Supporting Information for

A tolane-modified 5-ethynyluridine as universal and fluorogenic photochemical DNA crosslinker

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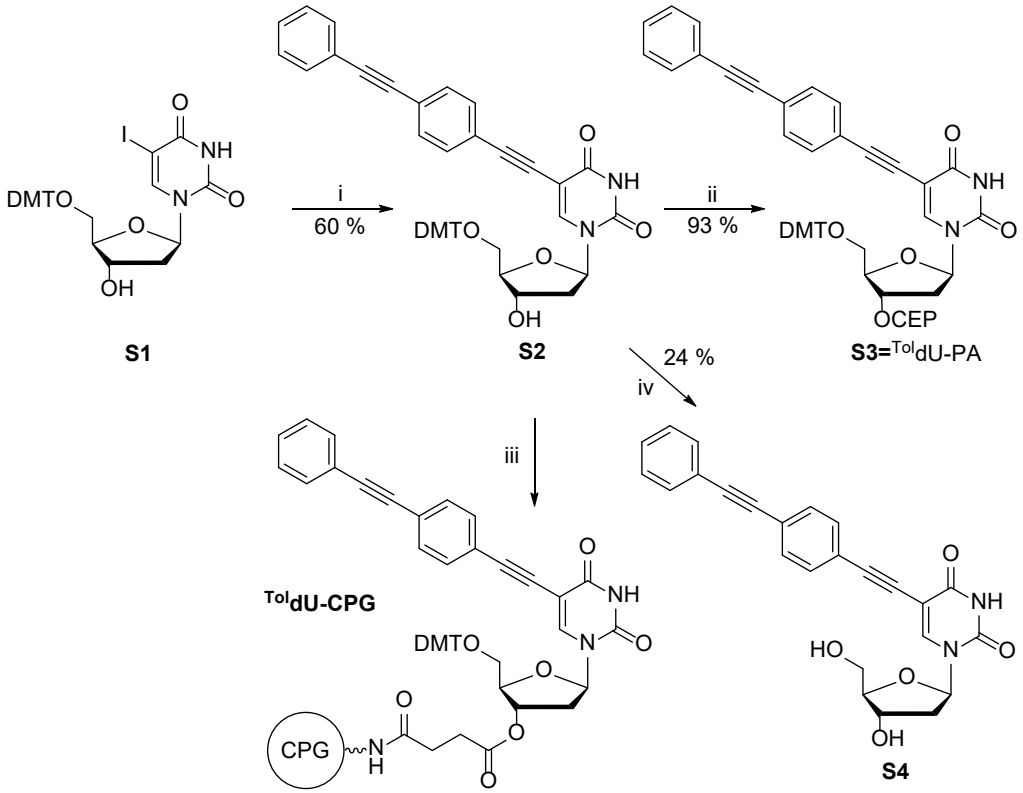


Scheme S1. Synthesis of the ^{Tol}dU nucleoside, phosphoramidite and solid support. i) 1-Ethynyl-4-(2-phenylethynyl)benzene, CuI, $Pd(PPh_3)_4$, Et_3N/DMF, 60 °C, over night; ii) CEP-Cl, DIPEA, DCM, r.t., 4 h; iii) 1.succinic anhydride, DMAP, DCM, r.t., 4 h, 2. LCAA-CPG, BOP, *N*-methylimidazole, MeCN r.t. over night; iv) TFA, DCM, r. t.,15 min.

Table S1. Sequences and ESI-MS results of the used DNA oligonucleotides.

Oligo	5'-Sequence-3'	Chemical formula	Calc. Mass	Measured Mass
ODN1_A	GATGATAGCTAG	$C_{119}H_{148}N_{49}O_{69}P_{11}$	3707.66977	3707.69796
ODN1_C3	GATGAT(C3)GCTAG	$C_{112}H_{143}N_{44}O_{68}P_{11}$	3532.61981	3532.62184
ODN1_C	GATGATCGCTAG	$C_{118}H_{148}N_{47}O_{70}P_{11}$	3683.65853	3683.68621
ODN1_G	GATGATGGCTAG	$C_{119}H_{148}N_{49}O_{70}P_{11}$	3723.66468	3723.66893
ODN1_T	GATGATTGCTAG	$C_{119}H_{149}N_{46}O_{71}P_{11}$	3698.65820	3698.70495
^{Tol}dU1_A	GATGA(^{Tol}dU)AGCTAG	$C_{134}H_{154}N_{49}O_{69}P_{11}$	3893.71617	3893.73041
Alk-^{Tol}dU1_A	Alk-GATGA(^{Tol}dU)AGCTAG	$C_{140}H_{163}N_{49}O_{72}P_{12}$	4053.74510	4053.74251
^{Tol}dU2_A	CTAGC(^{Tol}dU)ATCATC	$C_{131}H_{155}N_{40}O_{71}P_{11}$	3764.68616	3764.70338
^{Tol}dU1_C	GATGA(^{Tol}dU)CGCTAG	$C_{133}H_{154}N_{47}O_{70}P_{11}$	3869.70493	3869.70721
Alk-^{Tol}dU1_C	Alk-GATGA(^{Tol}dU)CGCTAG	$C_{139}H_{163}N_{47}O_{73}P_{12}$	4029.73387	4029.74938
^{Tol}dU2_C	CTAGC(^{Tol}dU)CTCATC	$C_{130}H_{155}N_{38}O_{72}P_{11}$	3740.67492	3740.68243
5'-^{Tol}dU-Lig/Hairpin	(^{Tol}dU)GCTATCATATG	$C_{133}H_{156}N_{41}O_{72}P_{11}$	3819.69197	3819.70602
Hairpin-3'-^{Tol}dU	Alk-CATATGATAGCC(^{Tol}dU)	$C_{148}H_{176}N_{47}O_{79}P_{13}$	4277.77939	4277.79279
Lig-3'-^{Tol}dU	Alk-AGGTCCGGACTG(^{Tol}dU)	$C_{148}H_{175}N_{50}O_{80}P_{13}$	4334.77515	4334.77052
Lig-Template	CATATGATAGCAACAGTCCGGACCT	$C_{243}H_{306}N_{96}O_{145}P_{24}$	7631.32246	7631.34777
H1-HCR	TAGCTTATCAGACCTATGTTGATCGGTCTGATAATCTAAAAGTC	$C_{432}H_{544}N_{159}O_{264}P_{43}$	13519.29063	13519.38774
H2-HCR	TCAACATCTGTCTGATAAGCTAGACTTTTATCTTATCAGACCGA	$C_{430}H_{544}N_{155}O_{264}P_{43}$	13433.26305	13433.26331
Initiator-HCR	TAGCTTATCAGACCGATGTTGA	$C_{216}H_{272}N_{81}O_{131}P_{21}$	6746.16076	6746.16580
H1-^{Tol}dU-HCR	TAGCTTATCAGACC(^{Tol}dU)ATGTTGATCGGTCTGATAA(^{Tol}dU)CTAAAAGTC	$C_{462}H_{556}N_{159}O_{264}P_{43}$	13891.38477	13892.57158
H2-^{Tol}dU-HCR	TCAACATC(^{Tol}dU)GTCTGATAAGCTAGACTTTTA(^{Tol}dU)CTTATCAGACCGA	$C_{460}H_{556}N_{155}O_{264}P_{43}$	13811.37254	13809.49174

Table S2. Thermodynamic data for DNA duplexes in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0).

Duplex	Oligo	Sequence	c_{total}[a] [µM]	T_m [°C]	ΔH^0 [kcal mol^{-1}]	ΔS^0 [cal mol^{-1}]	ΔG^{298}[b] [kcal mol^{-1}]
dA/^{Tol}dU	ODN1_A ^{Tol}dU2_A	5'…TAG…3' 3'…A^{Tol}dUC…5'	2.4 4.9 12.4 24.0 49.6	37.2 38.9 41.1 42.5 44.4	-83.3±0.5	-240±1.5	-11.9±0.7
C3/^{Tol}dU	ODN1_C3 ^{Tol}dU2_A	5'…TC3G…3' 3'…A^{Tol}dUC…5'	2.3 5.2 12.2 23.6 48.9	27.9 29.4 32.4 34.2 36.1	-65.9±1.5	-190±4.3	-9.2±1.9
dC/^{Tol}dU	ODN1_C ^{Tol}dU2_A	5'…TCG…3' 3'…A^{Tol}dUC…5'	2.4 5.0 13.0 24.6 50.5	23.8 26.4 28.6 31.7 33.8	-54.9±1.4	-157±4.1	-8.3±1.8
dG/^{Tol}dU	ODN1_G ^{Tol}dU2_A	5'…TGG…3' 3'…A^{Tol}dUC…5'	2.3 5.0 12.3 24.2 48.1	27.3 29.5 32.0 33.7 35.5	-68.5±0.5	-199±1.4	-9.1±0.6
dT/^{Tol}dU	ODN1_T ^{Tol}dU2_A	5'…TTG…3' 3'…A^{Tol}dUC…5'	2.3 4.7 11.9 23.2 47.4	26.7 28.6 31.1 32.8 34.8	-68.3±3.4	-199±1.0	-8.8±0.5

[a] Total concentration of DNA strands. Values were calculated with the absorption at 260 nm of the melted duplex [b] Calculated for T = 25 °C.

Table S3. Fluorescence data for DNA duplexes in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0).

Duplex	Oligo	Sequence	$\lambda_{ex,\,max}$ [a] [nm]	$\lambda_{em,\,max}$ [b] [nm]	Φ
dA/^{Tol}dU	ODN1_A/^{Tol}dU2_A	5'…TAG…3'/3'…A^{Tol}dUC…5'	333	405	0.037
C3/^{Tol}dU	ODN1_C3/^{Tol}dU2_A	5'…TC3G…3'/3'…A^{Tol}dUC…5'	328	419	0.010
dC/^{Tol}dU	ODN1_C/^{Tol}dU2_A	5'…TCG…3'/3'…A^{Tol}dUC…5'	331	413	0.017
dG/^{Tol}dU	ODN1_G/^{Tol}dU2_A	5'…TGG…3'/3'…A^{Tol}dUC…5'	328	423	0.021
dT/^{Tol}dU	ODN1_T/^{Tol}dU2_A	5'…TTG…3'/3'…A^{Tol}dUC…5'	334	415	0.022
ssDNA ^{Tol}dU	^{Tol}dU2_A	5'…C^{Tol}dUA…3'	331	420	0.021

[a] λ_{em}=405 nm , [b] λ_{ex}=320 nm.

Table S4. Lifetime data for DNA duplexes in phosphate buffer (1 µM DNA in 100 mM NaCl, 10 mM sodium phosphate, pH 7.0).

