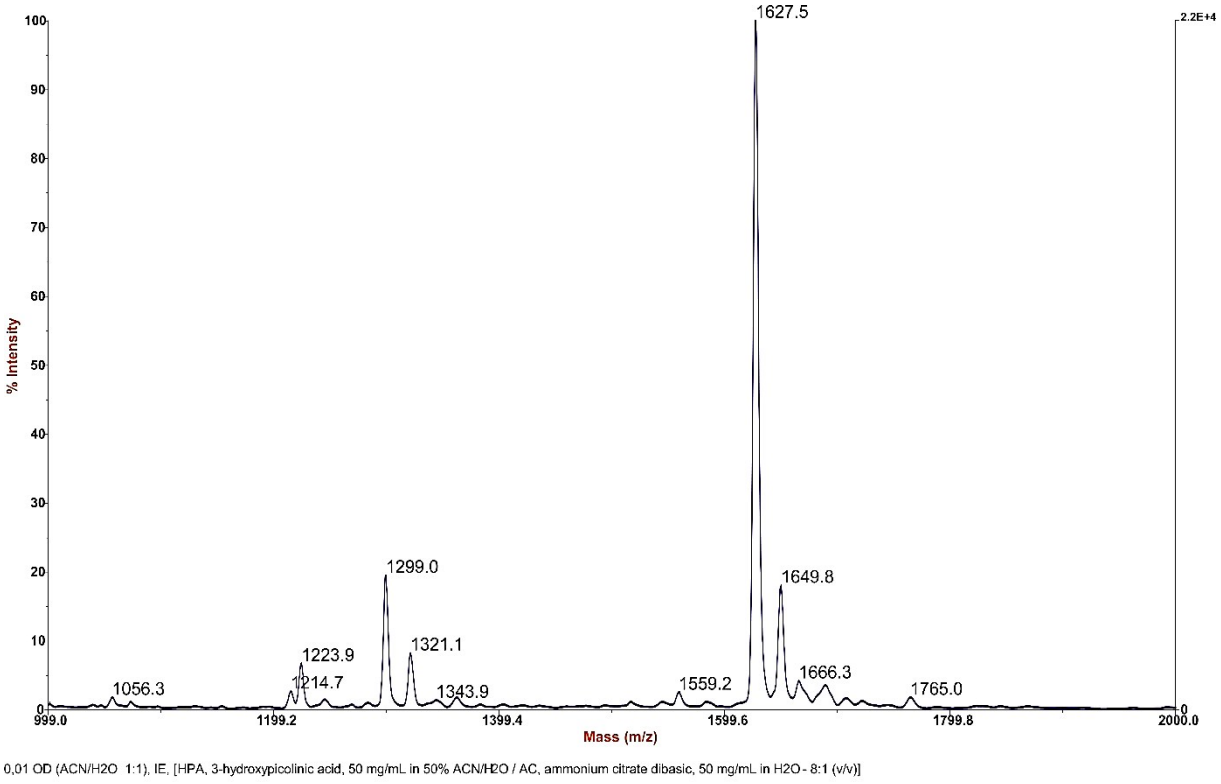



**Fig. S26.** The MALDI-TOF spectrum of 5'-GU**inm$^5$U**AC-3' (*m/z* calcd 1627, found 1628).

## f) Post-synthetic transformation of Pivom$^5$U → cnm$^5$U (Table 1, entry 6)

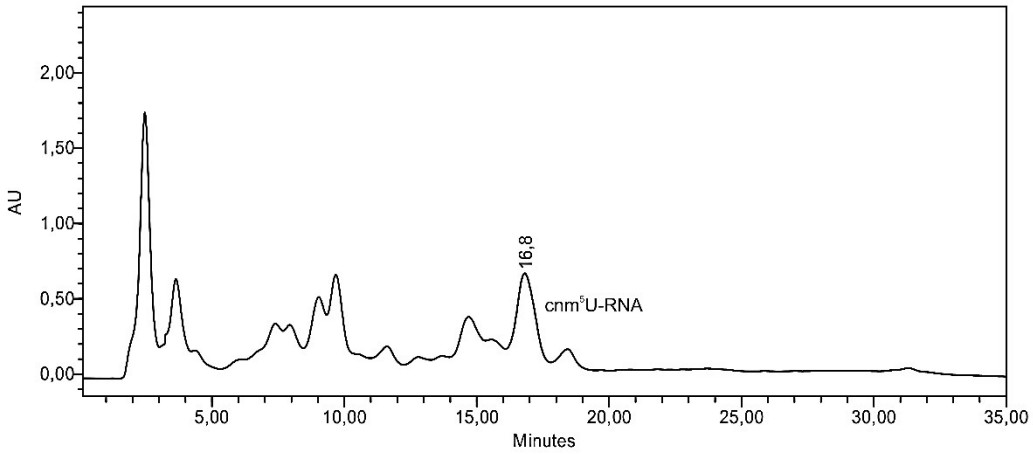



**Fig. S27.** IE-HPLC analysis of crude, deprotected 5'-GU**cnm$^5$U**AC-3'.

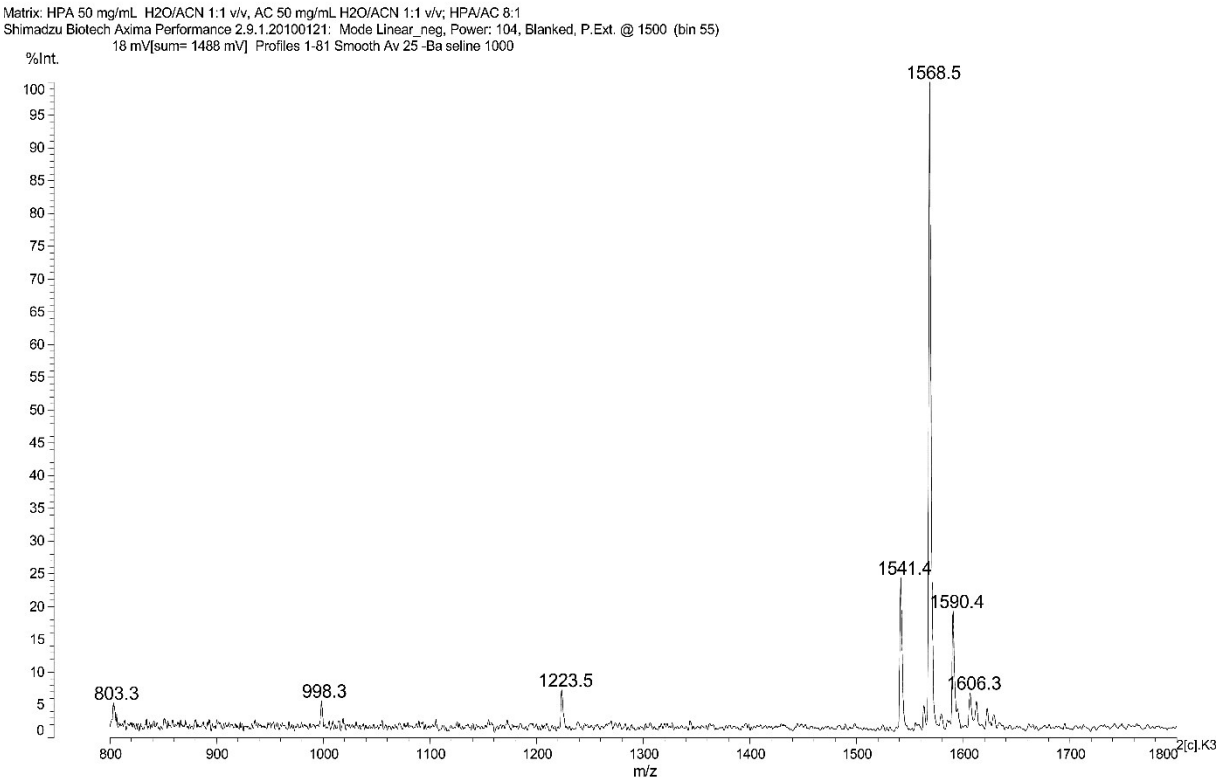



**Fig. S28.** The MALDI-TOF spectrum of 5'-GU**cnm$^5$U**AC-3' (*m/z* calcd 1569, found 1569).

## VII. Deprotection and characterization of Pivom$^5$U-RNA (17-mer; 5'-GUUGACU(Pivom$^5$U)UUAAUCAAC-3')

The "DMTr-off", CPG-linked Pivom$^5$U-RNA (0.1 μmol) was treated with $Et_3N$/acetonitrile mixture (136 μL, 1/1, v/v) for 20 min. The solution was removed and the support-bound RNA was washed with acetonitrile (3 x 100 μL), dried *in vacuo* for 30 min, and treated with 30% aq. $NH_3$ (150 μL) at rt for 3.5 h. The supernatant was removed and the support was washed with ethanol/water (3 x 150 μL, 1/1, v/v). The combined washings were evaporated on a Speed-Vac concentrator and the solid residue was treated with a solution of $Et_3N{\cdot}3HF$ in NMP (60 μL, 1/1, v/v) for 24 h at rt. The reaction was quenched by addition of 120 μL of ethoxytrimethylsilane and the crude RNA was precipitated using 300 μL of *tert*-butyl methyl ether. The fully deprotected RNA was purified by anion-exchange (IE) HPLC (Source 15Q 4.6/100PE) at constant flow rate of 1 mL/min. The column was eluted with a linear gradient 50 mM to 500 mM NaBr in 20 mM $Na_2HPO_4$-$NaH_2PO_4$ buffer solution pH 7.5, containing 50 μM EDTA and 10% ACN. Fractions containing the desired product (Pivom$^5$U-RNA, $R_t$ = 28.9 min., **Fig. S29**) was collected, concentrated, and desalted on a C-18 cartridge (Sep-Pak, Waters). The desalted Pivom$^5$U-RNA were lyophilized and analyzed by MALDI-TOF mass

spectrometry (Pivom$^5$U-RNA: *m/z* calcd 5447, found 5448; **Fig. S30)**. The total yield of Pivom$^5$U-incorporation into RNA chain was calculated as 81%.

