

Supplementary Information

for manuscript entitled

"Enzyme-free Ligation of Dimers and Trimers to RNA Primers"

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1. Materials and Methods

The following is an expanded version of the corresponding section of the main manuscript, focused on analytical methods.

NMR Spectroscopy. NMR-spectra were acquired on Avance 700 or 400 spectrometers (Bruker, Rheinstetten, Germany) in D₂O at 700 MHz for ¹H-NMR spectra and 284 MHz or 161 MHz for ³¹P-NMR spectra. Chemical shifts are given in δ values (ppm) that were relative to the peak of the residual solvent and coupling constants (J) are given in Hertz. Multiplicities in signals are given as: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet.

UV-Vis Spectroscopy. Samples were analyzed by UV-Vis spectrometer NanoDrop 1000 or NanoDrop 2000 from PeqLab. Samples were diluted in demineralized water such as their absorbance at 260 nm for 1 mm path length was in the range 0.1-1.0. The concentration of oligonucleotides and monomers were determined using molar extinction coefficients delivered by IDT or by the manufacturer with at least 3 measurements per sample.

Mass Spectrometry. MALDI-TOF mass spectra were measured on Microflex MALDI-TOF spectrometer (Bruker, Billerica, MA) with a N₂ laser (337 nm), using the Flex Control software in linear negative mode. A MSP 96 ground steel target was used to place samples for MALDI-TOF analysis. For samples with a mass higher than 1000 Da, the laser was set at 70% of its maximal intensity. One spectrum was the sum of 200 laser shots at 1.5 Hz frequency. For target compounds with a mass lower than 1000 Da, laser intensity was dropped to 50%, with a frequency of 2 Hz and accumulation 150 shots. Aliquots (0.7 μ L) were desalted with the ammonium form of cation exchange resin Dowex 50 WX8-200 in 25 μ L volume for 20 min. Sample of 0.5 μ L were dried on the MALDI target, and a mixture (0.4 μ L) of matrix and co-matrix solutions (0.3 M trihydroxyacetophenone in EtOH, and 0.1 M aqueous triammonium citrate; 2/1, v/v) was placed on the spot. The matrix has a pH value of 7 to suppress the cleavage of the phosphoramidate backbone in oligonucleotides. An external calibration was done with a mixture of oligonucleotides with known masses and the analysis of the spectra was done with FlexAnalysis, version 3.4 (Bruker). Analysis was performed on the [M-H]⁻ ions.

2. Yields and Analytical Data for Dimers and Trimers

Dimer 1aa; yield 0.6 μmol , 28%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: δ (ppm) = 8.24 (s, A1H8, 1H), 8.18 (s, A2H2, 1H), 8.06 (s, A1H2, 1H), 7.90 (s, A2H2, 1H), 5.88 (d, $J = 4.0$ Hz, A1H1', 1H), 5.79 (d, $J = 3.9$ Hz, A2H1', 1H), 4.58 (dd, $J = 4.3$ Hz, A2H2', 1H), 4.40 (dd, $J = 4.8$ Hz, A1H2', 1H), 4.36 (dd, $J = 5.2$ Hz, A2H3', 1H), 4.36-4.35 (m, A1H4', 1H), 4.24-4.22 (m, A2H4', A1H3', 2H), 4.08-3.99 (m, A1H5', A1H5'', A2H5', A2H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 0.2 (P1), -0.9 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{20}\text{H}_{25}\text{N}_{10}\text{O}_{13}\text{P}_2$ $[\text{M-H}]^-$: 675, found 675.

Dimer 1ca; yield 0.7 μmol , 33%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: δ (ppm) = 8.37 (s, A2H8, 1H), 8.15 (s, A2H2, 1H), 7.85 (d, $J = 7.0$ Hz, C1H6, 1H), 6.00 (d, $J = 4.9$ Hz, A2H1', 1H), 5.95 (d, $J = 7.0$ Hz, C1H5) 5.70 (d, $J = 4.4$ Hz, C1H1', 1H), 4.56 (dd, $J = 5.0$ Hz, A2H2', 1H), 4.45 (td, $J = 5.0$ Hz, $J = 8.0$ Hz, C1H3', 1H), 4.40 (dd, $J = 4.7$ Hz, A2H3', 1H), 4.25-4.23 (m, A2H4', C1H4', 2H), 4.20 (dd, $J = 4.7$ Hz, C1H2', 1H), 4.13-4.01 (m, A2H5', A2H5'', 2H), 4.01-3.92 (m, C1H5'-H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 0.0 (P1), -0.8 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{19}\text{H}_{25}\text{N}_8\text{O}_{14}\text{P}_2$, $[\text{M-H}]^-$: 651, found 651.

Dimer 1ga, yield 0.7 μmol , 33%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: δ (ppm) = 8.31 (s, A2H8, 1H), 8.14 (s, A2H2, 1H), 7.93 (s, G1H8, 1H), 6.01 (d, $J = 4.5$ Hz, A2H1', 1H), 5.64 (d, $J = 4.8$ Hz, G1H1', 1H), 4.58 (m, G1H2', A2H2', 2H), 4.44 (dd, $J = 5.0$, A2H3', 1H), 4.35-4.33 (m, G1H4', 1H), 4.30-4.29 (m, A2H4', 1H), 4.25-4.08 (m, A2H5', A2H5'', 2H), 4.06-3.99 (m, G1H5', G1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 0.2 (P1), -0.8 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{20}\text{H}_{25}\text{N}_{10}\text{O}_{14}\text{P}_2$, $[\text{M-H}]^-$: 691, found 691.

Dimer 1ua, yield 1.0 μmol , 50%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: δ (ppm) = 8.46 (s, A2H8, 1H), 8.27 (s, A2H2, 1H), 7.78 (d, $J = 8.2$ Hz, U1H6, 1H), 6.07 (d, $J = 5.0$ Hz, A2H1', 1H), 5.79 (d, $J = 8.2$ Hz, U1H5, 1H), 5.76 (d, $J = 5.7$ Hz, U1H1', 1H), 4.69 (dd, $J = 4.8$ Hz, A2H2', 1H), 4.51 (td, $J = 3.8$ Hz, $J = 7.5$ Hz, U1H3', 1H), 4.47 (dd, $J = 4.8$ Hz, A2H3', 1H), 4.30 (m, A2H4', 1H), 4.26 (td, $J = 2.5$ Hz, $J = 6.1$ Hz, U1H4', 1H), 4.24 (dd, $J = 5.0$ Hz, U1H2', 1H), 4.17-4.06 (m, A2H5', A2H5'', 2H), 3.96 (m, U1H5', U1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 0.0 (P1), -0.8 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{19}\text{H}_{24}\text{N}_7\text{O}_{15}\text{P}_2$, $[\text{M-H}]^-$: 652, found 651.

Dimer 1ac, yield 0.8 μmol , 40%; **$^1\text{H NMR}$ (700 MHz, D_2O)**: δ (ppm) = 8.39 (s, A1H8, 1H), 8.12 (s, A1H2, 1H), 7.76 (d, $J = 7.8$ Hz, C2H6, 1H), 5.96 (d, $J = 3.1$ Hz, A1H1', 1H), 5.83 (d, $J = 7.8$ Hz, C2H5, 1H) 5.57 (d, $J = 2.5$ Hz, C2H1', 1H), 4.75 (dd, $J = 3.2$ Hz, $J = 4.6$ Hz, A1H2', 1H), 4.44 (m, A1H4', 1H), 4.25-4.23 (m, C2H5', 1H), 4.22-4.19 (m, A1H5', 1H), 4.14-4.11 (m, C2H4', C2H3', 2H), 4.07-4.03 (m, A1H5'', C2H2', 2H), 4.02-3.99 (m, C2H5'', 1H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 0.0 (P1), -1.1 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{19}\text{H}_{25}\text{N}_8\text{O}_{14}\text{P}_2$, $[\text{M-H}]^-$: 651, found 651.

Dimer 1cc, yield 0.8 μmol , 40%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: δ (ppm) = 7.97 (d, $J = 7.6$ Hz, C1H6, 1H), 7.86 (d, $J = 7.6$ Hz, C2H6, 1H), 5.97 (d, $J = 7.6$ Hz, C1H5, 1H), 5.96 (d, $J = 7.6$ Hz, C2H5, 1H), 5.84 (d, $J = 3.3$ Hz, C2H1', 1H), 5.78 (d, $J = 3.0$ Hz, C1H1', 1H), 4.51-4.49 (m, C1H3', 1H), 4.38 (dd, $J = 3.2$ Hz, $J = 4.4$ Hz, C1H2', 1H), 4.36 (m, C1H4', 1H), 4.24 (dd, $J = 5.0$ Hz, $J = 6.4$ Hz, C2H3', 1H), 4.23 (m, C2H4', 1H) 4.19-4.18 (m, C2H5', C1H5', 2H), 4.12 (dd, $J = 3.4$ Hz, $J = 4.6$ Hz, C2H2', 1H), 4.06-4.01 (m, C2H5'', C1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: δ (ppm) = 1.0 (P1), -1.0 (P2); **MALDI-TOF MS** m/z : calculated for $\text{C}_{18}\text{H}_{25}\text{N}_6\text{O}_{15}\text{P}_2$, $[\text{M-H}]^-$: 627, found 627.

Dimer 1gc, yield 0.7 μmol , 35%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 8.18$ (s, G1H8, 1H), 7.75 (d, $J = 7.4$ Hz, C2H6, 1H), 5.81 (d, $J = 4.8$ Hz, C2H1', 1H), 5.79 (d, $J = 6.2$ Hz, G1H1', 1H), 5.73 (d, $J = 7.4$ Hz, C2H5, 1H), 4.75 (dd, $J = 4.3$ Hz, G1H2', 1H), 4.67 (m, G1H3', 1H), 4.42 (m, G1H4', 1H), 4.31-4.04 (m, C2H5' C2H5'', 2H), 4.22 (dd, $J = 6.1$ Hz, C2H3', 1H), 4.18-4.17 (dd, $J = 3.7$ Hz, C2H4', 1H), 4.11 (dd, $J = 3.7$ Hz, C2H2', 1H) 4.06-3.95 (m, G1H5'', G1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.0$ (P1), -1.0 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{19}\text{H}_{25}\text{N}_8\text{O}_{15}\text{P}_2$, $[\text{M-H}]^-$: 667, found 667.

Dimer 1uc, yield 0.9 μmol , 45%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 8.00$ (d, $J = 7.9$ Hz, C2H6, 1H), 7.85 (d, $J = 8.2$ Hz, U1H6, 1H), 6.15 (d, $J = 7.9$ Hz, C2H5, 1H), 5.82 (d, $J = 5.2$ Hz, U1H1', 1H), 5.80 (d, $J = 3.1$ Hz, C2H1', 1H) 5.80 (d, $J = 8.2$ Hz, U1H5, 1H), 4.50 (td, $J = 4.6$, $J = 8.3$ Hz, U1H3', 1H), 4.34 (m, U1H4', 1H), 4.32 (dd, $J = 4.6$ Hz, U1H2', 1H), 4.19 (dd, $J = 5.7$ Hz, C2H2', 1H), 4.19 (dd, $J = 5.1$, C2H3', 1H) 4.18-4.15 (m, C2H4', C2H5', 2H), 4.08-3.97 (m, U1H5', U1H5'', 2H), 4.01-3.97 (m, C2H5', C2H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.0$ (P1), -0.9 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{18}\text{H}_{24}\text{N}_5\text{O}_{16}\text{P}_2$, $[\text{M-H}]^-$: 628, found 628.