Duplex	Oligo	Sequence	a_1	τ_1 [ns]	a_2	τ_2 [ns]	a_3	τ_3 [ns]	$<\tau>$ [ns]
dA/^{Tol}dU	ODN1_A/^{Tol}dU2_A	5'…TAG…3'/3'…A^{Tol}dUC…5'	0.96	0.34	0.04	1.27	-	-	0.37
C3/^{Tol}dU	ODN1_C3/^{Tol}dU2_A	5'…TC3G…3'/3'…A^{Tol}dUC…5'	0.67	0.21	0.30	0.87	0.02	2.32	0.46
dC/^{Tol}dU	ODN1_C/^{Tol}dU2_A	5'…TCG…3'/3'…A^{Tol}dUC…5'	0.75	0.21	0.23	0.81	0.02	2.02	0.39
dG/^{Tol}dU	ODN1_G/^{Tol}dU2_A	5'…TGG…3'/3'…A^{Tol}dUC…5'	0.64	0.23	0.34	0.71	0.02	2.07	0.43
dT/^{Tol}dU	ODN1_T/^{Tol}dU2_A	5'…TTG…3'/3'…A^{Tol}dUC…5'	0.73	0.18	0.25	0.76	0.02	2.00	0.36
ssDNA ^{Tol}dU	^{Tol}dU2_A	5'…C^{Tol}dUA…3'	0.65	0.17	0.33	0.78	0.03	1.95	0.42

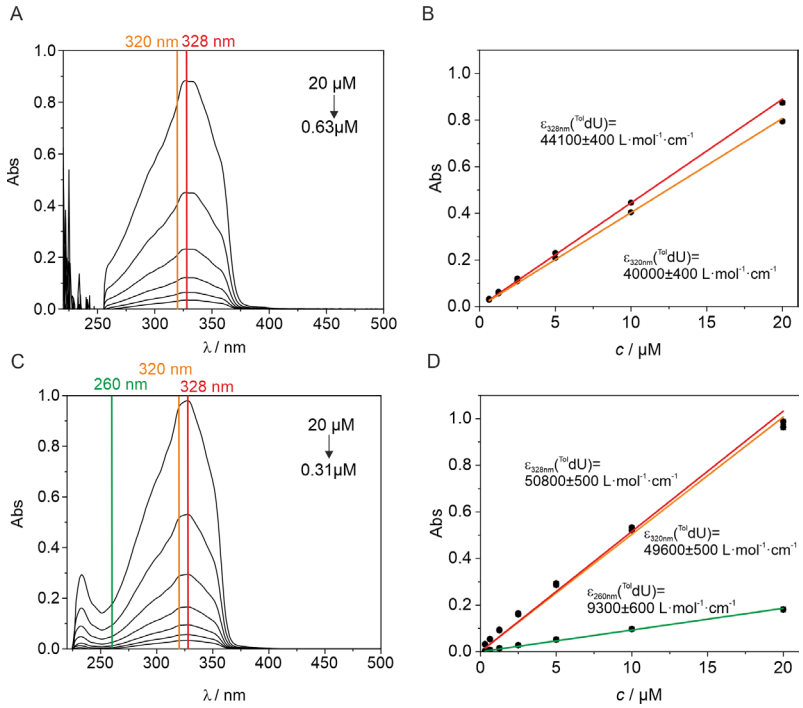


Figure S1 (A) UV/Vis spectrum of ^{Tol}dU in DMSO upon serial dilution starting from a 10 mM stock solution in DMSO. The extinction coefficient ε was determined at (B) 320 nm and 328 nm from a linear fit of the absorbance versus concentration. (C) UV/Vis spectrum of ^{Tol}dU in methanol starting from a 10 mM stock solution in DMSO. The extinction coefficient ε was determined (D) at 260 nm 320 nm and 328 nm from a linear fit of the absorbance versus concentration.

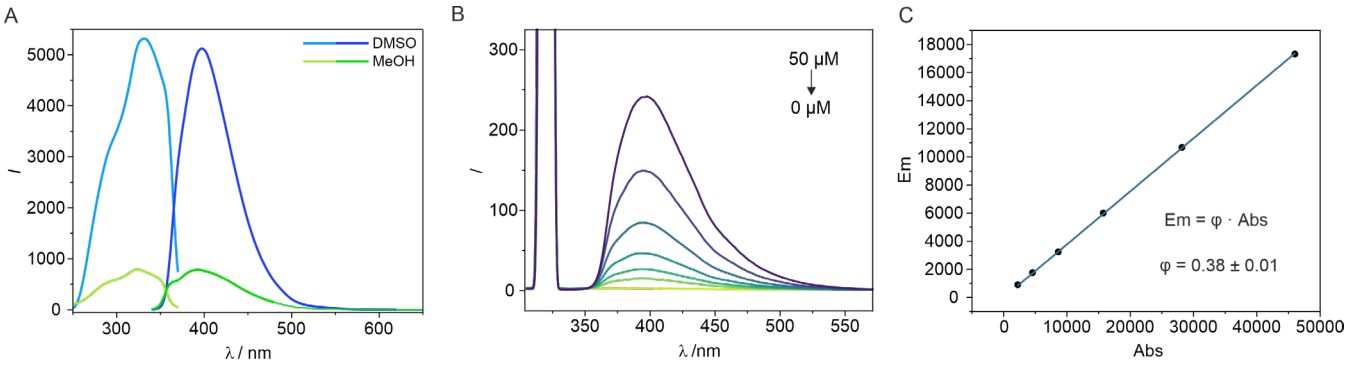


Figure S2 (A) Fluorescence excitation (λ_{em} = 390 nm) and emission (λ_{ex} = 320 nm) spectra of the nucleoside in DMSO and MeOH. (B) Fluorescence spectra for absolute quantum yield determination (λ_{ex}=320nm) of the ^{Tol}dU nucleoside in DMSO at different concentrations. The scatter peak has been cropped for clarity. (C) Determination of the absolute quantum yield from a linear fit of the integrated emission vs absorbed light.

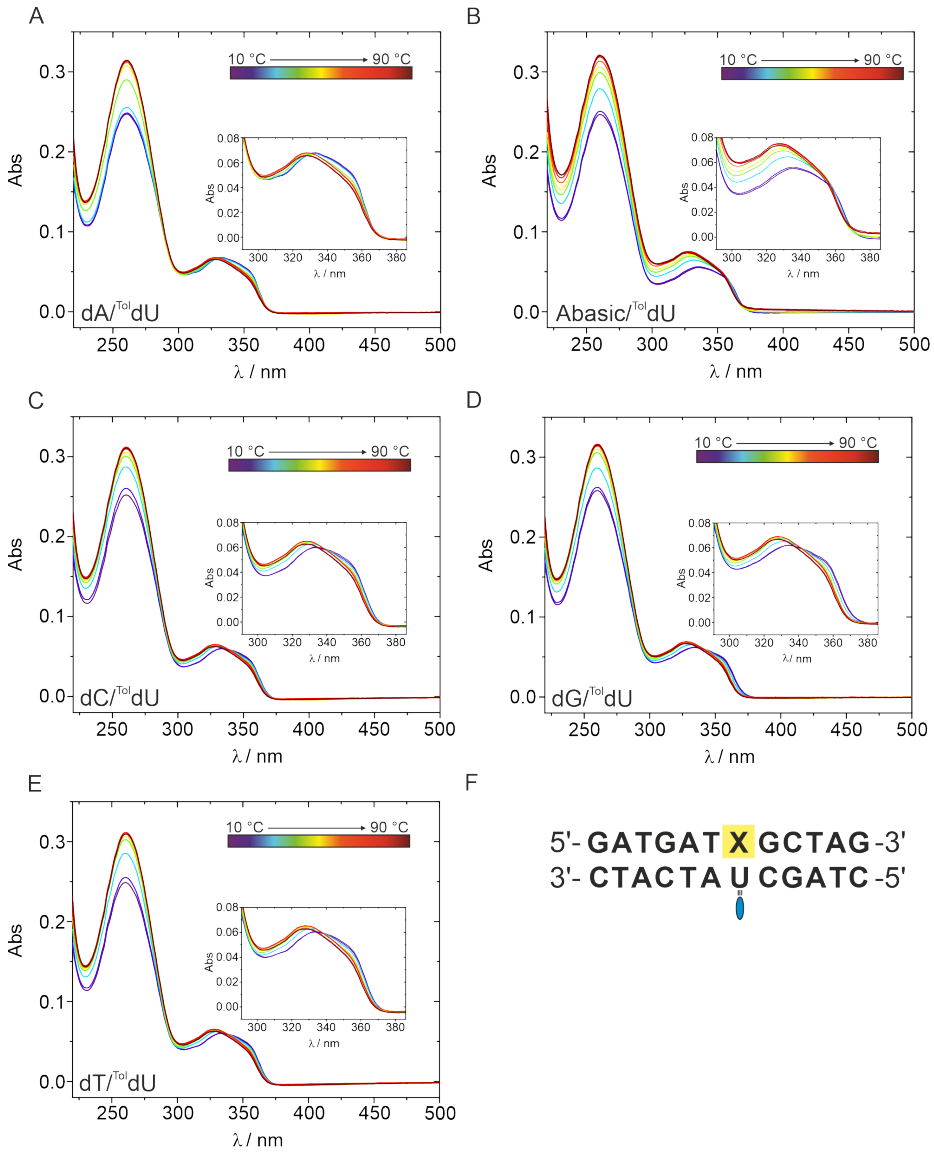


Figure S3 UV/Vis spectrum of ^{Tol}dU opposite of (A) deoxyadenosine, (B) abasic linker, (C) deoxycytidine, (D) deoxyguanosine and (E) thymidine in a DNA duplex at different temperatures (1 µM DNA in 100 mM NaCl, 10 mM sodium phosphate, pH 7.0). The inset shows the absorption of ^{Tol}dU. (F) Sequence of the DNA duplex. Different nucleosides have been incorporated at the yellow highlighted positon X opposite of ^{Tol}dU unit.

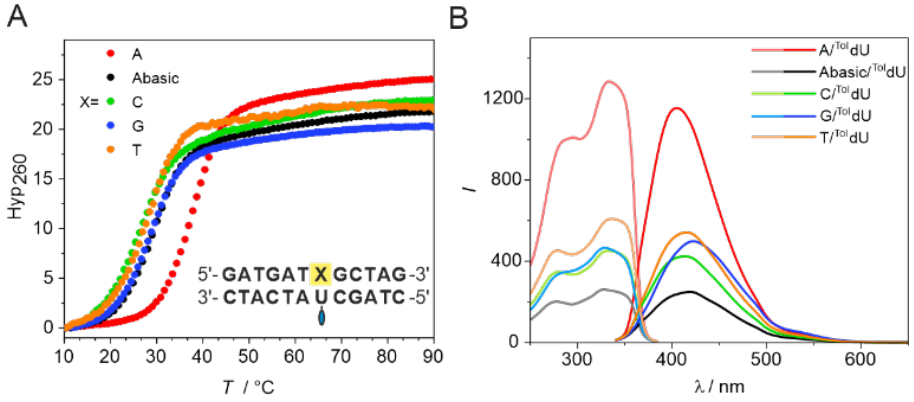


Figure S4 (A) UV melting curves and (B) fluorescence spectra of ^{Tol}dU DNA duplexes containing different nucleobases or an alkyl-linker opposite of ^{Tol}dU (X=A, C, G, T, or *n*-propyl), (1 µM DNA in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0)

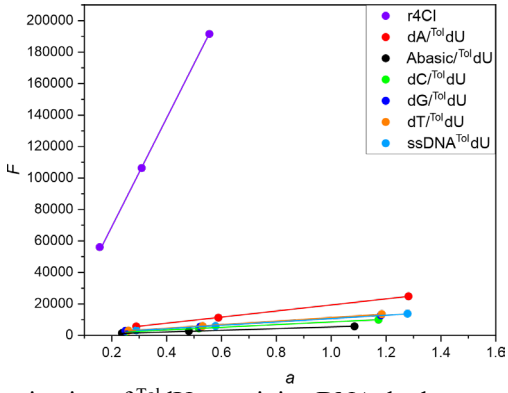


Figure S5 Relative quantum yield determination of ^{Tol}dU containing DNA duplexes and single strand with r4CI as comparison (1 µM DNA in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0). Results are summarized in Table S3.