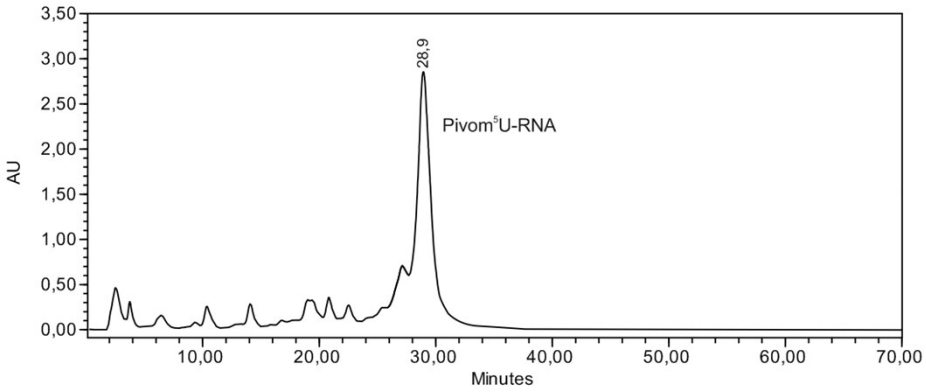



**Fig. S29.** IE-HPLC analysis of crude, deprotected 5'-GUUGACU**Pivom$^5$U**UUUAAUCAAC-3'.

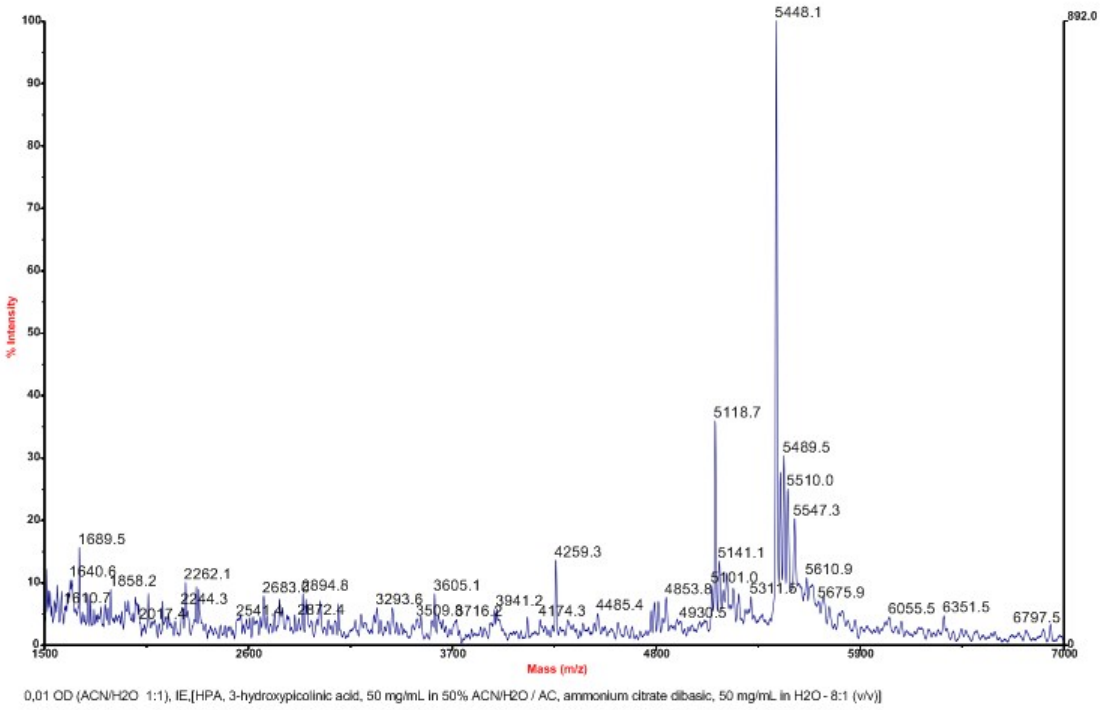



**Fig. S30.** The MALDI-TOF spectrum of 5'-GUUGACU**Pivom$^5$U**UUUAAUCAAC-3' (*m/z* calcd 5447, found 5448).

## VIII. IE-HPLC analysis and spectral identification of xm$^5$U-RNA (17-mers)