Dimer 1ag, yield 0.7 μmol , 35%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 8.46$ (s, A1H8, 1H), 8.23 (s, A1H2, 1H), 8.00 (s, G2H8, 1H), 6.04 (d, $J = 4.3$ Hz, A1H1', 1H), 5.84 (d, $J = 5.1$ Hz, G2H1', 1H), 4.71 (dd, $J = 5.2$ Hz, G2H2', 1H) 4.50 (m, G2H3', A1H4', 2H), 4.36-4.34 (m, G2H4', 1H), 4.33-4.18 (m, G2H5', G2H5'', 2H), 4.18-4.11 (m, A1H5', A1H5'', 2H), **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.0$ (P1), -0.8 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{20}\text{H}_{25}\text{N}_{10}\text{O}_{14}\text{P}_2$, $[\text{M-H}]^-$: 691, found 690.

Dimer 1cg, yield 0.7 μmol , 33%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 7.87$ (s, G2H8, 1H), 7.82 (d, $J = 7.8$ Hz, C1H6, 1H), 5.94 (d, $J = 7.8$ Hz, C1H5, 1H), 5.73 (d, $J = 5.6$ Hz, G2H1', 1H), 5.68 (d, $J = 5.6$ Hz, C1H1', 1H), 4.44 (td, $J = 5.1$, $J = 8.0$ Hz, C1H3', 1H) 4.34 (dd, $J = 4.0$ Hz, G2H3', 1H), 4.20 (m, C1H4', 1H), 4.18 (dd, $J = 4.6$ Hz, G2H2', 1H), 4.18-4.16 (m, G2H4', 1H), 4.08-3.99 (m, G2H5', G2H5'', 2H), 3.93-3.87 (m, C1H5', C1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.0$ (P1), -0.7 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{19}\text{H}_{25}\text{N}_8\text{O}_{15}\text{P}_2$, $[\text{M-H}]^-$: 667, found 666.

Dimer 1gg, yield 0.6 μmol , 28%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 7.94$ (s, G1H8, 1H), 7.90 (s, G2H8, 1H), 5.78 (d, $J = 5.6$ Hz, G2H1', 1H), 5.73 (d, $J = 5.6$ Hz, G1H1', 1H), 4.72-4.70 (m, G1H3', 1H), 4.65-4.63 (m, G1H2', 1H) 4.41 (dd, $J = 4.2$ Hz, $J = 5.0$ Hz, G2H3', 1H), 4.34-4.33 (m, G1H4', 1H), 4.26-4.24 (m, G2H4', 1H), 4.18-4.08 (m, G2H5', G2H5'', 2H), 4.00-3.94 (m, G1H5', G1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.3$ (P1), -0.7 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{20}\text{H}_{25}\text{N}_{10}\text{O}_{15}\text{P}_2$, $[\text{M-H}]^-$: 707, found 707.

Dimer 1ug, yield 0.8 μmol , 43%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 8.04$ (s, G2H8, 1H), 7.73 (d, $J = 8.1$ Hz, U1H6, 1H), 5.79 (d, $J = 5.3$ Hz, G2H1', 1H), 5.74 (d, $J = 8.2$ Hz, U2H5, 1H), 5.73 (d, $J = 5.5$ Hz, U1H1', 1H), 4.59 (dd, $J = 5.6$ Hz, U1H2', 1H), 4.48 (td, $J = 4.6$, $J = 8.6$ Hz, U1H3', 1H) 4.38 (dd, $J = 4.7$ Hz, G1H3', 1H), 4.22-4.20 (m, G2H4', U1H4', G2H3', 3H), 4.10-4.02 (m, G2H5', G2H5'', 2H), 3.90-3.87 (m, U1H5', U1H5'', 2H); **$^{31}\text{P-NMR}$ (284 MHz, D_2O)**: $\delta(\text{ppm}) = 0.0$ (P1), -0.7 (P2); **MALDI-TOF MS m/z** : calculated for $\text{C}_{19}\text{H}_{24}\text{N}_7\text{O}_{16}\text{P}_2$, $[\text{M-H}]^-$: 668, found 667.

Dimer 1au, yield 0.8 μmol , 38%; **$^1\text{H-NMR}$ (700 MHz, D_2O)**: $\delta(\text{ppm}) = 8.52$ (s, A1H8, 1H), 8.30 (s, A1H2, 1H), 7.77 (d, $J = 8.1$ Hz, U2H6, 1H), 6.14 (d, $J = 4.1$ Hz, U2H1', 1H), 5.80 (d, $J = 3.9$ Hz, A1H1', 1H), 5.69 (d, $J = 8.1$ Hz, U2H5, 1H), 4.84 (dd, $J = 4.5$ Hz, U2H2', 1H), 4.76 (td, $J = 5.1$ Hz, $J = 8.4$ Hz, A1H3', 1H), 4.57 (m, A1H4', 1H), 4.32-4.23 (m, U2H5', U2H2', U2H3', U2H4', A1H5', 5H),

4.17-4.12 (m, A1H5", U2H5", 1H); ³¹P-NMR (284 MHz, D₂O): δ(ppm) = 0.3 (P1), -0.8 (P2); MALDI-TOF MS *m/z*: calculated for C₁₉H₂₄N₇O₁₅P₂, [M-H]⁻ : 652, found 651.

Dimer 1cu, yield 0.8 μmol, 40%; ¹H-NMR (700 MHz, D₂O): δ(ppm) = 8.10 (d, *J* = 7.8 Hz, C1H6, 1H), 7.85 (d, *J* = 8.1 Hz, U2H6, 1H), 6.17 (d, *J* = 7.84 Hz, C1H5, 1H), 5.90 (d, *J* = 4.1 Hz, U2H1', 1H), 5.88 (d, *J* = 4.1 Hz, C1H1', 1H), 5.85 (d, *J* = 8.1 Hz, U2H5, 1H), 4.58 (td, *J* = 5.2 Hz, *J* = 8.1 Hz, C1H3', 1H), 4.44-4.43 (m, C1H4', 1H), 4.40-4.39 (dd, *J* = 4.5 Hz, U2H2', 1H), 4.28-4.26 (m, C1H2', U2H3', 2H), 4.21-4.05 (m, U2H4', U2H5', U2H5", C1H5', C1H5", 5H); ³¹P-NMR (284 MHz, D₂O): δ(ppm) = 0.0 (P1), -0.8 (P2); MALDI-TOF MS *m/z*: calculated for C₁₈H₂₄N₅O₁₆P₂, [M-H]⁻ : 628, found 628.

Dimer 1gu, yield 0.9 μmol, 45%; ¹H-NMR (700 MHz, D₂O): δ(ppm) = 8.06 (s, G1H8, 1H), 7.71 (d, *J* = 8.0 Hz, U2H6, 1H), 5.81 (d, *J* = 4.8 Hz, U2H1', 1H), 5.79 (d, *J* = 6.2 Hz, G1H1', 1H), 5.66 (d, *J* = 8.0 Hz, U2H5), 4.73 (dd, *J* = 5.3 Hz, G1H2', 1H), 4.34 (m, G1H4', 1H), 4.21 (dd, *J* = 5.0 Hz, U2H3', 1H), 4.16 (dd, *J* = 5.1 Hz, U2H2'), 4.13-3.99 (m, U2H4', U2H5', U2H5", 3H), 3.89-3.81 (m, G1H5', G1H5", 2H); ³¹P-NMR (284 MHz, D₂O): δ(ppm) = 3.4 (P1), -0.5 (P2); MALDI-TOF MS *m/z*: calculated for C₁₉H₂₄N₇O₁₆P₂, [M-H]⁻ : 668, found 668.

Dimer 1uu, yield 0.5 μmol, 25%; ¹H-NMR (700 MHz, D₂O): δ(ppm) = 7.95 (d, *J* = 7.8 Hz, U2H6, 1H), 7.89 (d, *J* = 7.7 Hz, U1H6, 1H), 5.96 (d, *J* = 5.3 Hz, U2H1', 1H), 5.95 (d, *J* = 4.0 Hz, U1H1', 1H), 5.92 (d, *J* = 7.8 Hz, U2H5, 1H), 5.91 (d, *J* = 7.7 Hz, U1H5, 1H), 4.65-4.63 (m, U1H3', 1H), 4.49-4.46 (m, U1H4', 1H), 4.44 (dd, *J* = 5.3 Hz, U1H2', 1H), 4.34-4.32 (m, U2H2', U2H3', 2H), 4.26-4.07 (m, U2H4', U2H5', U2H5", U1H5', U1H5", 5H); ³¹P-NMR (284 MHz, D₂O): δ(ppm) = 0.1 (P1), -0.7 (P2); MALDI-TOF MS *m/z*: calculated for C₁₈H₂₃N₄O₁₇P₂, [M-H]⁻ : 629, found 629.

Trimer 2aaa, yield 1.4 μmol, 14%; ¹H-NMR (700 MHz, D₂O): δ(ppm) = 8.33 (s, 1H), 8.19 (s, 1H), 8.14 (s, 1H), 8.10 (s, 1H), 7.99 (s, 1H), 7.88 (s, 1H), 5.94 (d, *J* = 3.86 Hz, A3H1', 1H), 5.89 (d, *J* = 3.28 Hz, A1H1', 1H), 5.82 (d, *J* = 2.97 Hz, A2H1', 1H), 4.80 (m, A1H2', A1H3', 2H), 4.74 (m, A2H3', 1H), 4.54 (t, *J* = 3.78 Hz, A2H2', 1H), 4.50 (m, A1H4', A2H4', 2H), 4.44 (t, *J* = 5.11 Hz, A3H3', 1H), 4.40 (m, A3H2', A2H5', 2H), 4.36-4.34 (m, A3H5', A3H4', 2H), 4.23 (m, A1H3', A2H5', 2H), 4.16-4.15 (m, A3H5', A1H5', 2H); ³¹P-NMR (284 MHz, D₂O): δ(ppm) = 0.4 (P1), -0.9 (P2); MALDI-TOF MS *m/z*: calculated for C₃₀H₃₇N₁₅O₁₉P₃, [M-H]⁻ : 1004, found 1003.

Trimer 2ccc, yield 0.5 μmol, 25%; MALDI-TOF MS *m/z*: calculated for C₂₇H₃₇N₉O₂₂P₃, [M-H]⁻ : 932, found 932. This compound was prepared on too small a scale to allow for characterization by NMR.