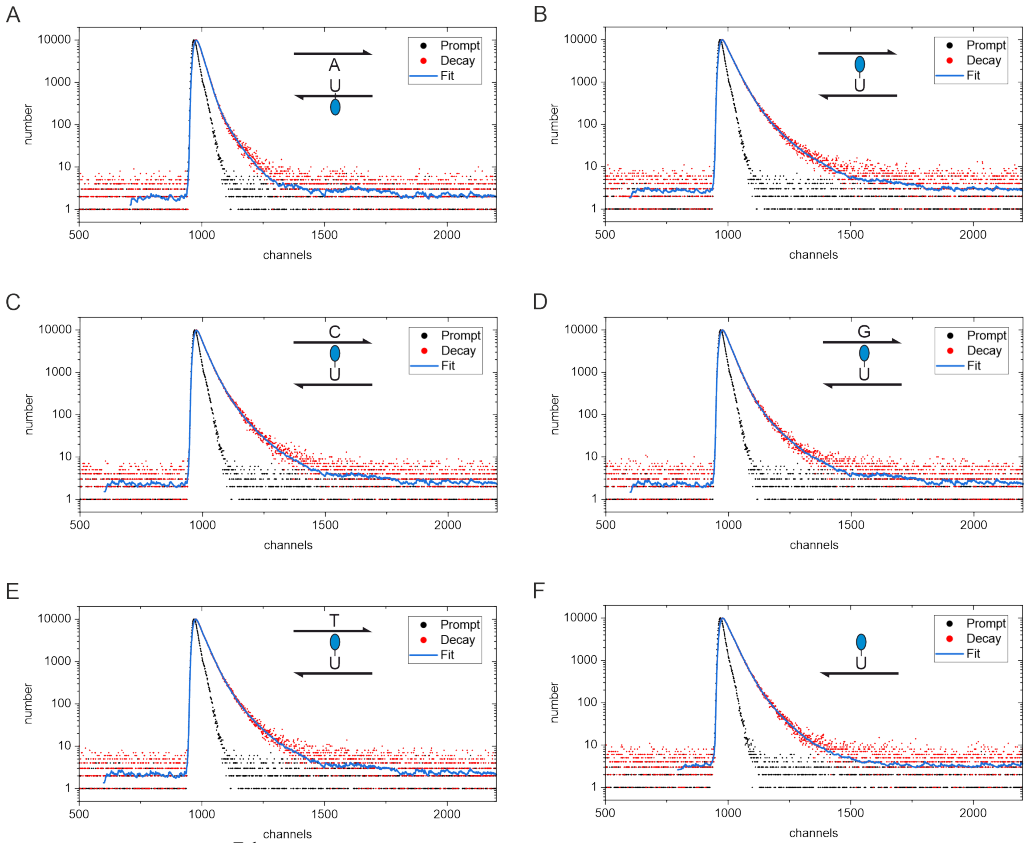


Figure S6 Fluorescence lifetime of ^{Tol}dU of (A) deoxyadenosine, (B) abasic linker, (C) deoxycytidine, (D) deoxyguanosine, (E) thymidine in a DNA duplex and (F) in a ssDNA (1 µM DNA in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0). Results are summarized in Table S4. Time calibration = 0.0257 ns channel^{-1}

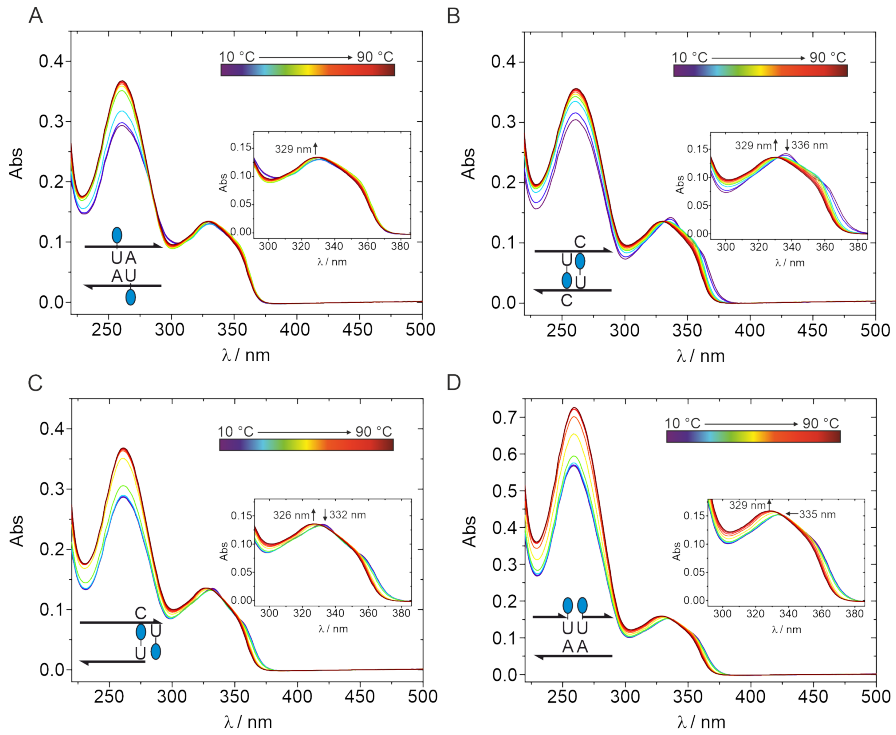


Figure S7 UV/Vis spectrum of two ^{Tol}dU units incorporated in DNA scaffold. ^{Tol}dU units arranged in a (A) ICL dA/^{Tol}dU, (B) ICL dC/^{Tol}dU, (C) hairpin CL and (D) ligation CL arrangement at different temperatures (1 µM DNA in 100 mM NaCl, 10 mM sodium phosphate, pH 7.0). The inset shows the absorption of ^{Tol}dU. Shifted maxima are indicated with an arrow.

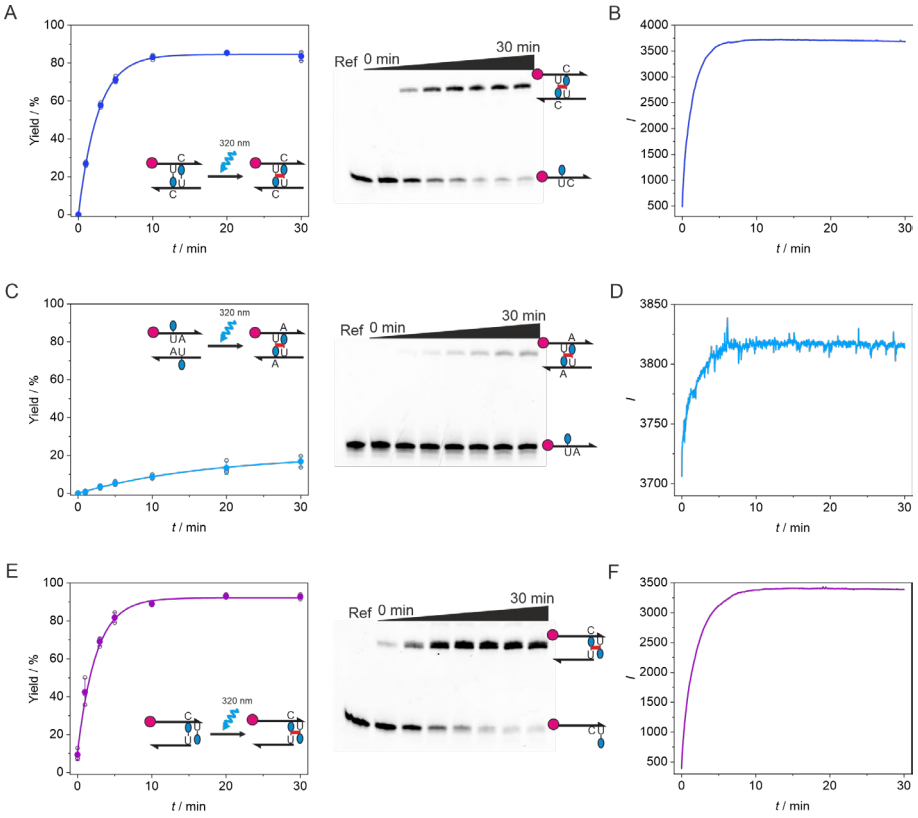


Figure S8 Kinetic characterization of CL formation using the fluorescence spectrometer light source with PAGE analysis and fluorescence increasement at 410 nm of the DNA mixture for (A and B) ICL dC/^{Tol}dU, (C and D) ICL dA/^{Tol}dU and (E and F) hairpin CL arrangement. Cy3 for quantification is shown as a magenta circle (1 µM DNA in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0).

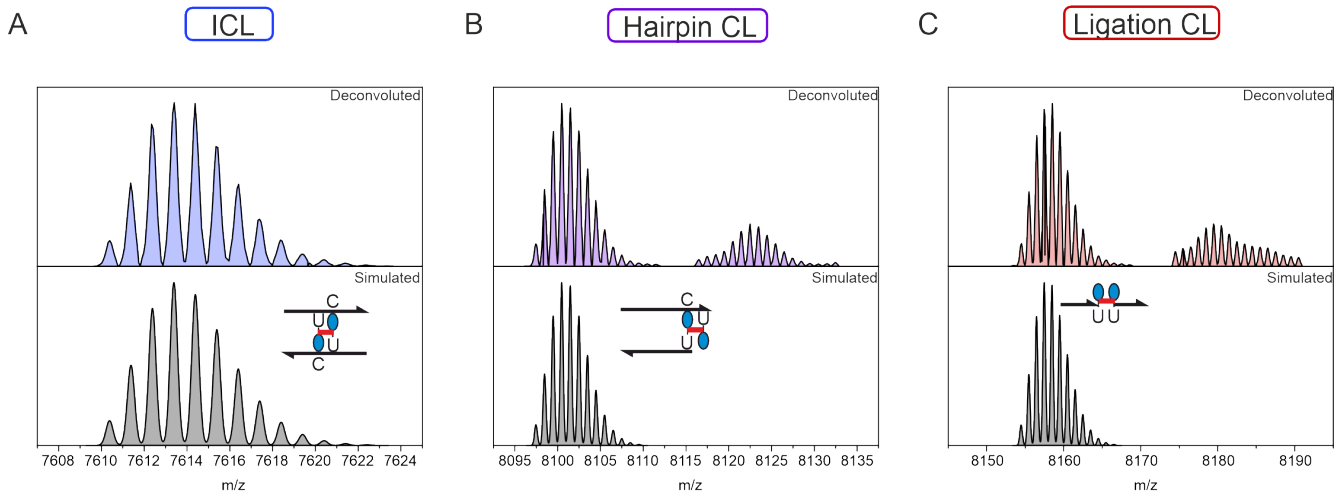


Figure S9 HR-ESI-MS analysis of the crosslinked DNA duplex for (A) ICL, (B) hairpin CL and (C) ligation CL. The simulated spectra correspond to the mass of the combined single strands.

