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 Et₃N (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 dissol vinyl 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; Rt = 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 Rf = 0.58 (iPrOH/ammonia/H₂O 8:1:1 v/v/v). NMR (δ [ppm], D₂O): 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); ¹³C (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⁺) calcld for C₁₅H₂₄N₃O₆ [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; Rt = 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 Rf = 0.36 (CHCl₃/MeOH 8:2 v/v). NMR (δ [ppm], DMSO-d₆): 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); 13C (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 C11H13N3O6 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 combined organic layers were dried over MgSO4, filtered and concentrated under reduced pressure. The crude product was purified on silica gel column with 4% MeOH in CHCl3 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 °C and neutralized with 0.5 M aqueous HCl (4 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried over MgSO4, filtered and concentrated under reduced pressure. The crude product 7 was purified on a silica gel column with 6% MeOH in CHCl3 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$_2$O)

![NMR spectrum of 5](image)

**Fig. S2.** $^{13}$C NMR spectrum of 5 (176 MHz, D$_2$O)

![NMR spectrum of 5](image)
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$_3$)$_2$CO)
**Fig. S11.** $^{31}$P NMR spectrum of 14 (101 MHz, (CD$_3$)$_2$CO)

![NMR spectrum](image)

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$_3$N/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$_3$N·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$_2$HPO$_4$-NaH$_2$PO$_4$ 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 $\rightarrow$ 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\textsuperscript{5}U-RNA and nm\textsuperscript{5}U-RNA were lyophilized and analyzed by MALDI-TOF mass spectrometry (Pivom\textsuperscript{5}U-RNA: \textit{m/z} calcd 1644, found 1642; Fig. S13; the spectral analyses of nm\textsuperscript{5}U-RNA was identical to that shown on page S11). The total yield of Pivom\textsuperscript{5}U-incorporation into RNA chain was calculated as 85%.

Fig. S12. IE-HPLC analysis of crude, deprotected Pivom\textsuperscript{5}U-RNA (5'-GUPivom\textsuperscript{5}UAC-3').

Fig. S13. The MALDI-TOF spectrum of 5'-GU\textsuperscript{5}UAC-3' (\textit{m/z} calcd 1644, found 1642).
VI.  IE-HPLC analysis and spectral identification of \textit{xm}^5\textit{U}-RNA (5-mers)

a)  Post-synthetic transformation of Pivom\textit{5}U-RNA $\rightarrow$ nm\textit{5}U-RNA (Table 1, entry 1)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure_s14}
\caption{IE-HPLC analysis of crude, deprotected nm\textit{5}U-RNA (5'-GUnm\textit{5}UAC-3').}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure_s15}
\caption{The MALDI-TOF spectrum of 5'-GUnm\textit{5}UAC-3' ($m/z$ calcd 1559, found 1558).}
\end{figure}
**Fig. S16.** RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of 5'-GU
\textit{nm}^5\textit{U}AC-3'. (B) RP-HPLC of the reference of \textit{nm}^3\textit{U} nucleoside.

\textbf{b) Post-synthetic transformation of Pivom^5U-RNA \rightarrow mnm^5U-RNA (Table 1, entry 2)}

**Fig. S17.** IE-HPLC analysis of crude, deprotected 5'-GU
\textit{nm}^5\textit{U}AC-3'.
Fig. S18. The MALDI-TOF spectrum of 5'-GU\textsubscript{cmnm}\textsuperscript{5}UAC-3' (m/z calcd 1573, found 1571).

c) Post-synthetic transformation of Pivom\textsuperscript{5}U-RNA → cmnm\textsuperscript{5}U-RNA (Table 1, entry 3)

Fig. S19. IE-HPLC analysis of crude, deprotected 5'-GUcmnm\textsuperscript{5}UAC-3'.
Fig. S20. The MALDI-TOF spectrum of 5'-GU\textsubscript{cmnm}UAC-3' (m/z calcd 1617, found 1616).

(A)

(B)

Fig. S21. RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of cmnm\textsubscript{U}-RNA. (B) RP-HPLC of the reference of cmnm\textsuperscript{2}U nucleoside.
d) Post-synthetic transformation of Pivom$^5$U-RNA $\rightarrow$ $\tau m^5$U-RNA (Table 1, entry 4)

Fig. S22. IE-HPLC analysis of crude, deprotected 5'-GU$\tau m^5$UAC-3'.

Fig. S23. The MALDI-TOF spectrum of 5'-GU$\tau m^5$UAC-3' ($m/z$ calcd 1667, found 1667).
Fig. S24. RNA enzymatic digestion. (A) RP-HPLC analysis of nucleoside composition of \( \tau m^5 U \)-RNA. (B) RP-HPLC of the reference of \( \tau m^5 U \) nucleoside.

e) Post-synthetic transformation of Pivom\(^5\)U-RNA \( \rightarrow \) inm\(^5\)U-RNA (Table 1, entry 5)

Fig. S25. IE-HPLC analysis of crude, deprotected 5'-GUinm\(^5\)UAC-3'
Fig. S26. The MALDI-TOF spectrum of 5'-GU\textsuperscript{mnm}UAC-3' (m/z calcd 1627, found 1628).

\textbf{f) Post-synthetic transformation of Pivom\textsuperscript{5}U → cnm\textsuperscript{5}U (Table 1, entry 6)}

Fig. S27. IE-HPLC analysis of crude, deprotected 5'-GUcnm\textsuperscript{5}UAC-3'.
Fig. S28. The MALDI-TOF spectrum of 5'-GUcnm$^5$UAC-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$_3$N/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$_3$N·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$_2$HPO$_4$-NaH$_2$PO$_4$ 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 (Pivom₅U-RNA: \(m/z\) calcd 5447, found 5448; **Fig. S30**). The total yield of Pivom₅U-incorporation into RNA chain was calculated as 81%.

**Fig. S29.** IE-HPLC analysis of crude, deprotected 5'-GUUGACUPivom₅UUAAUCAAC-3'.

**Fig. S30.** The MALDI-TOF spectrum of 5'-GUUGACUPivom₅UUAAUCAAC-3' \((m/z\) calcd 5447, found 5448).
VIII. IE-HPLC analysis and spectral identification of \( \text{xm}^5\text{U-RNA} \) (17-mers)

a) Post-synthetic transformation of Pivom\( ^5\text{U-RNA} \rightarrow \text{mn}m^5\text{U-RNA} \) (Table 1, entry 7)

![IE-HPLC analysis of crude, deprotected 5'-GUUGACUmn5UUUUAAUCAAC-3'].

Fig. S31. IE-HPLC analysis of crude, deprotected 5'-GUUGACUmn5UUUUAAUCAAC-3'.

![The MALDI-TOF spectrum of 5'-GUUGACUmn5UUUUAAUCAAC-3' (m/z calcd 5376, found 5374).]

Fig. S32. The MALDI-TOF spectrum of 5'-GUUGACUmn5UUUUAAUCAAC-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$^5$UUAAUCAAC-3'.

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