NMR spectra

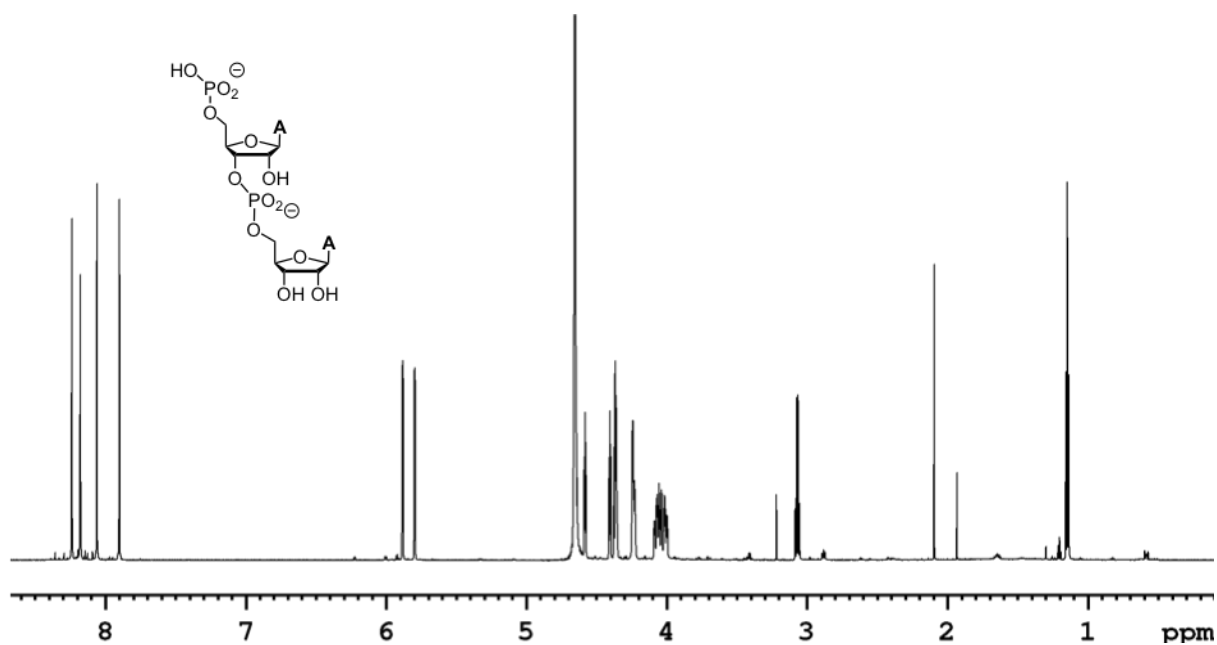


Figure S1. ¹H-NMR spectra (700 MHz, D₂O) of dimer 1aa

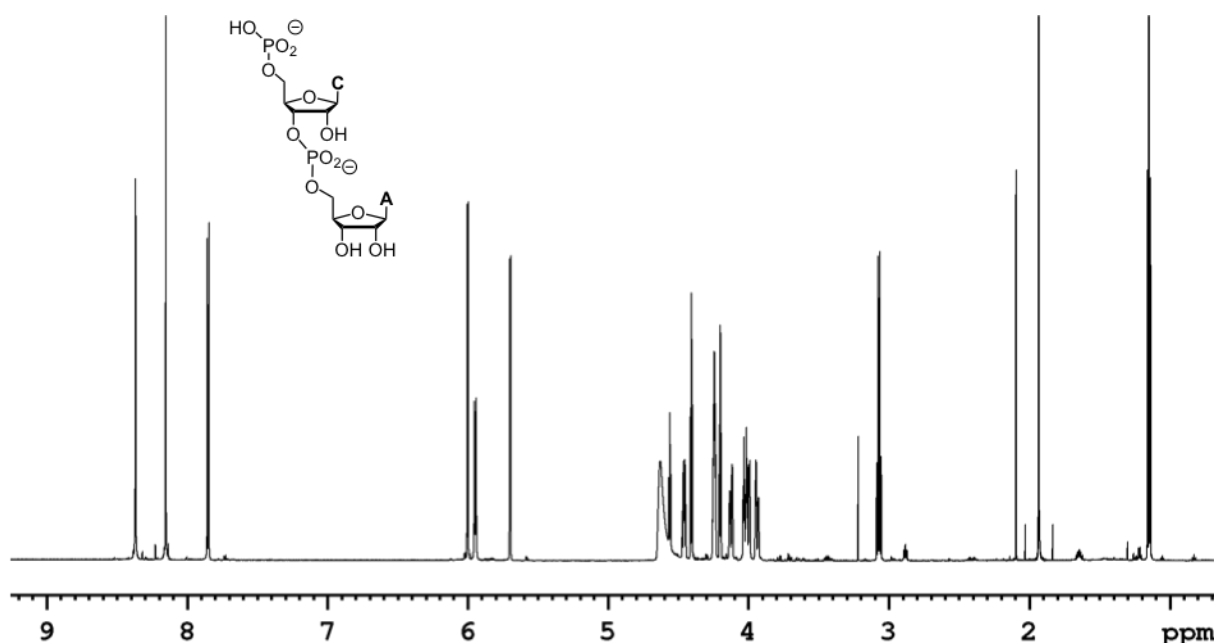


Figure S2. ¹H-NMR spectra (700 MHz, D₂O) of dimer 1ca

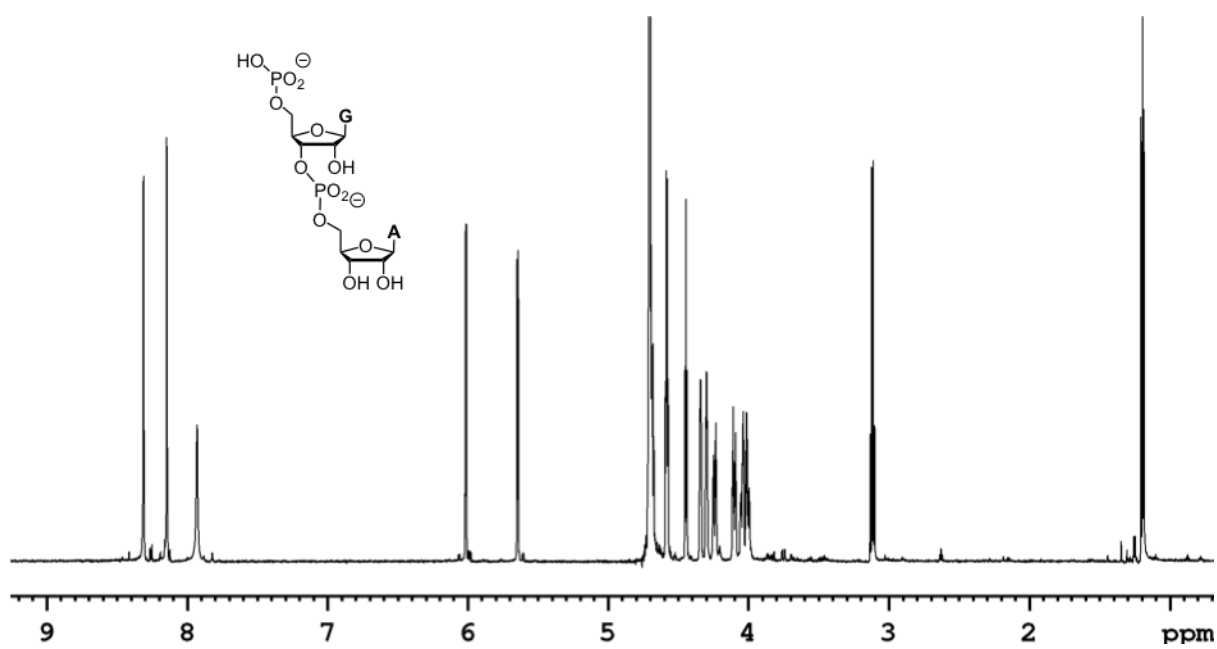


Figure S3. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1ga**

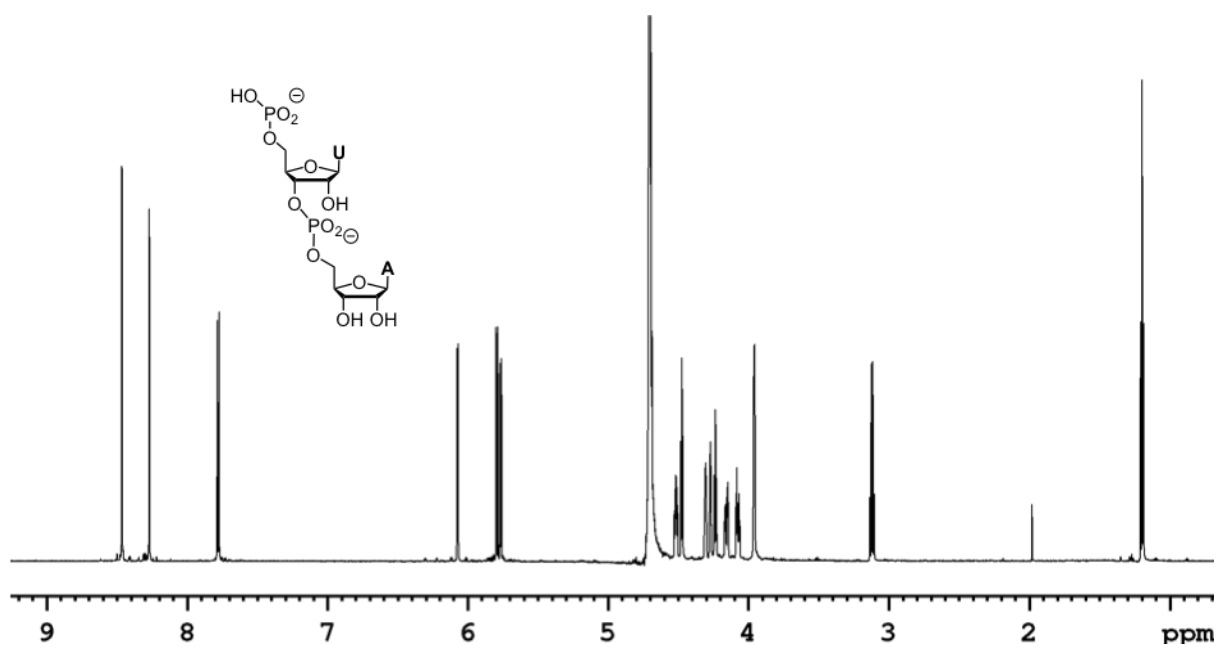


Figure S4. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1ua**

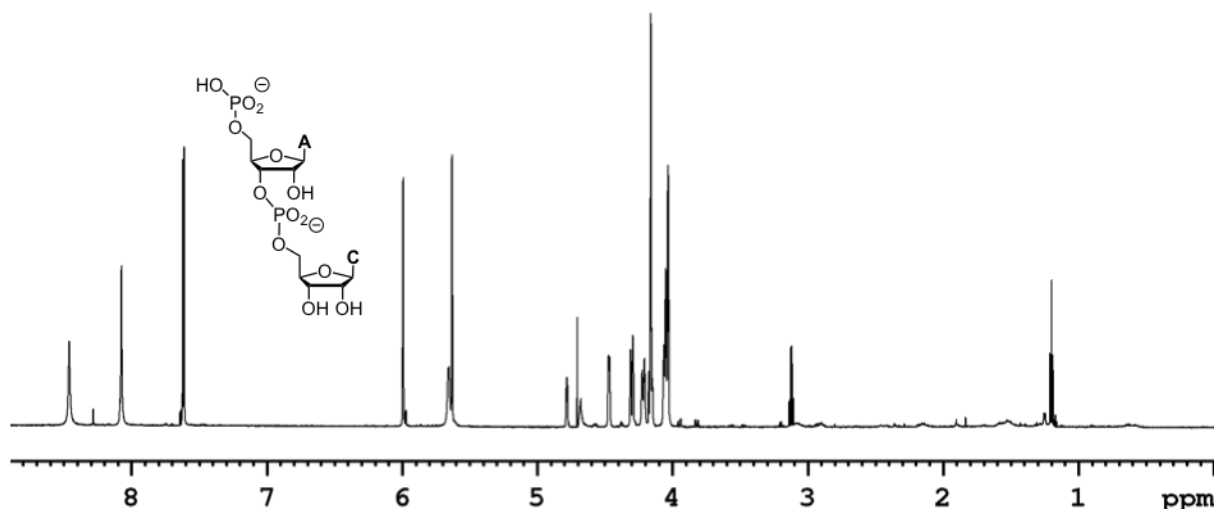


Figure S5. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1ac**

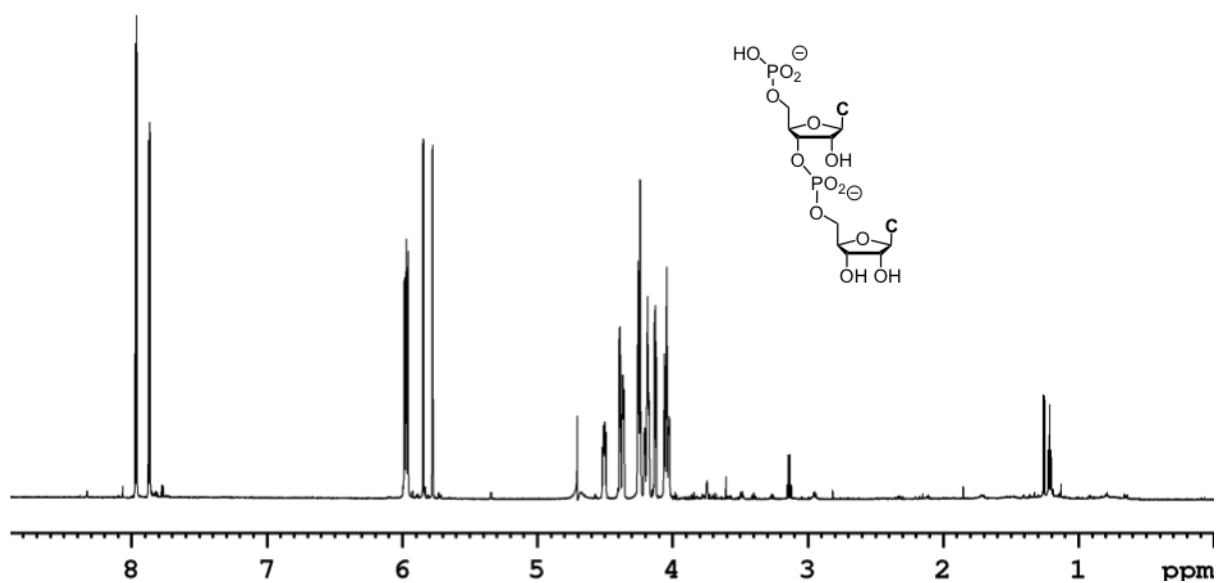


Figure S6. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1cc**

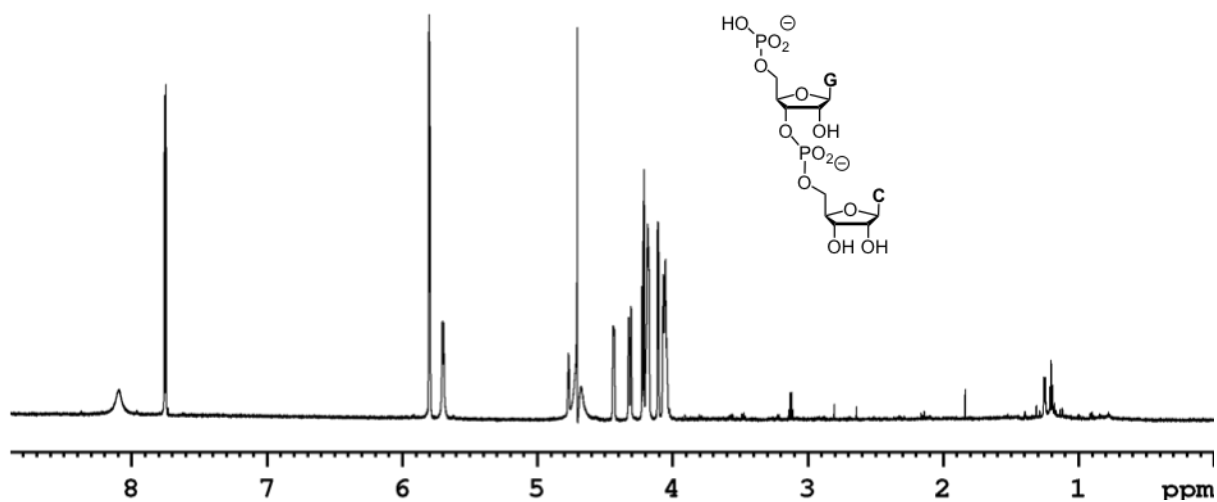


Figure S7. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1gc**

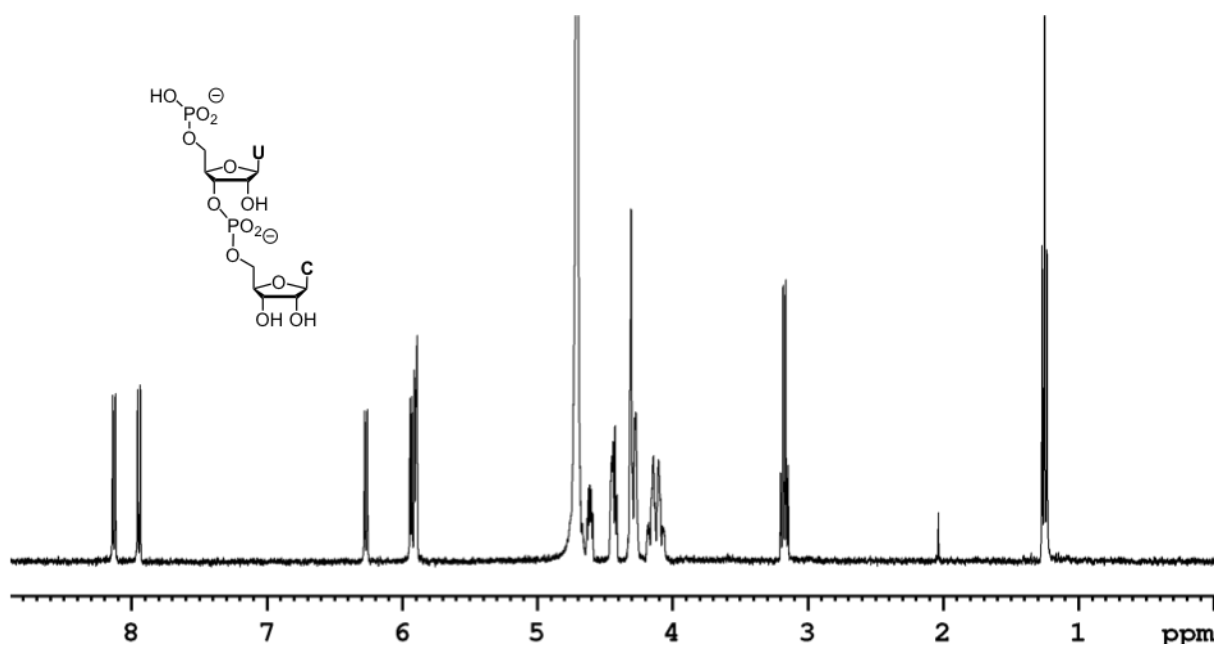


Figure S8. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1uc**

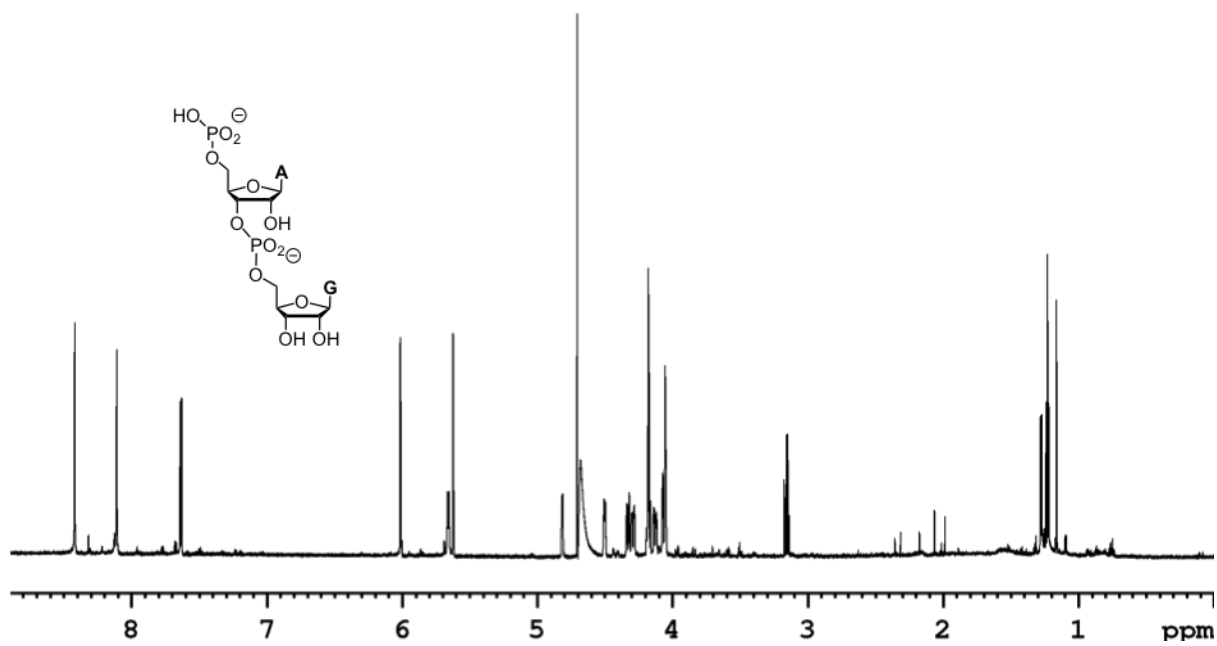


Figure S9. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1ag**

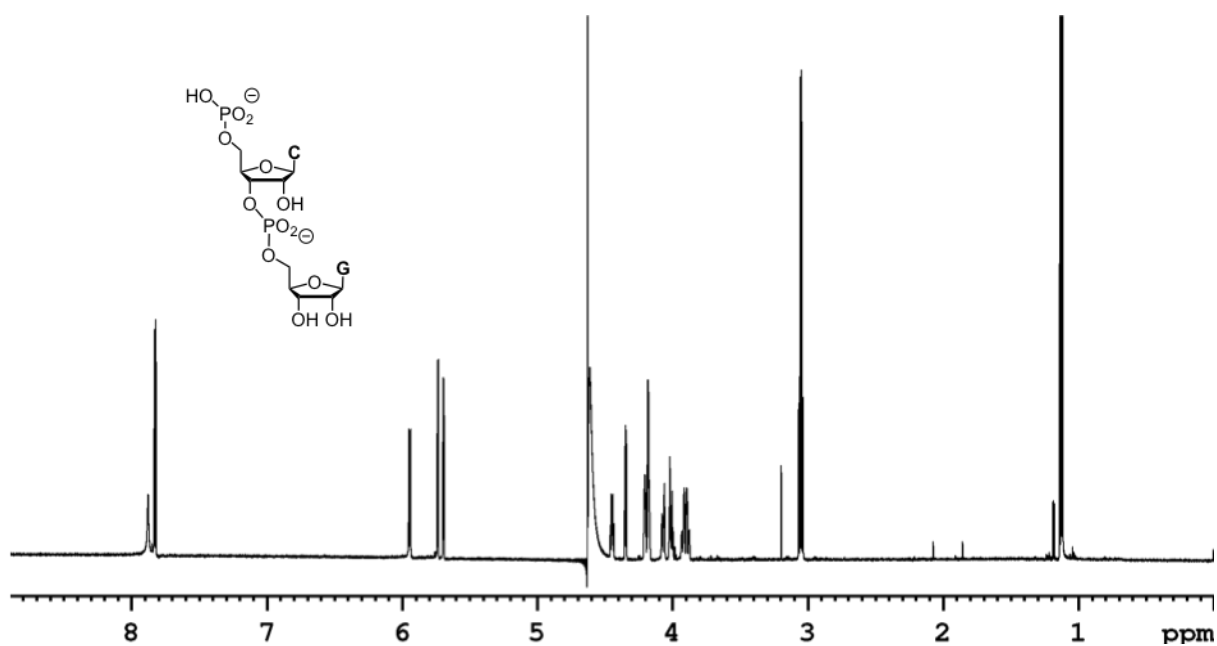


Figure S10. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1cg**

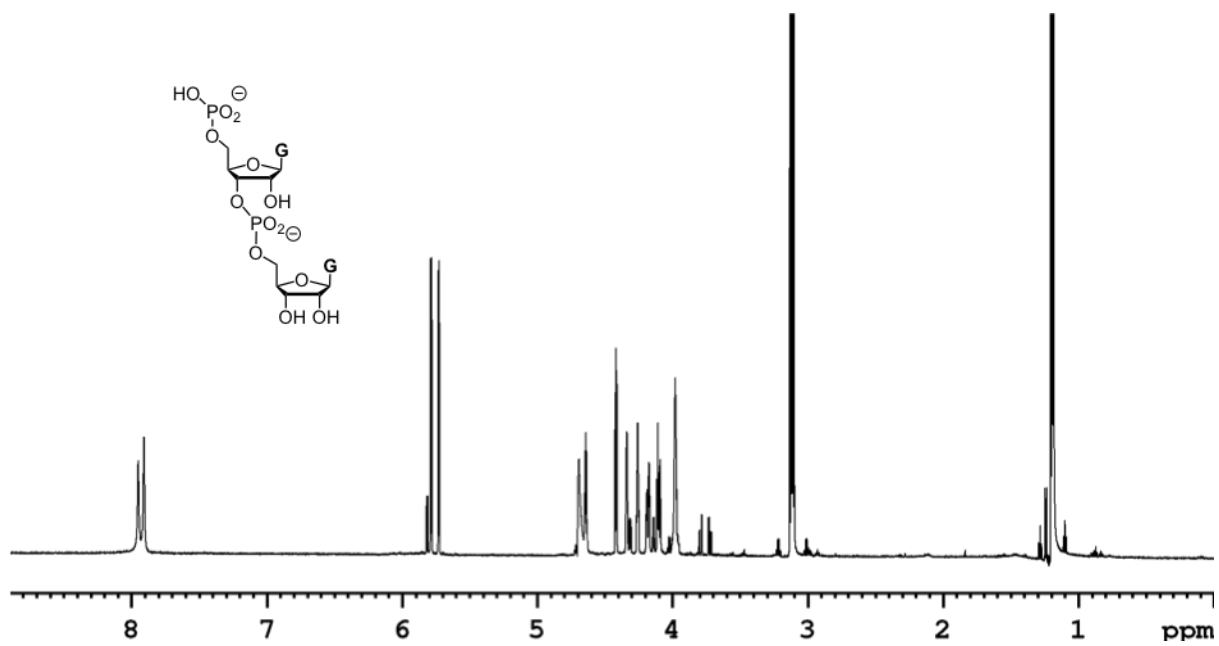


Figure S11. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1gg**