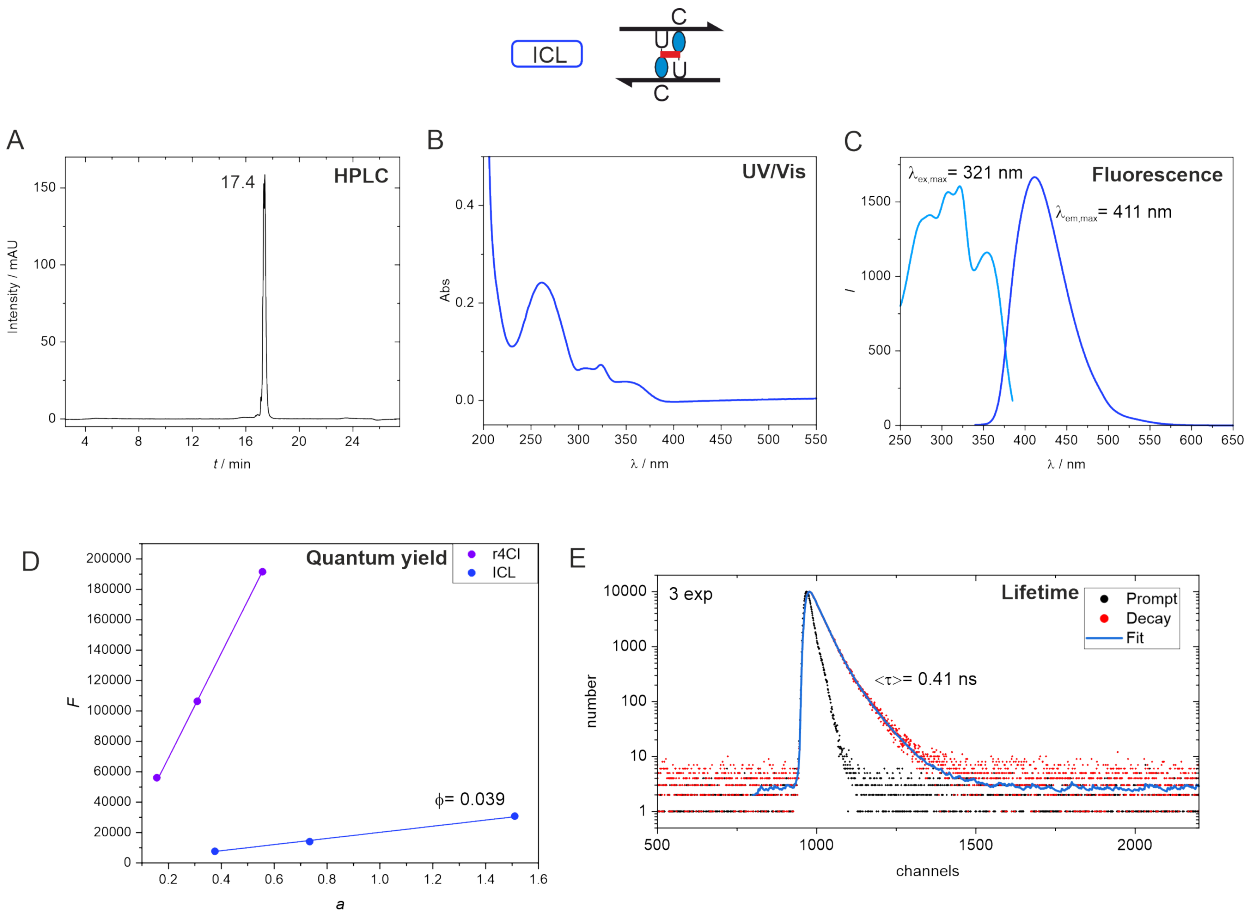


Figure S10 (A) Analytical anion exchange HPLC chromatogram, (B) UV/Vis spectra, (C) fluorescence excitation (λ_{em} = 405 nm) and emission (λ_{ex}=320 nm) spectra, (D) quantum yield determination and (E) life time measurement of the ICL containing duplex (10 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0).

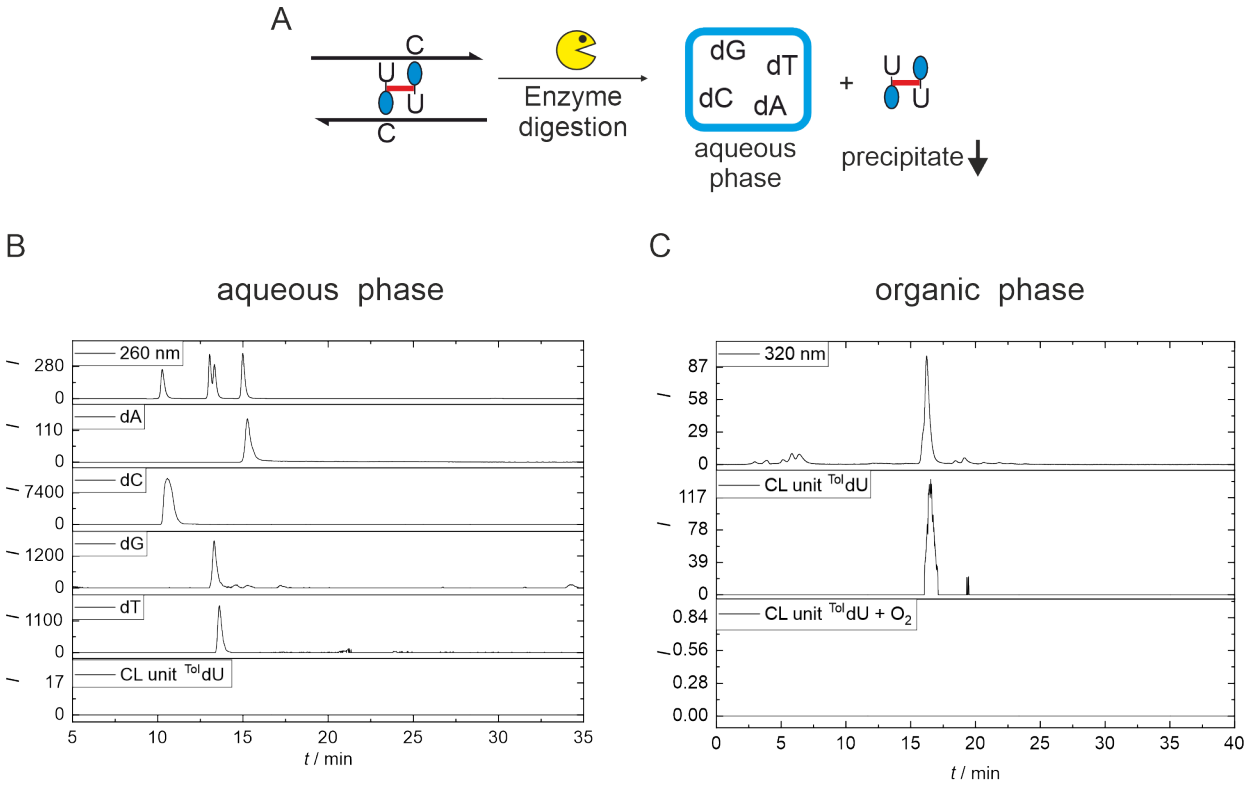


Figure S11 (A). Schematic drawing of the enzymatic digesting of the DNA duplex containing the ICL. The formed CL unit is water insoluble and was dissolved in MeCN/10 mM NH_4OAc (4/6, *v*/*v*) (B). LC-MS analysis of the aqueous phase. Extracted ion chromatograms (EIC) for CL unit ^{Tol}dU showing MH+ (m/z 857.28 ± 0.05) or MNa+ (m/z 879.26 ± 0.05) corresponding to two ^{Tol}dU units. (C) LC-MS analysis of the organic phase. EIC for CL unit ^{Tol}dU showing MH^+ (m/z 857.28 ± 0.05) or MNa^+ (m/z 879.26 ± 0.05) corresponding to two ^{Tol}dU units and EIC for CL unit ^{Tol}dU+O_2 showing MH^+ (m/z 889.271 ± 0.05) or MNa^+ (m/z 911.25 ± 0.05) corresponding to two ^{Tol}dU units with additional O_2. Incorporation of O_2 is not observed.