### a) Post-synthetic transformation of Pivom$^5$U-RNA → mnm$^5$U-RNA (Table 1, entry 7)

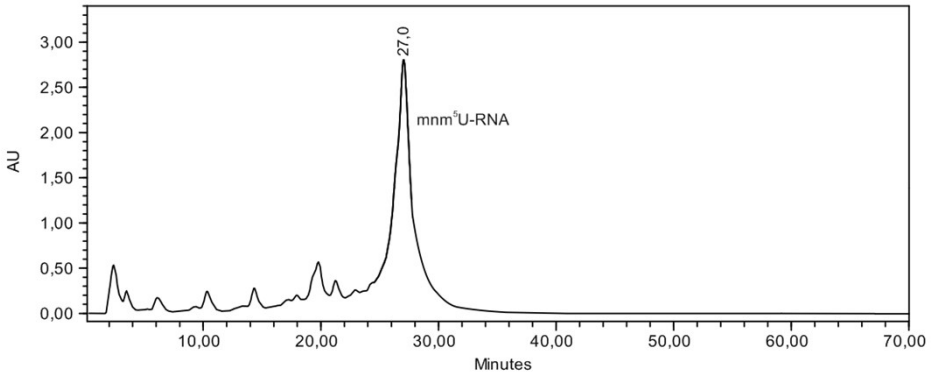



**Fig. S31.** IE-HPLC analysis of crude, deprotected 5'-GUUGACU**mnm$^5$U**UUUAAUCAAC-3'.

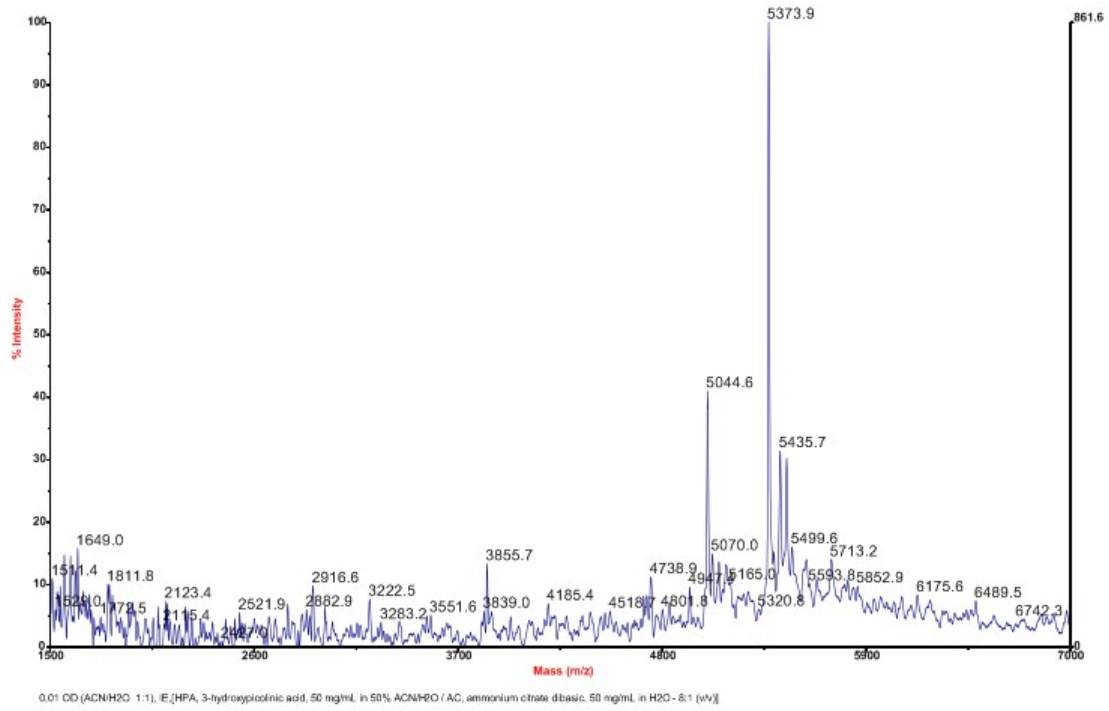



**Fig. S32.** The MALDI-TOF spectrum of 5'-GUUGACU**mnm$^5$U**UUUAAUCAAC-3' (*m/z* calcd 5376, found 5374).