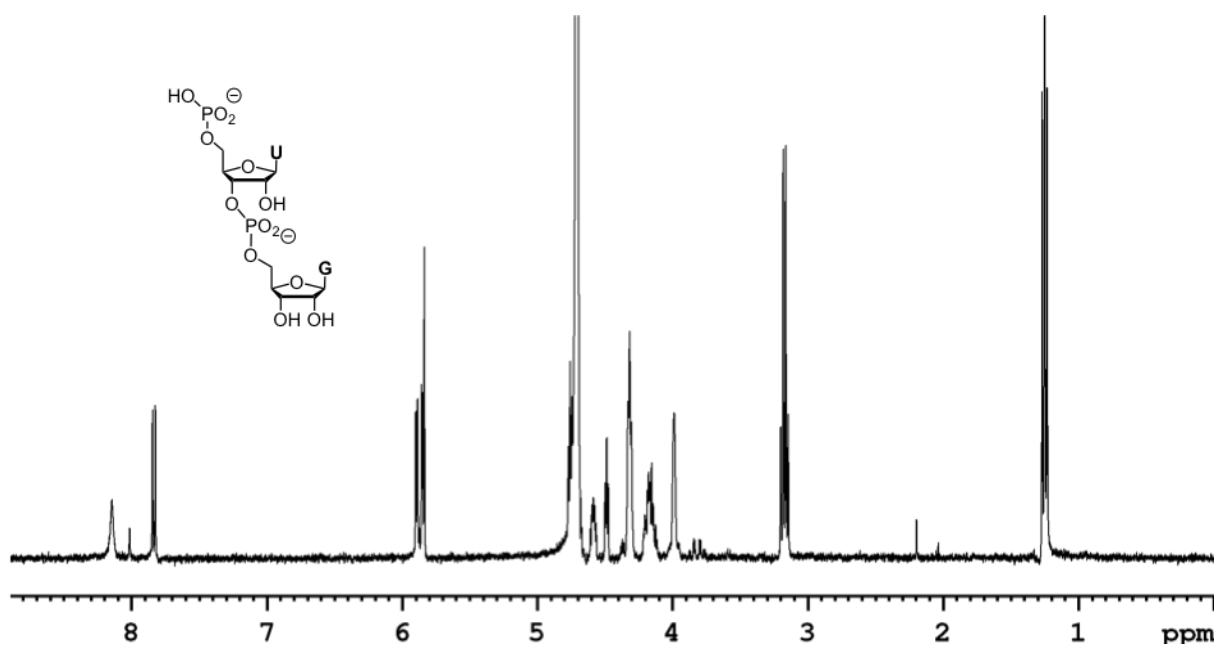


Figure S12. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1ug**

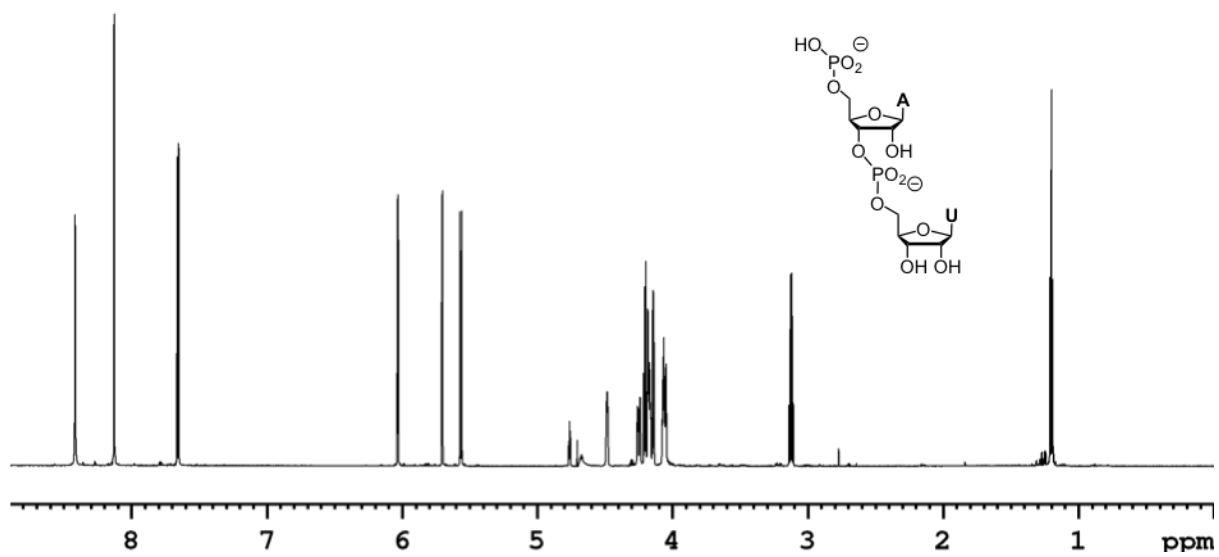


Figure S13. ¹H-NMR spectra (700 MHz, D₂O) of dimer 1au

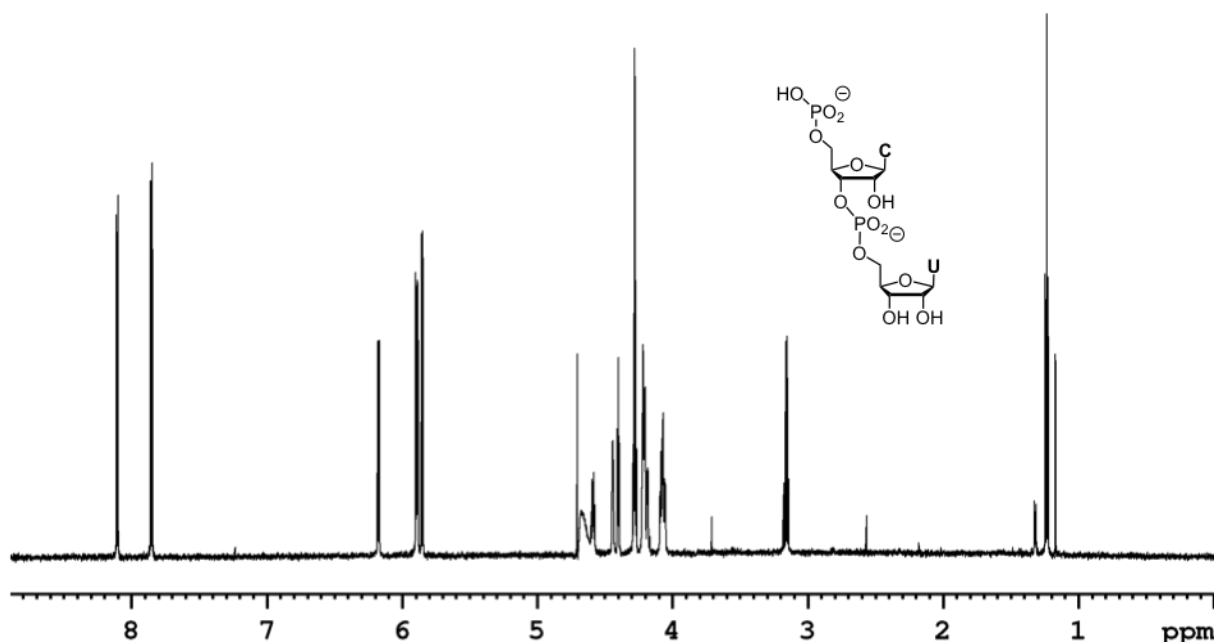


Figure S14. ¹H-NMR spectra (700 MHz, D₂O) of dimer 1cu

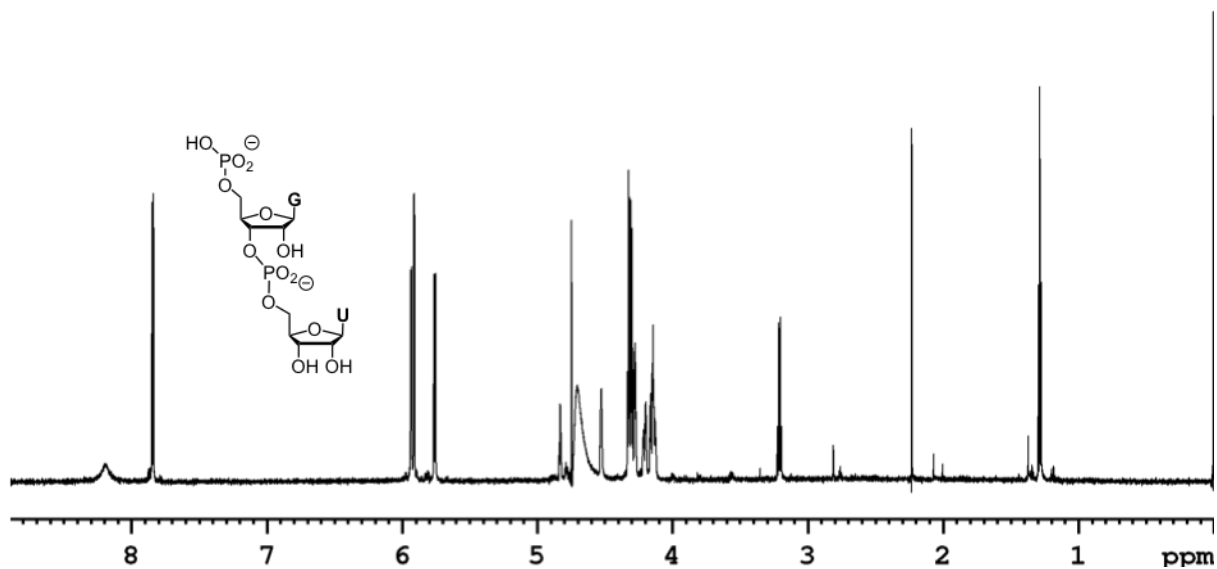


Figure S15. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1gu**

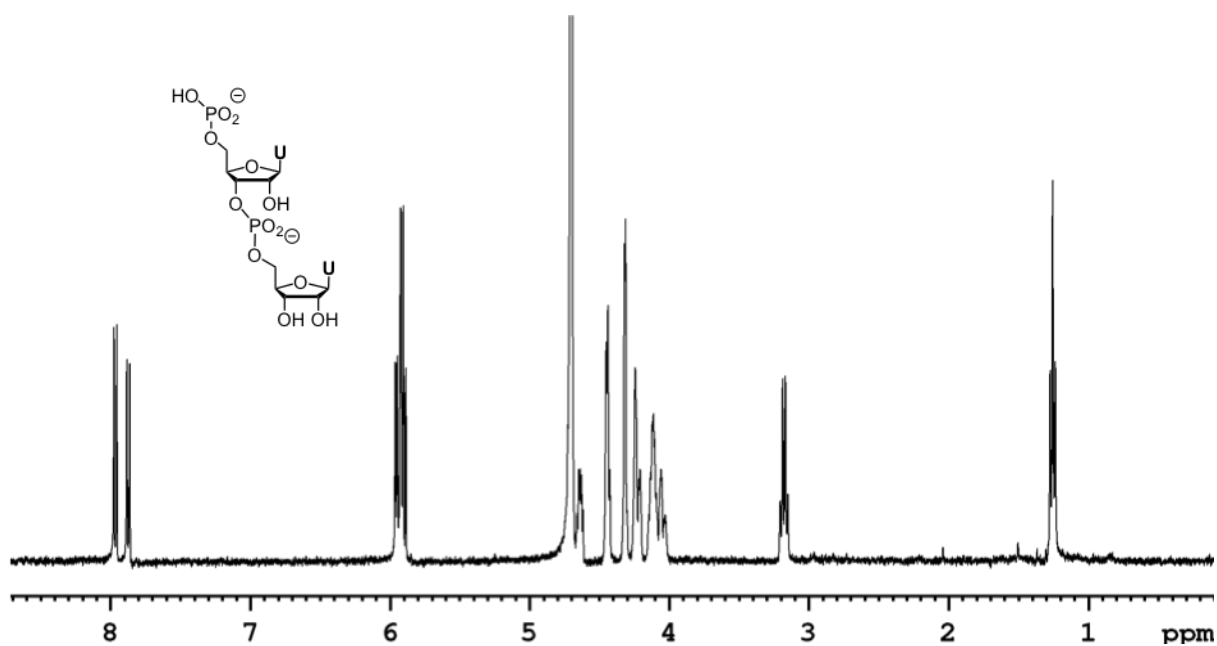


Figure S16. ¹H-NMR spectra (700 MHz, D₂O) of dimer **1uu**

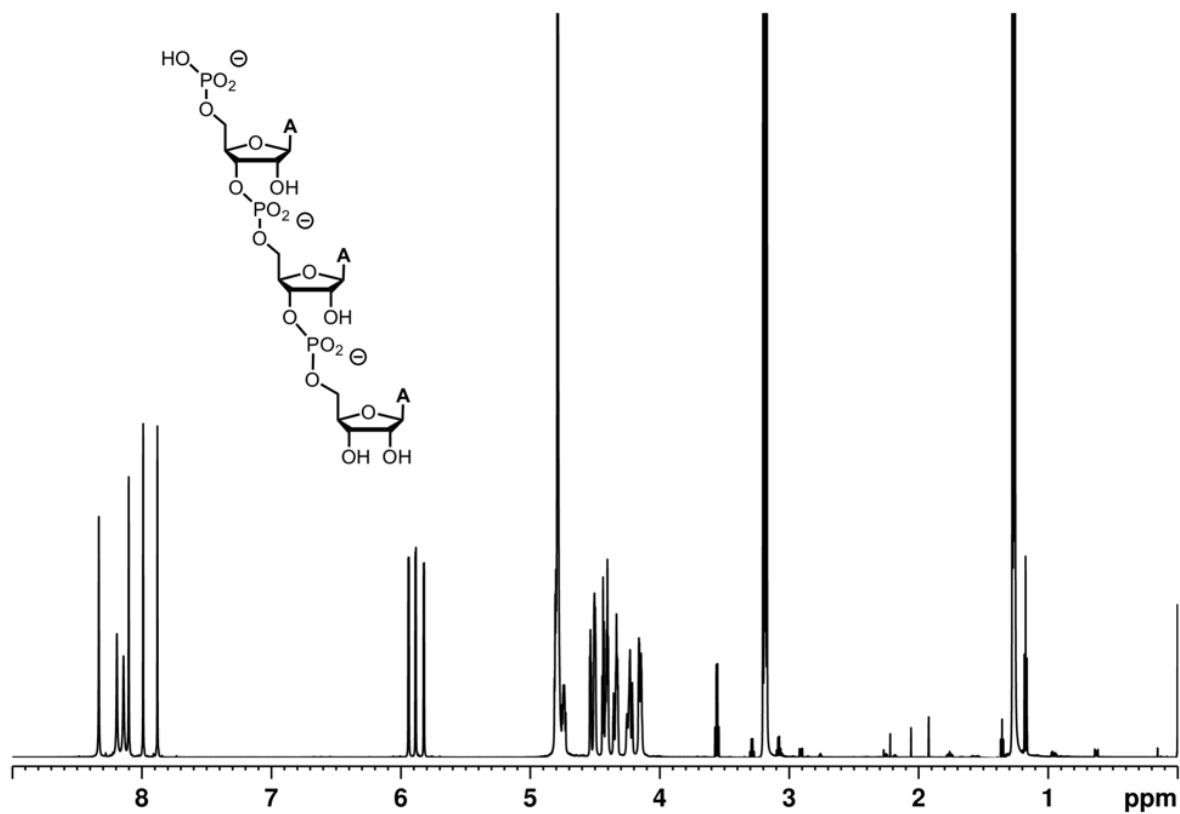


Figure S17. ¹H-NMR spectra (700 MHz, D₂O) of trimer **2aaa**

3. Helper Tetramers

The tetramer helper strands were prepared by automated RNA synthesis using an ABI 8909 Expedite synthesizer and commercial, 2'-TBDMS-protected phosphoramidite building blocks, following the protocol recommended by the manufacturer. The purification was achieved via cartridge chromatography on Sep-Pak Vac C18, 3cc cartridges (1 g; Waters, Milford, MA, USA), using a gradient of CH₃CN in water. All sequences were freed of acetate salts via ion exchange to the sodium form on RP cartridges (1 g). Failure to remove acetate from the assay solution may cause acetylation of the primer and early termination of ligation.