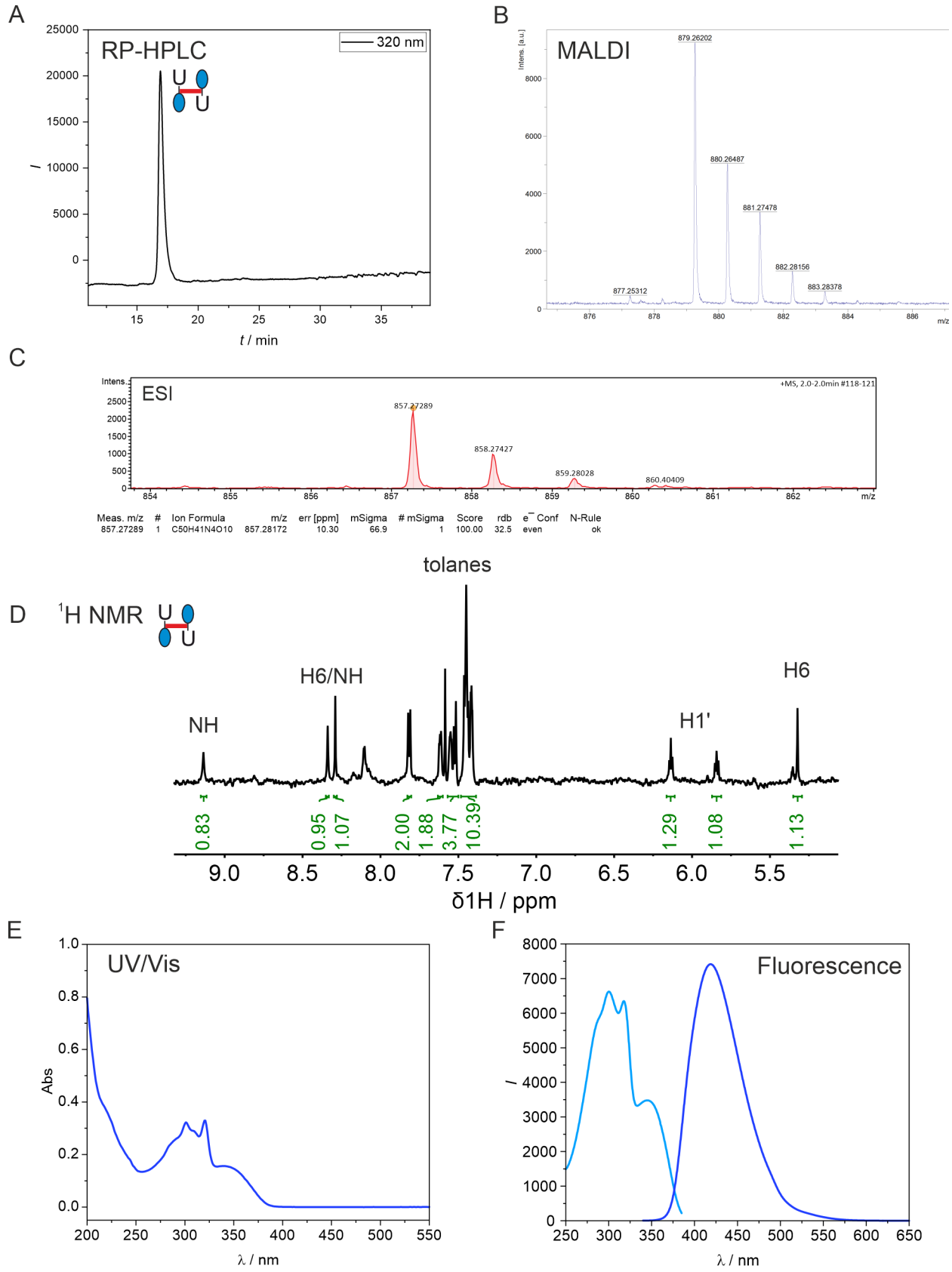
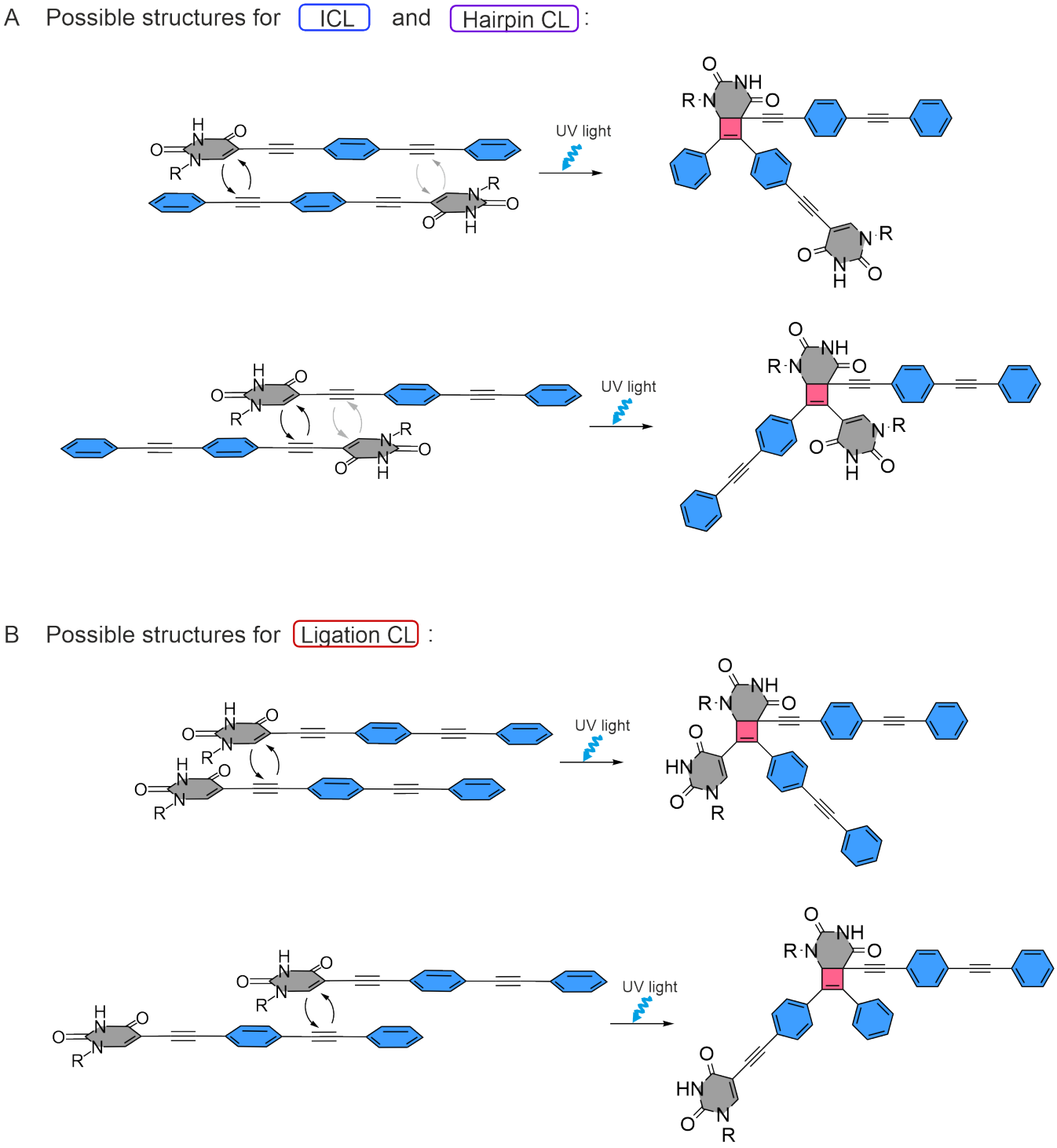


Figure S12 (A). Characterization of the RP-HPLC-purified crosslinked dimer unit by (A) analytical RP-HPLC using RP-18 column (Synergi, 4-µm Fusion-RP C18 80 Å, 250x2 mm) from Phenomenex on a JASCO HPLC system, (B) MALDI MS showing Na^+-adduct (879.262 Da), (C) HR-ESI-MS (857.272 Da), (D) ^{1}H-NMR (600 MHz in MeCN-d_3) with tentative assignment, (E) UV/Vis and (F) fluorescence excitation (λ_{em} = 405 nm) and emission (λ_{ex}=320 nm) spectra in MeCN.



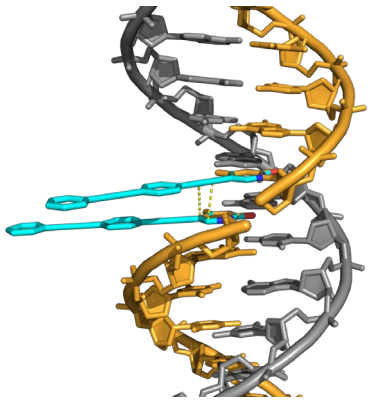


Figure S13. (A) Schematic drawing of possible structures for ICL and hairpin CL. The C5-C6 double bond could react with the inner or outer triple bond of the other ^{Tol}dU. In each case two isomers are possible (indicated by the light gray arrows for the second option). However, exact arrangement of the ^{Tol}dU units is not known in either case, and the spectroscopic results of the crosslinked products do not allow us to unambiguously distinguish the constitutional isomers.
(B) Schematic drawing of possible structures for nicked duplex ligation CL, involving either inner or outer triple bond. Left: A model of a nicked duplex with two ^{Tol}dU units (generated from an idealized B-form duplex) suggests a plausible parallel arrangement for the alkyne alkene cycloaddition.

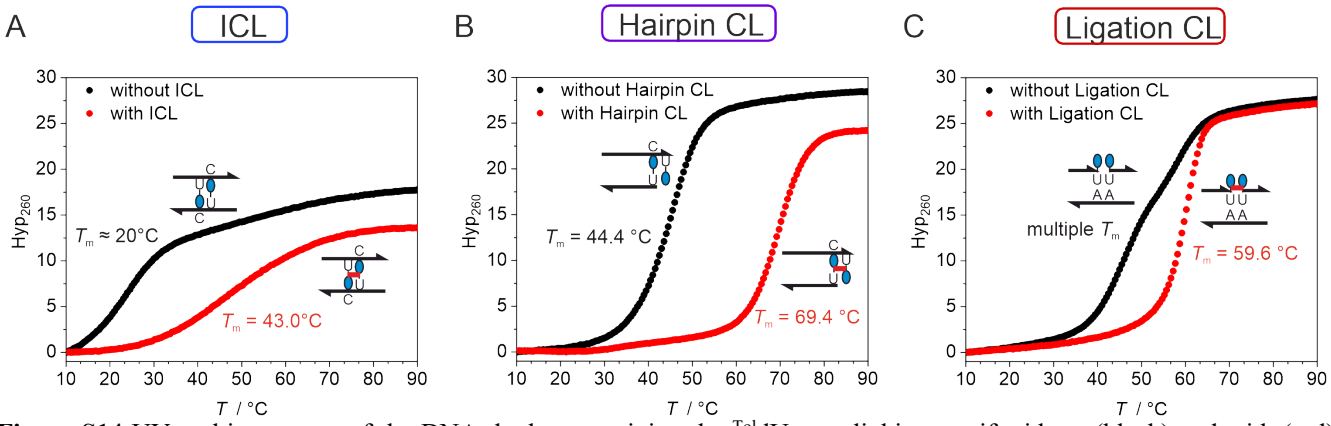


Figure S14 UV melting curves of the DNA duplex containing the ^{Tol}dU crosslinking motif without (black) and with (red) crosslinking for (A) ICL, (B) hairpin CL and (C) ligation CL (1 µM DNA in 10 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0).

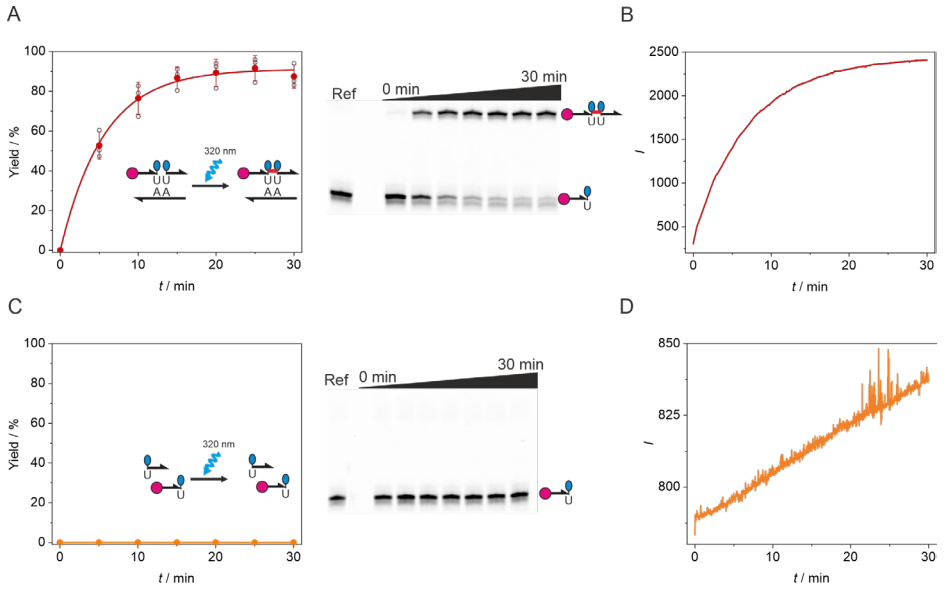


Figure S15 (A) Kinetic characterization of CL formation using the fluorescence spectrometer light source with PAGE analysis and fluorescence increasement at 410 nm of the DNA mixture for ligation CL (A and B) with and (C and D) without template. Cy3 for quantification is shown as a magenta circle (1 µM DNA in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0).