**b) Post-synthetic transformation of Pivom$^5$U-RNA → τm$^5$U-RNA (Table 1, entry 8)**

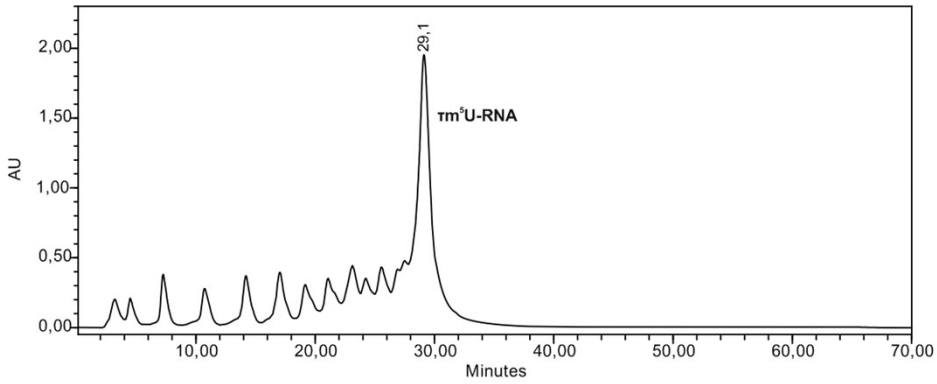



**Fig. S33.** IE-HPLC analysis of crude, deprotected 5'-GUUGACU**τm$^5$U**UUUAAUCAAC-3'.

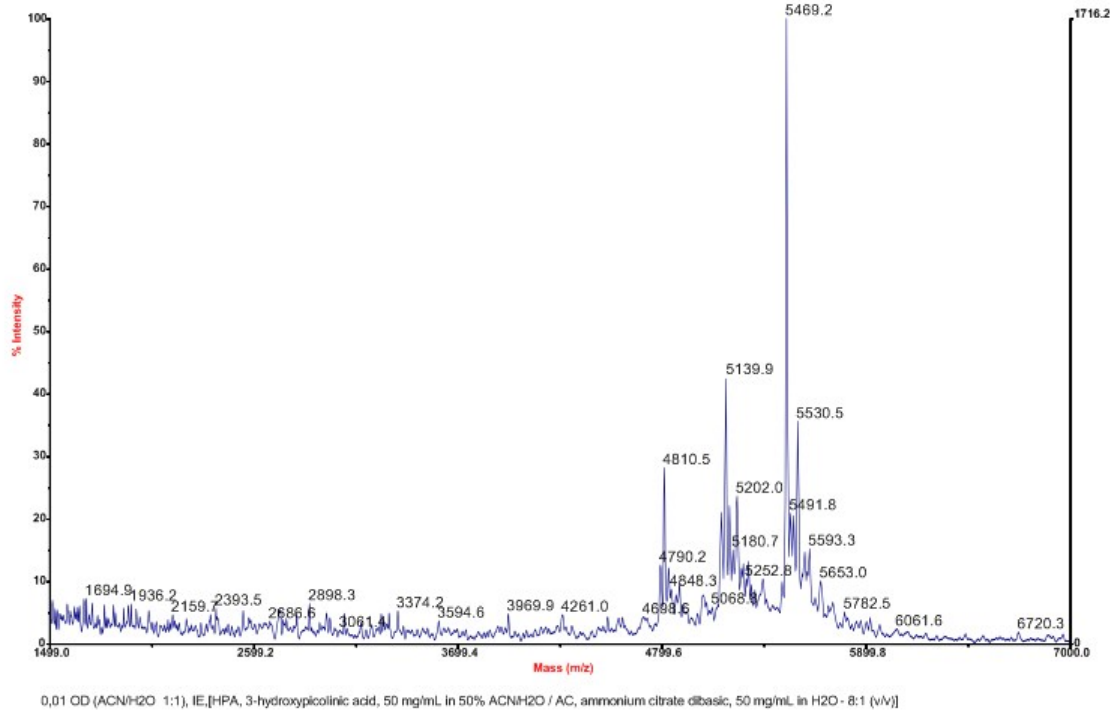



**Fig. S34.** The MALDI-TOF spectrum of 5'-GUUGACU**τm$^5$U**UUUAAUCAAC-3' (*m/z* calcd 5470, found 5469).