Analytical Data

Oligonucleotide helper strand 8; yield 0.5 μmol, 16%; **MALDI-TOF MS** *m/z*: calculated for C₃₇H₄₆N₁₂O₂₈P₃ [M-H]⁻: 1199, found 1200.

Oligonucleotide helper strand 9; yield 0.4 μmol, 25%; **MALDI-TOF MS** *m/z*: calculated for C₃₈H₄₇N₁₅O₂₇P₃ [M-H]⁻: 1238, found 1239.

4. MALDI-TOF Detection of Ligation Products

The detection of ligation products relied on MALDI-TOF mass spectrometry, using desalting and ionization conditions known to allow for quantitative detection (S1). To assess the effect of the elongation of the primer by two or three nucleotides, correction factors for desorption and ionization were determined for representative cases. The following is a protocol for measuring the correction factor for the extension product formed by the reaction with dimer **1ag** and is representative. Stock solutions of the synthetic control compound of **10ag** were prepared in distilled water at concentrations ranging from 5 μM to 200 μM . A solution of primer **3** (50 μM) was also prepared in distilled water. All concentrations were confirmed by UV absorption analysis. Aliquots of 1 μL of the stock solution of primer **3** and 1 μL of one of the stock solutions of synthetic control compound of **10ag** were mixed, treated with a few beads of cation exchange resin (Dowex 50 WX8-200, ammonium form, 25 μL suspension), and aliquots of the supernatant of the resulting suspension were employed for MALDI-TOF analysis. The peak ratios of extension product versus primer were plotted against the concentration of the analyte to obtain a calibration curve. Figure S18, below shows representative plots, and Table S1 reports the correction factors obtained for dimer and trimer extension.

Table S1. Correction factors for MALDI-TOF analysis of ligation with di- and trinucleotides. Slopes and R^2 were determined by fitting the data with via linear regression in Microsoft Excel.

Extended Primer	Slope	R²	Correction Factor
P+AG	0.87	0.993	1.2
P+CG	0.73	0.992	1.4
P+GG	0.19	0.992	5.3
P+UG	0.82	0.997	1.2
P+AAA	1.08	0.997	0.9
P+CCC	0.99	0.862	1.0

For all extended sequences but **10gg**, the correction factors were small, so that no additional control experiments were performed for other extension products. The quantification was performed by relative peak intensities (peak heights) in all cases.