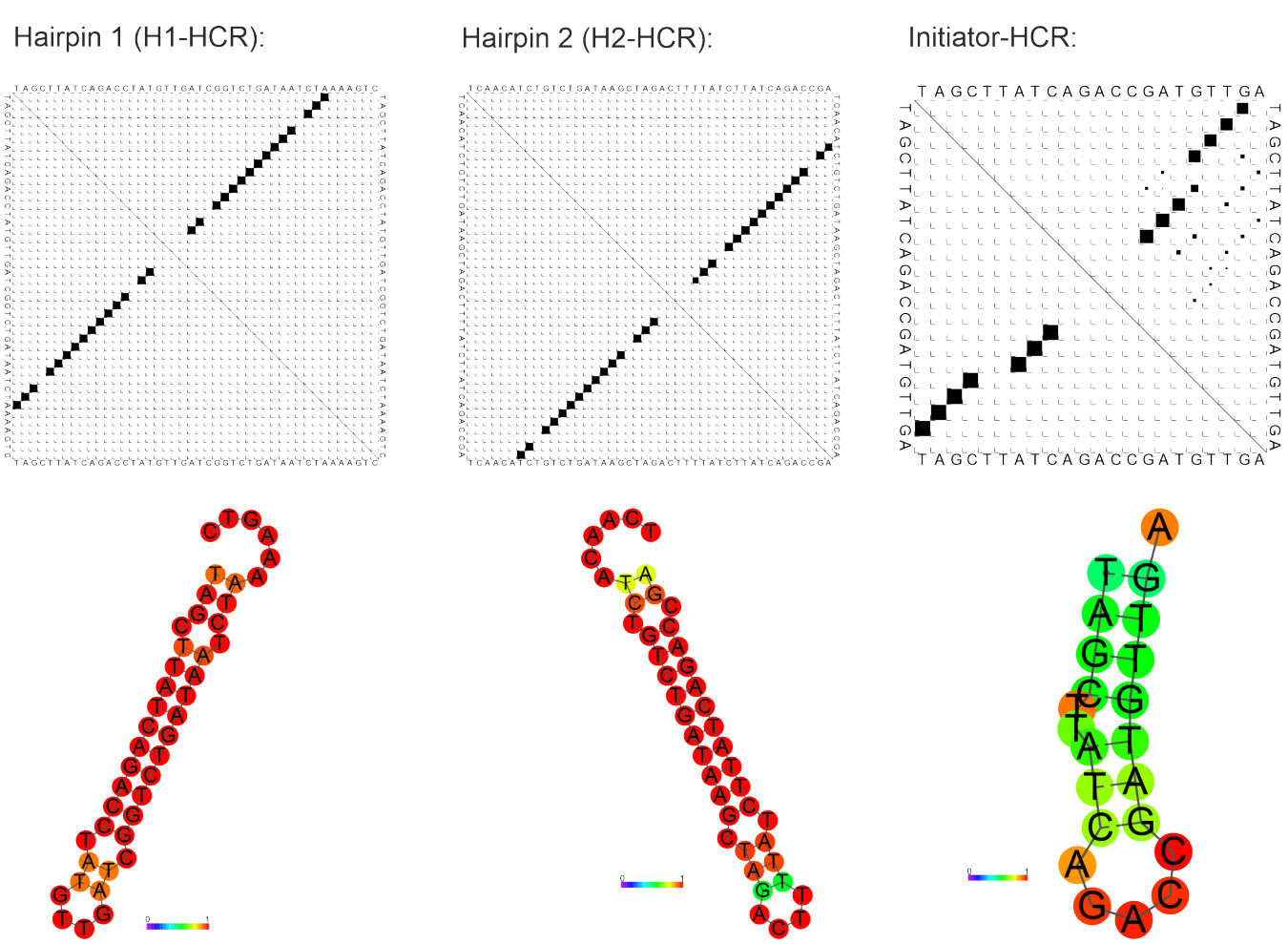
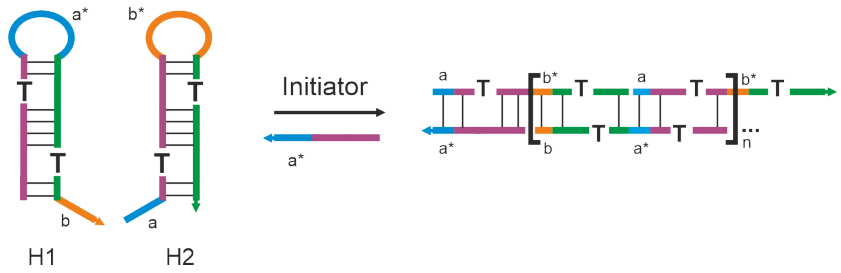


Figure S16. Secondary structure prediction of the DNA hairpins H1 and H2 and the initiator strand used for the HCR. Structure predictions and dot plots were generated using the ViennaRNA Web Service (http://rna.tbi.univie.ac.at/).

A



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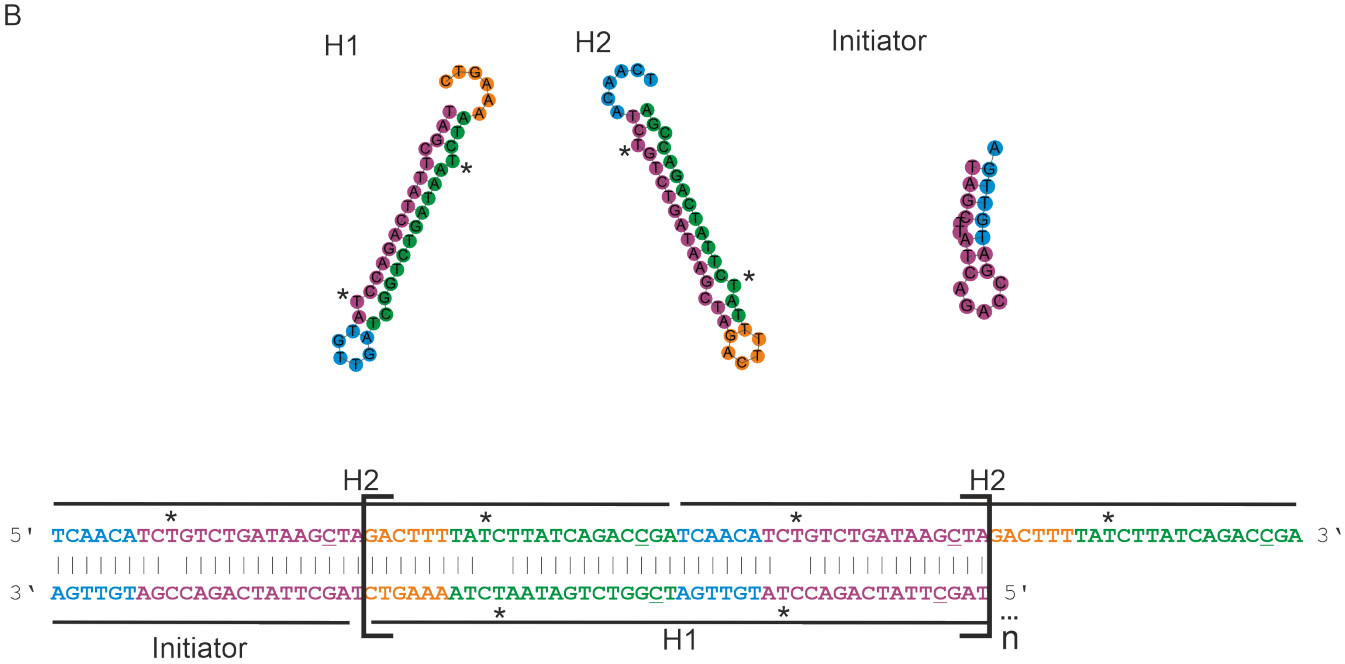

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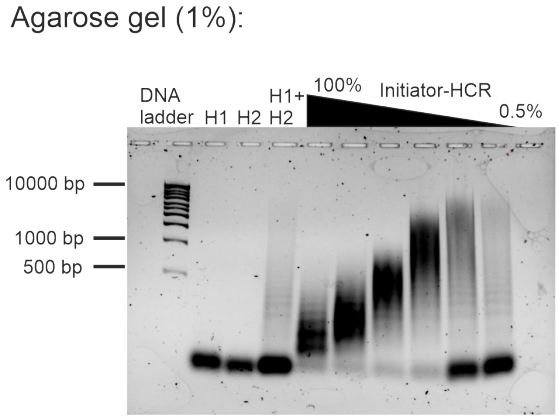


Figure S17. (A) Illustration of HCR with dC/dT mismatch in H1 and H2 and (B) sequence of hairpins, initiator, and HCR product. Nucleotides are labelled according to the schematic representation. Mismatched dC, which are base paired in the HCR product are underlined. Asterisks indicate mismatched Ts in the hairpins and in the HCR product. (C) The test of hairpins with two mismatches and no ^{Tol}dU modifications shows the formation of HCR products (0.5 µM DNA in 75 mM sodium citrate and 750 mM NaCl, pH 7.0).

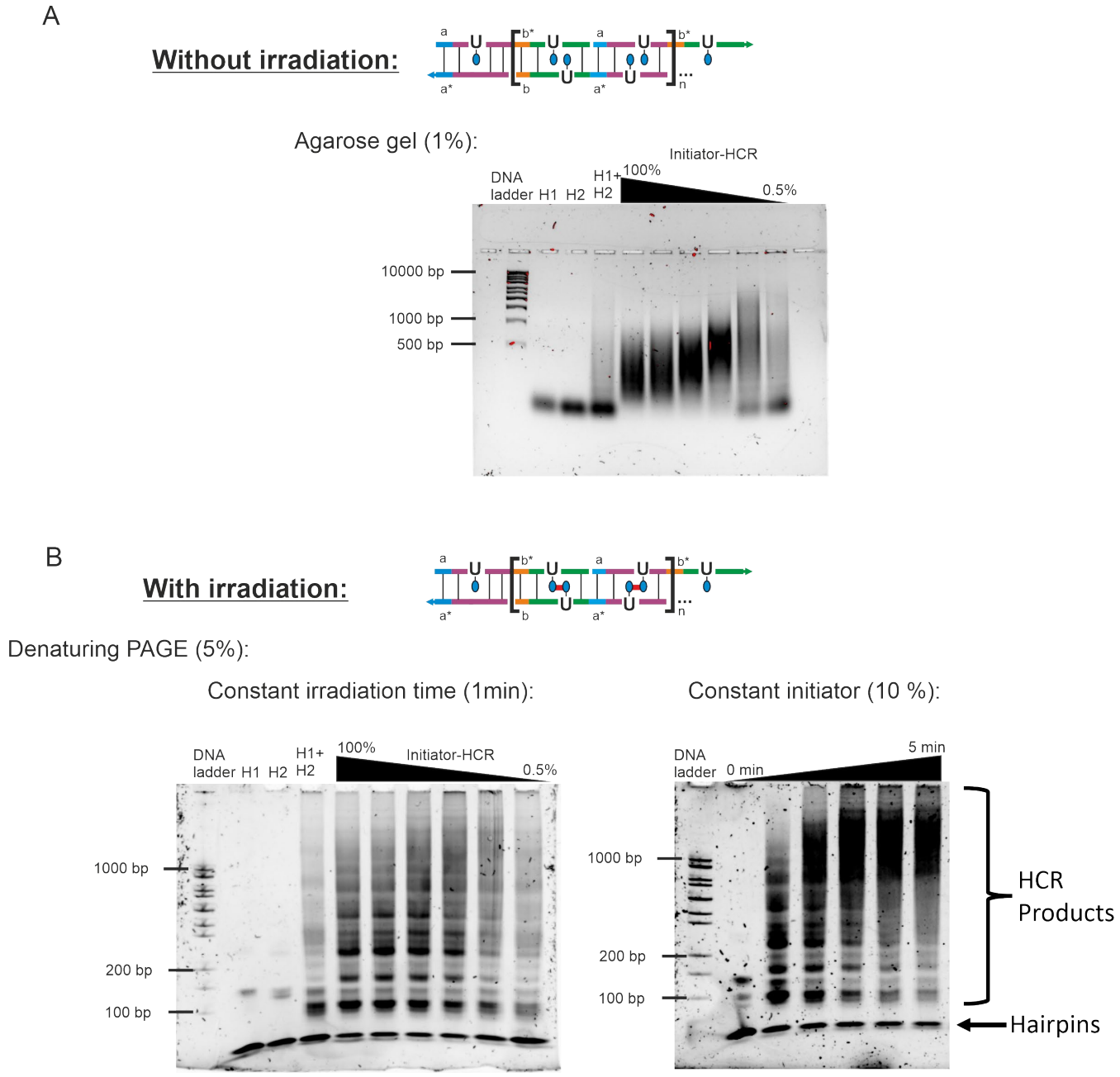


Figure S18 (A) HCR reaction with ^{Tol}dU containing crosslinks. (B) Analysis of HCR products after light irradiation with a transilluminator (312 nm). Denaturing PAGE showed the formation of covalently linked HCR products. (0.5 µM DNA in 75mM sodium citrate and 750 mM NaCl, pH 7.0).

Experimental Procedures

General Material and Methods

All standard chemicals were purchased from commercial suppliers. Trimethylsilylacetylene was obtained from Tokyo Chemical Industry and 2'-deoxy-5-iodouridine was purchased from Biosynth. Phenylacetylene, 1-bromo-4-iodobenzene and tetrakis(triphenylphosphine)palladium(0) were obtained from Sigma Aldrich. 4,4'-Dimethoxytrityl chloride and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite were obtained from ChemGenes Corporation.

2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-5-iodouridine (**S1**)[1] and 1-ethynyl-4-(2-phenylethynyl)benzene[2] were synthesized according to previously published protocols.

Dry solvents DCM, DMF and MeCN were obtained via a solvent purification system (SPS) from Inert Corporation. Pyridine for DMT-protection was obtained from Acros Organics and dried over activated molecular sieves. Technical grade solvents were used for column chromatography and distilled prior to use. All other organic solvents were used in pro analysis or for synthesis quality without further purification.

Column chromatography was carried out on silica gel (Kieselgel 60, Merck, 0.063 – 0.200 mm). Thin layer chromatography (TLC) was performed on aluminum-backed plates coated with silica gel and a fluorescent indicator (Alugram SIL G/UV254, Macherey-Nagel, UV visualization, 254 nm).

NMR-Spectroscopy and mass spectrometry

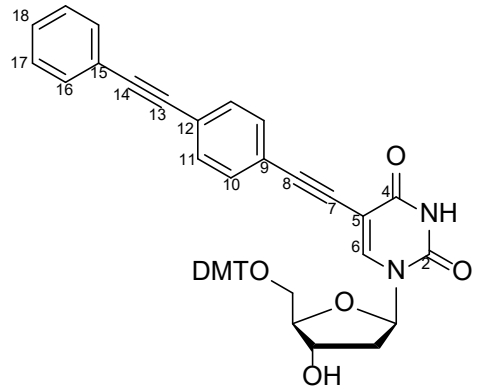
^{1}H-, ^{13}C- and ^{31}P-NMR spectra after chemical synthesis were recorded on a Bruker Avance HD III spectrometer at 400 MHz. Spectra were calibrated to the residual solvent peak of $CDCl_3$ (δ = 7.26 (^{1}H) and δ = 77.16 (^{13}C)) or DMSO-d_6 (δ = 2.50 (^{1}H) and δ = 39.52 (^{13}C)). Chemical shifts δ are given in ppm and coupling constants *J* are given in Hz. Multiplicities are denoted as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), ddd (doublet of doublet of doublet), m (multiplet).

NMR spectra of the building blocks were evaluated using MestReNova v12.0.4.

High resolution ESI mass spectra were measured on a Bruker micrOTOF-Q III spectrometer.