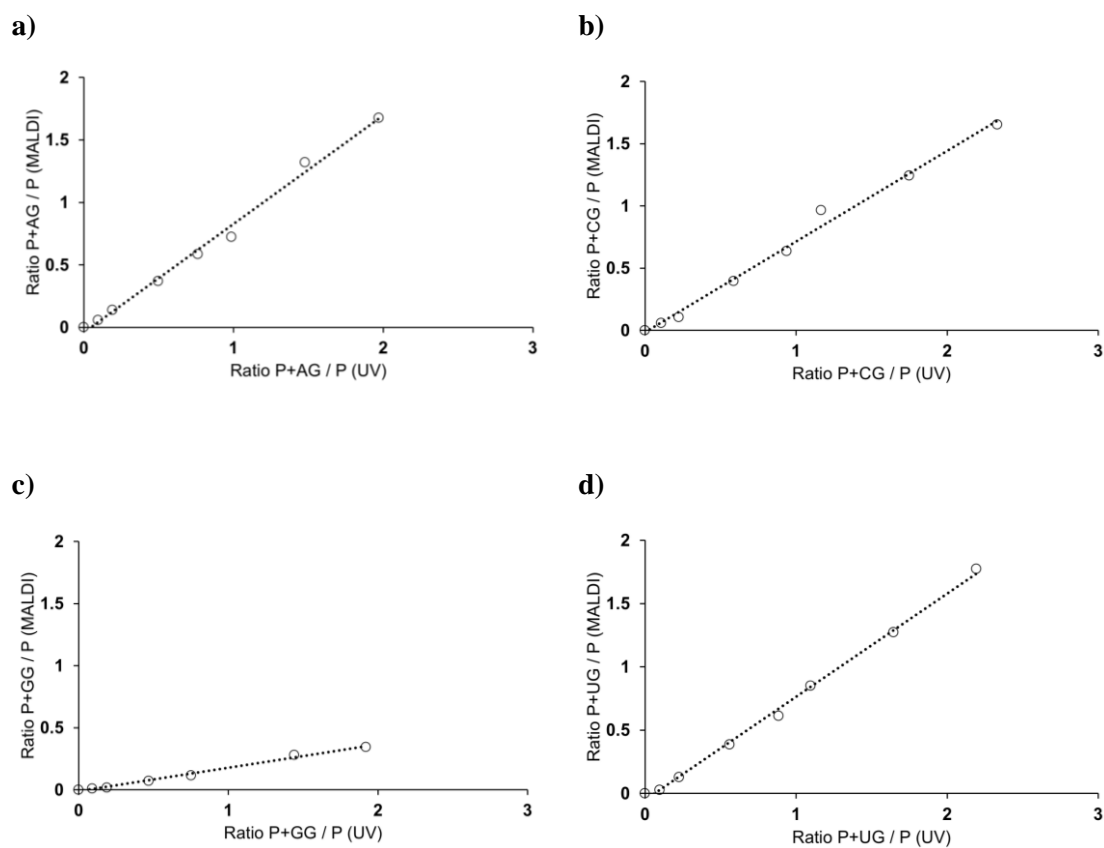


Figure S18. Representative calibration plots for the detection of elongated forms of primer **3**, based on relative peak intensities in MALDI-TOF mass spectra with synthetic control compounds **10nn**. Relative peak heights are plotted vs. relative intensities, as determined by absorption spectroscopy; a) for primer elongated with **1ag** (P+AG); b) for primer elongated with **1cg** (P+CG); c) for primer elongated with **1gg** (P+GG), and d) for primer elongated with **1ug** (P+UG). The dashed black line is the fit from linear regression, calculated in Excel.

5. Additional Data for NMR Titration, Inhibitor Assay, and Kinetics of Cyclization

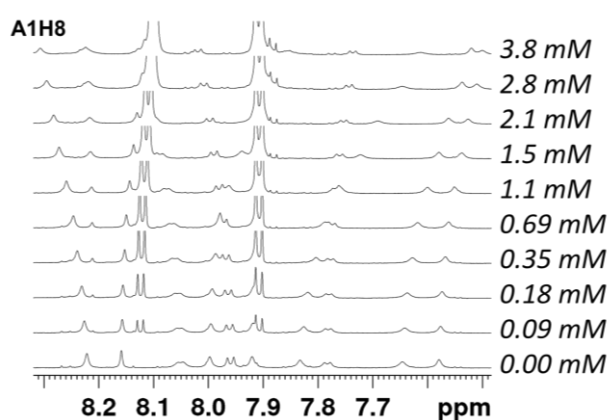
5.1 NMR Titration

The protocol for determining binding or dissociation constants was adapted from that recently published by us (S2). In the following, it is briefly described for the titration of hairpin **13aa** with dimer **1uu** and is representative. Hairpin **13aa** (0.1 μmol) was dissolved in 200 μL of deuterated buffer (200 mM phosphate, 400 mM NaCl, pH 7.0; uncorrected for deuterium effect) to reach a final concentration of 0.5 mM. A sample of 3-(trimethylsilyl)propionic-2,2,3,3 acid-d4 (TPS) was added as calibration standard (0.05 mM). A stock solution of **1uu** (50 mM) was prepared in 50 μL of the same buffer. Spectra were recorded on a Bruker Avance 700 spectrometer with helium-cooled probe. The assignment of the proton signals of the hairpin had previously been established based on 2D NMR spectra. Aliquots of the dimer stock solution were added to the solution containing the hairpin oligonucleotide. Displacement of the chemical shift of proton C6H6 (or A1H8 for **13ag/1cu**) was monitored in the ^1H -channel (700 MHz) using Topspin 3.2. software. For this, spectra were recorded with presaturation at the HDO frequency of 4.708 ppm. The spectra in Figure S19, below, are overlays of expansions of the spectral region given on the x-axis. For **13ag/1cu**, two independent titrations were performed over slightly different concentration ranges.

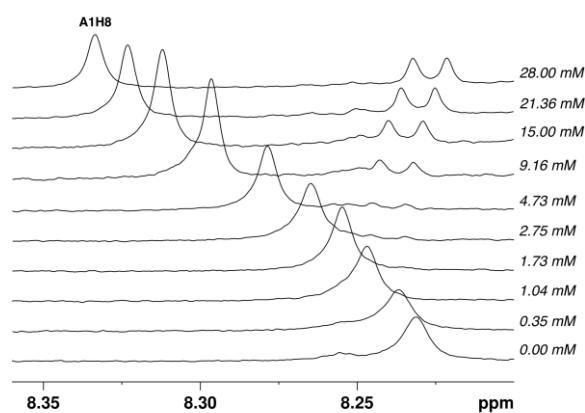
a)

$$\Delta\delta_{obs} = \frac{\Delta\delta_{M:PT}}{K_d} * \frac{[M]}{1 + \frac{[M]}{K_d}}$$

b) **13ag** and dimer **1cu** (first titration)



13ag and dimer **1cu** (second titration, expansion)



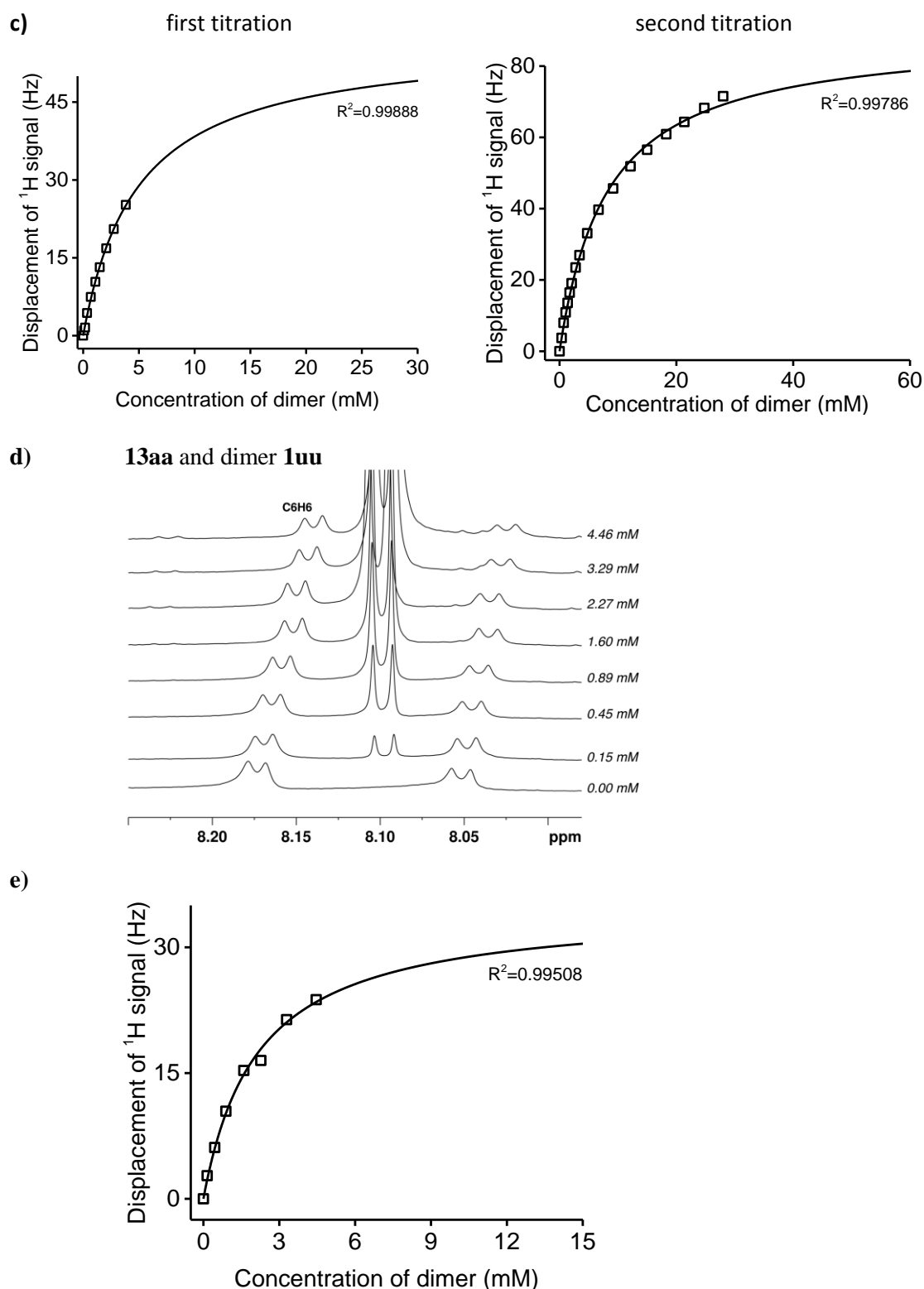


Figure S19. Determination of binding constant via ^1H -NMR titration. a) Equation linking the K_d value to the concentration of monomer $[M]$ and the displacement of chemical shift ($\Delta\delta$) of the ^1H signal, as well as the maximal displacement of the chemical shift ($\Delta\delta_{M:P}$) (S2). b) Overlay of selected ^1H -NMR spectra of hairpin **13ag** at increasing concentrations of dimer **1cu** (two runs). c) Plot of the displacement of the chemical shift of proton A1H8 of hairpin **13ag** versus the concentration of dimer **1cu** for the two runs. Curves are fits, giving K_d 's of 5 mM and 8 mM. The agreement between experimental data and the fit is given as R^2 value. d) Overlay of ^1H -NMR spectra of hairpin **13aa** at increasing concentrations of dimer **1uu**. e) Plot of the displacement of the chemical shift of proton C6H6 of **13aa** versus the concentration of dimer **1uu**.; the curve is the fit, giving a K_d of 2 mM.

5.2 Inhibitor Assay

Synthesis of **14ag**

Dimer **1ag** (2 μmol) was suspended in demineralized water (20 μL) containing EDC (10 μmol , 5 eq) and HOAt (10 μmol , 5 eq). The pH of the solution was adjusted to 5.0 with NaOH. The reaction was allowed to proceed for 2 h with mixing in an ultrasound bath at room temperature. The resulting solution was then used as stock solution of the activated dimer without further purification. To confirm that the activation conditions induce the formation of OAt ester **14ag**, a control reaction was performed with a more dilute solution. This control reaction was monitored by NMR, as shown in Figure S20, below.

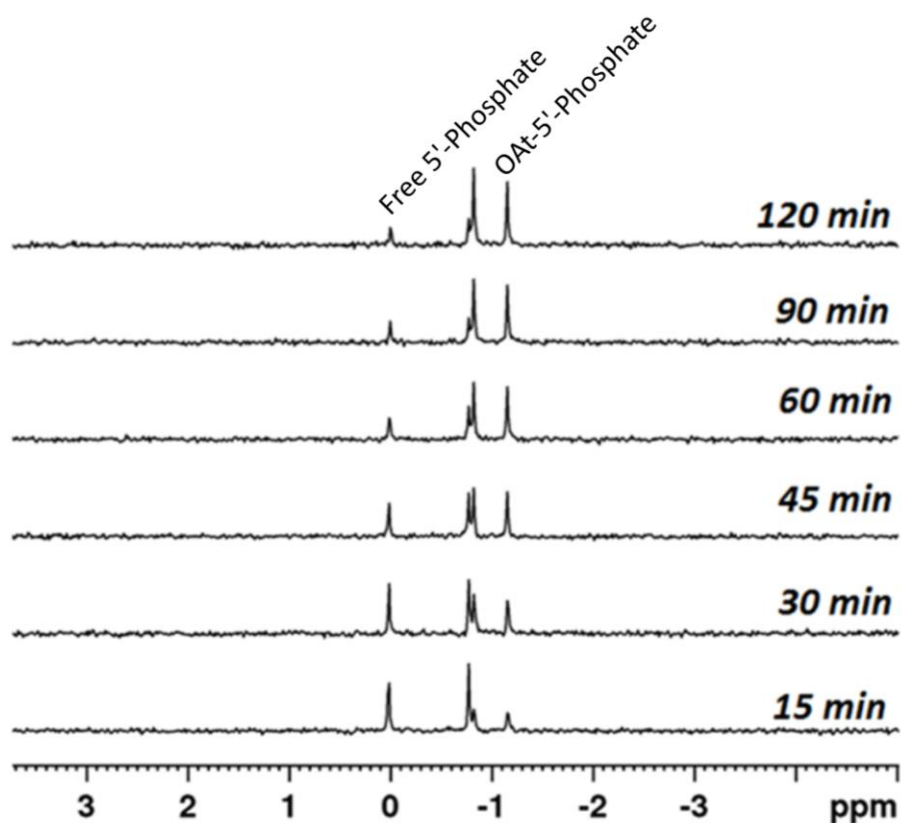


Figure S20. Model reaction to study the activation of **1cg** to **14ag** with HOAt/EDC at pH 5.0 and room temperature, performed at 0.5 mM concentration of the dimer and monitored by ^{31}P -NMR (161 MHz, D_2O). It is assumed that the activation is higher yielding at the 100 mM concentration used to prepare the stock solution of the activated dimer for the assay, so that the activation level detected here is the lower limit of that in the activation mixture.

5.3 Cyclization

Representative MALDI-TOF mass spectra from assay solutions (low mass region).

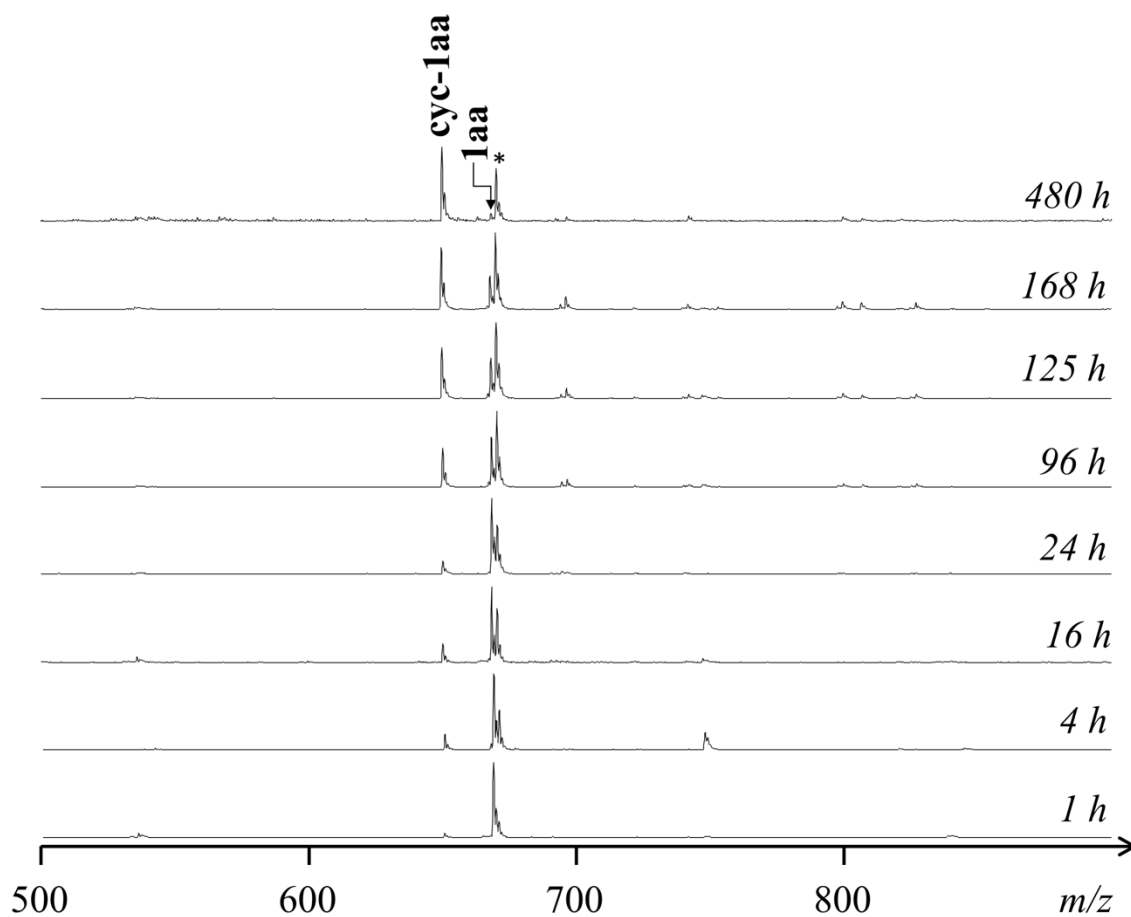


Figure S21. Cyclization of dimer **1aa**. Expansion of the low-mass region of MALDI-TOF mass region of spectra from ligation assays with primer **4** (30 μ M), template **6uu** (45 μ M) and helper **9** (45 μ M). Conditions : 500 mM MOPS, 800 mM EDC, 150 mM 1-ethylimidazole, 80 mM MgCl_2 , pH 6, 4 $^\circ\text{C}$. The asterisk labels a peak for a compound of unknown structure with a mass 2 Da higher than that of the starting material.

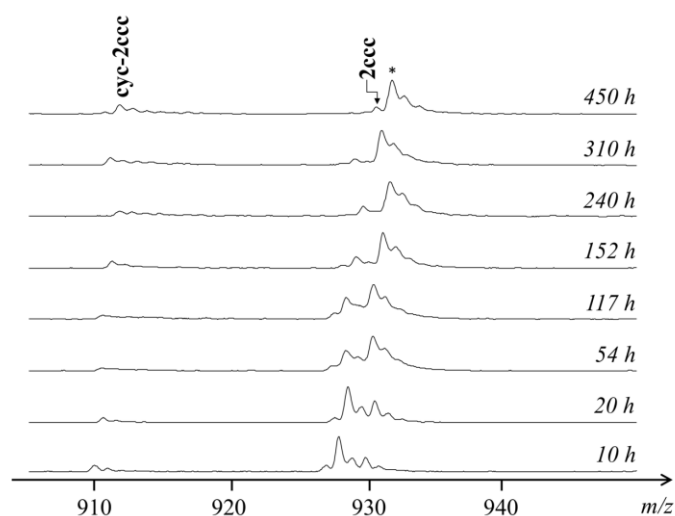


Figure S22. Cyclization of trimer **2ccc**. Expansion of the low-mass region of MALDI-TOF mass spectra from ligation assays with primer **4** (30 μ M), template **7ggg** (45 μ M) and helper **9** (60 μ M). Conditions : 500 mM MOPS, 800 mM EDC, 150 mM 1-ethylimidazole, 80 mM $MgCl_2$, pH 6 at 4 $^{\circ}C$. The asterisk labels a peak for a compound of unknown structure with a mass 2 Da higher than that of the starting material.

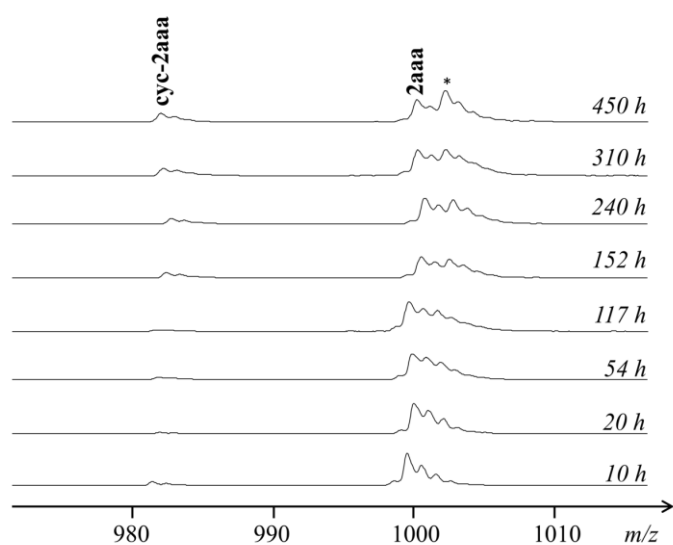


Figure S23. Cyclization of trimer **2aaa**: Low-mass region of MALDI-TOF mass spectra from a ligation assay with primer **4** (30 μ M), template **7uuu** (45 μ M), and helper **9** (60 μ M). Conditions : 500 mM MOPS, 800 mM EDC, 150 mM 1-ethylimidazole, 80 mM $MgCl_2$, pH 6, 4 $^{\circ}C$. The asterisk labels a peak for a compound of unknown structure with a mass 2 Da higher than that of the starting material and the other labels are for the peak of the linear and cyclic form of **2aa**.

Additional Data on Cyclization

The data shown in Table S2 were obtained by re-analyzing the low mass range of spectra acquired in the context of ligation assays. They are based on uncorrected signal intensities in spectra acquired with parameters optimized for a higher mass range, so this must be considered exploratory work, awaiting independent validation.

Table S2. Cyclization of dimers and trimers, as detected in MALDI-TOF mass spectra from ligation assays.^a

Dimer/Trimer	Cyclized after 20 d [%]
1aa	57
1ca	63
1ua	70
1ac	58
1cc	70
1gc	55
1uc	82
1ag	52
1cg	50
1gg	52
1ug	77
1au	65
1cu	76
1gu	60
1uu	90
2aaa	13
2ccc	20

^a As detected by MALDI-TOF MS after 20 d in condensation buffer containing 2 mM dimer or 1 mM trimer and 500 mM MOPS, 800 mM EDC, 150 mM 1-EtIm, 80 mM MgCl₂ at pH 6.0 and 4 °C.

6. References for Supplementary Information

- S1. Sarracino,D., Richert,C. (1996) Quantitative MALDI-TOF spectrometry of oligonucleotides and a nuclease assay. *Bioorg. Med. Chem. Lett.*, **6**, 2543-2548.
- S2. Kervio,E., Claasen,B., Steiner,U.E., Richert,C. (2014) The strength of the template effect attracting nucleotides to naked DNA. *Nucleic Acids Res.*, **42**, 7409–7420.