Synthetic procedures

Compound **S2**

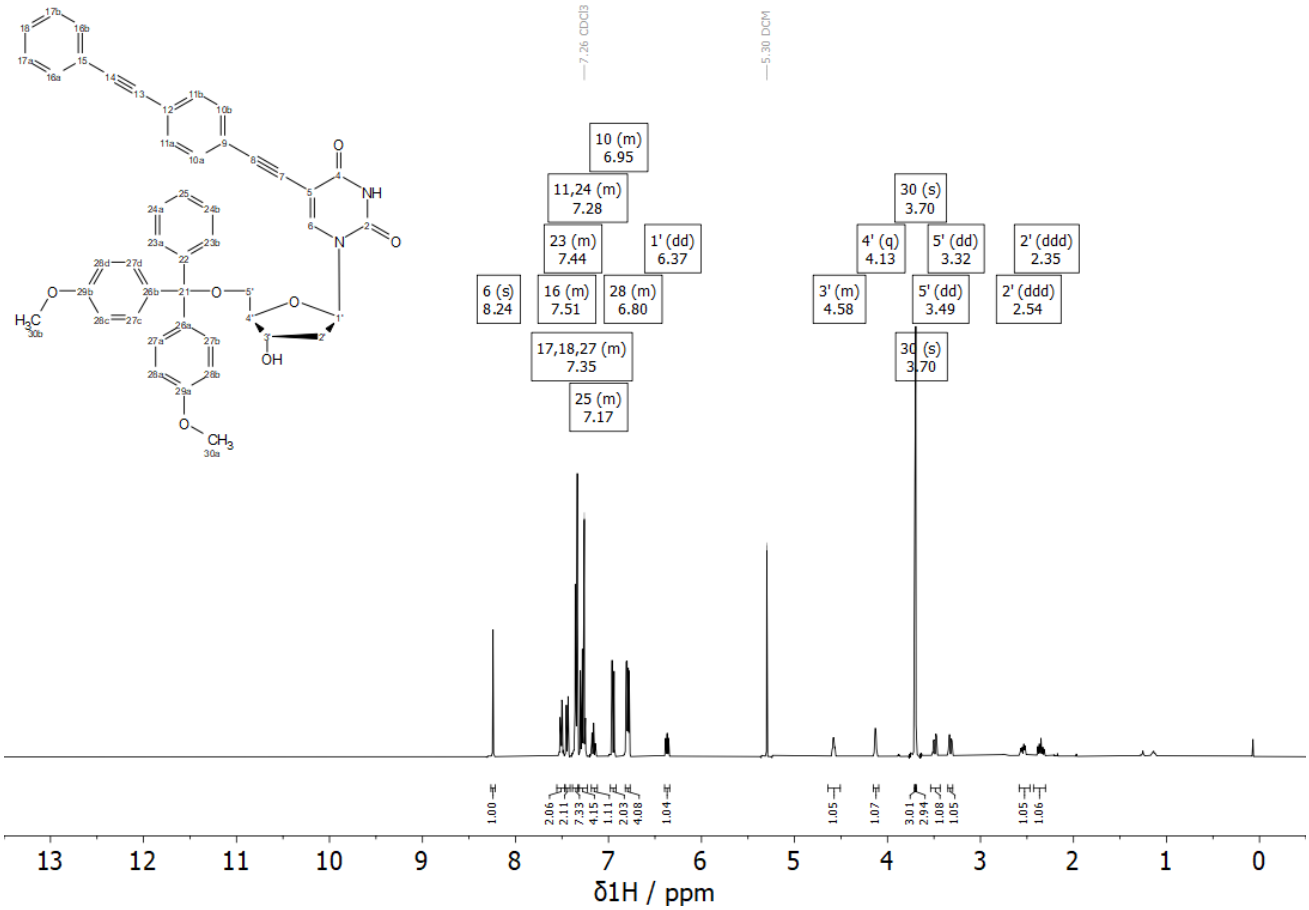


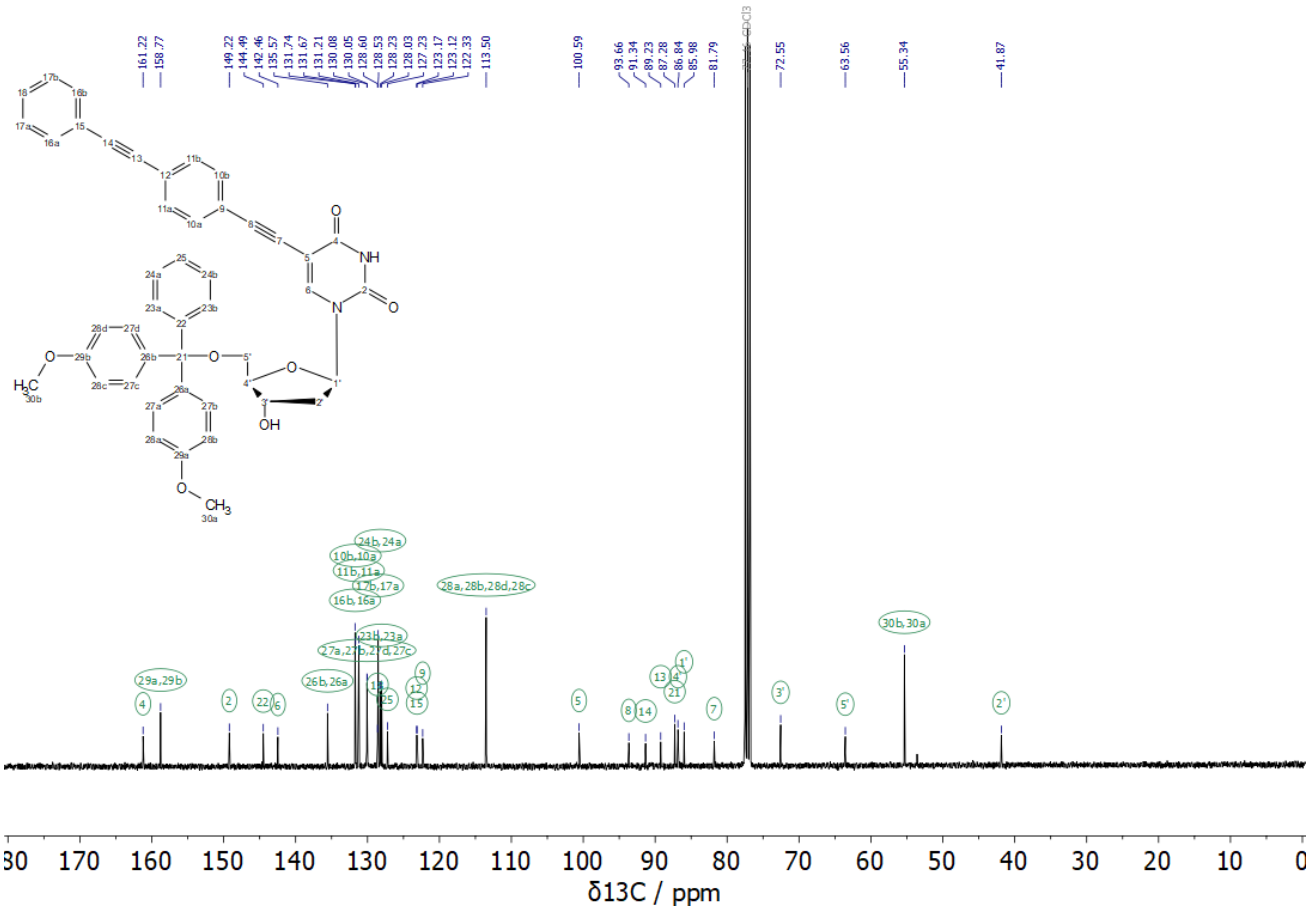
Under nitrogen atmosphere, compound **S1** (649 mg, 989 µmol, 1.00 eq), CuI (18.8 mg, 96.7 µmol, 10 mol%) and $Pd(PPh_3)_4$ (114 mg, 98.7 µmol, 10.0 mol%) were dissolved in argon-purged Et_3N/DMF (1:1) (50 mL). 1-Ethynyl-4-(2-phenylethynyl)benzene (300 mg, 1.50 mmol, 1.50 eq.) was added and stirring was continued over night at 60 °C. The reaction mixture was washed with a saturated solution of ammonium chloride (80 mL). The aqueous phase was extracted with DCM (4 x 150 mL). The combined organic phases were washed with water (80 mL) and brine (80 mL), dried over Na_2SO_4, evaporated and the residue was purified by column chromatography (DCM + 1 % Et_3N → DCM + 2 % MeOH + 1 % Et_3N) to afford compound **S2** as a yellow foam (437 mg, 598 µmol, 60 %).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.24 (s, 1H, 6-H), 7.56 – 7.47 (m, 2H, 16-H), 7.47 – 7.41 (m, 2H, DMT-H), 7.39 – 7.31 (m, 7H, 17-H, 18-H, DMT-H), 7.33 – 7.23 (m, 4H, 11-H, DMT-H), 7.18 – 7.12 (m, 1H, DMT-H), 6.98 – 6.92 (m, 2H, 10-H), 6.82 – 6.77 (m, 4H, DMT-H), 6.37 (dd, *J* = 7.6, 5.7 Hz, 1H, 1'-H), 4.64 – 4.51 (m, 1H, 3'-H), 4.13 (q, *J* = 2.9 Hz, 1H, 4'-H), 3.70 (s, 3H, DMT-H), 3.70 (s, 3H, DMT-H), 3.49 (dd, *J* = 10.8, 2.9 Hz, 1H, 5'-H), 3.32 (dd, *J* = 10.8, 3.3 Hz, 1H, 5'-H), 2.54 (ddd, *J* = 13.7, 5.9, 2.7 Hz, 1H, 2'-H), 2.35 (ddd, *J* = 13.6, 7.7, 5.9 Hz, 1H, 2'-H)

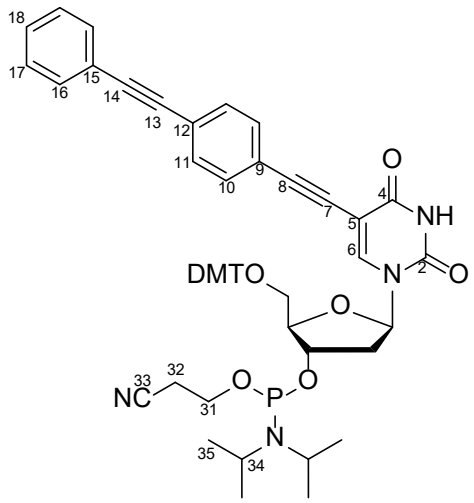
^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 161.22 (4-C), 158.77 (DMT -C), 149.22 (2-C), 144.49 (DMT -C), 142.46 (6-C), 135.57 (DMT -C), 131.74 (16-C), 131.67 (10-C), 131.21 (11-C), 130.08 (DMT -C), 130.05 (DMT -C), 128.60 (18-C), 128.53 (17-C), 128.23 (DMT -C), 128.03 (DMT -C), 127.23 (DMT -C), 123.17 (12-C), 123.12 (15-C), 122.33 (9-C), 113.50 (DMT -C), 100.59 (5-C), 93.66 (8-C), 91.34 (14-C), 89.23 (13-C), 87.28 (DMT -C), 86.84 (4'-C), 85.98 (1'-C), 81.79 (7-C), 72.55 (3'-C), 63.56 (5'-C), 55.34 (DMT-C), 41.87 (2'-C);

HR-MS (ESI+): *m/z* calc. ($C_{46}H_{38}N_2O_7Na$, $[M+Na]^+$): 753.25712, found: 753.25754.





Compound **S3**



Under nitrogen atmosphere, compound **S2** (350 mg, 479 µmol, 1.00 eq.) was dissolved with DIPEA (491 µL, 372 mg, 2.88 mmol, 6.00 eq.) in anhydrous DCM (14 mL). After 10 min CEP-Cl (162 mg, 684 µmol, 1.30 eq.) was added. After 1.5 h stirring at room temperature additional CEP-Cl (40.0 mg, 169 µmol, 0.35 eq.) was added in two portions in a 1.5 h interval. The reaction mixture was stirred additionally at ambient temperature for 1 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (EtOAc + 1% Et_3N) to afford compound **S3** as a yellow foam (415 mg, 445 µmol, 93%).

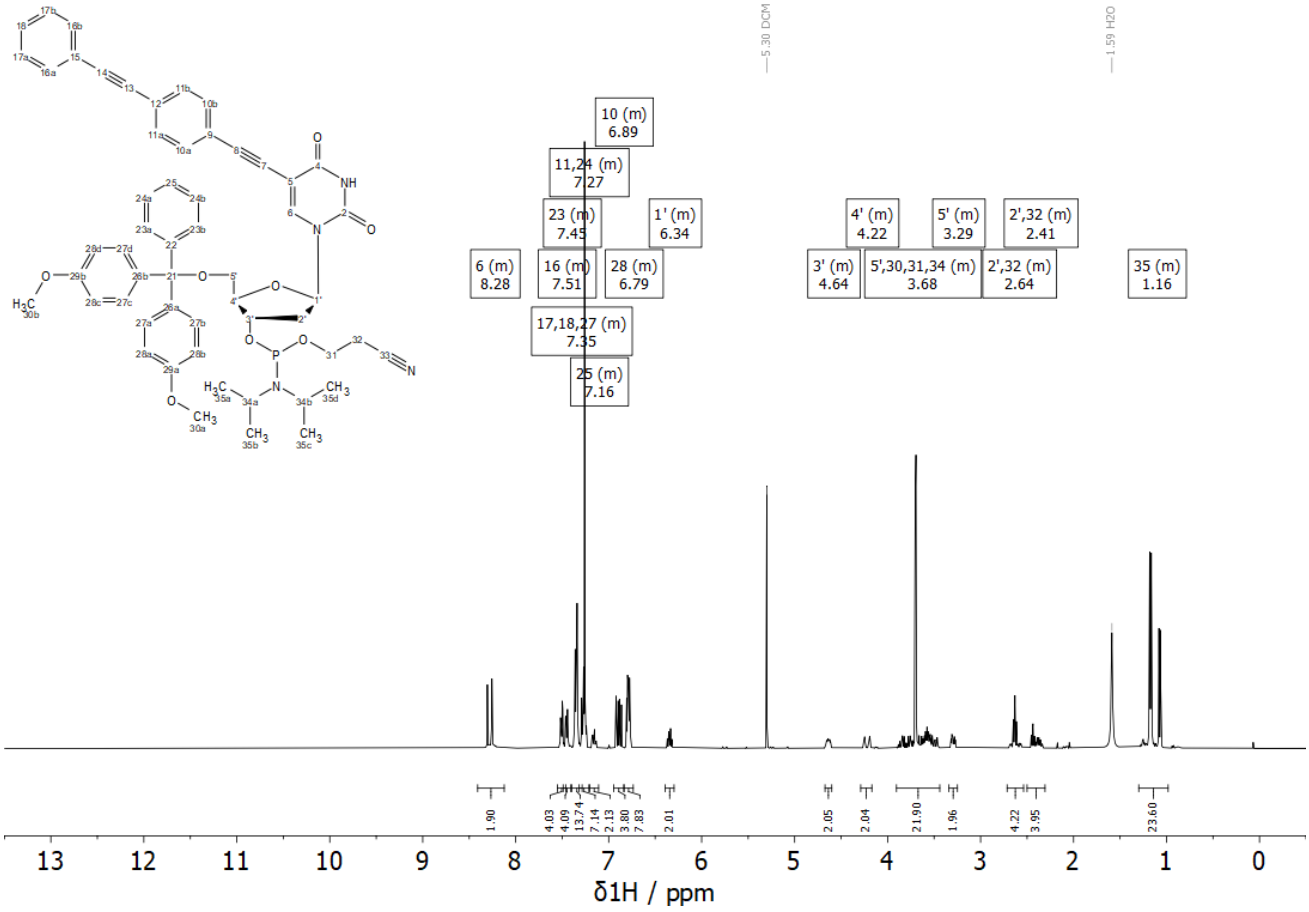
^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.41 – 8.12 (m, 2H, 6-H), 7.55 – 7.46 (m, 4H, 16-H), 7.49 – 7.41 (m, 4H, DMT-H), 7.40 – 7.29 (m, 14H, 17-H, 18-H, DMT-H), 7.32 – 7.21 (m, 6H, 11-H, DMT-H), 7.20 – 7.11 (m, 2H, DMT-H), 6.95 – 6.84 (m, 4H, 10-H), 6.84 – 6.74 (m, 8H, DMT-H), 6.39 – 6.30 (m, 2H, 1'-H), 4.67 – 4.60 (m, 2H, 3'-H), 4.29 – 4.17 (m, 2H, 4'H),

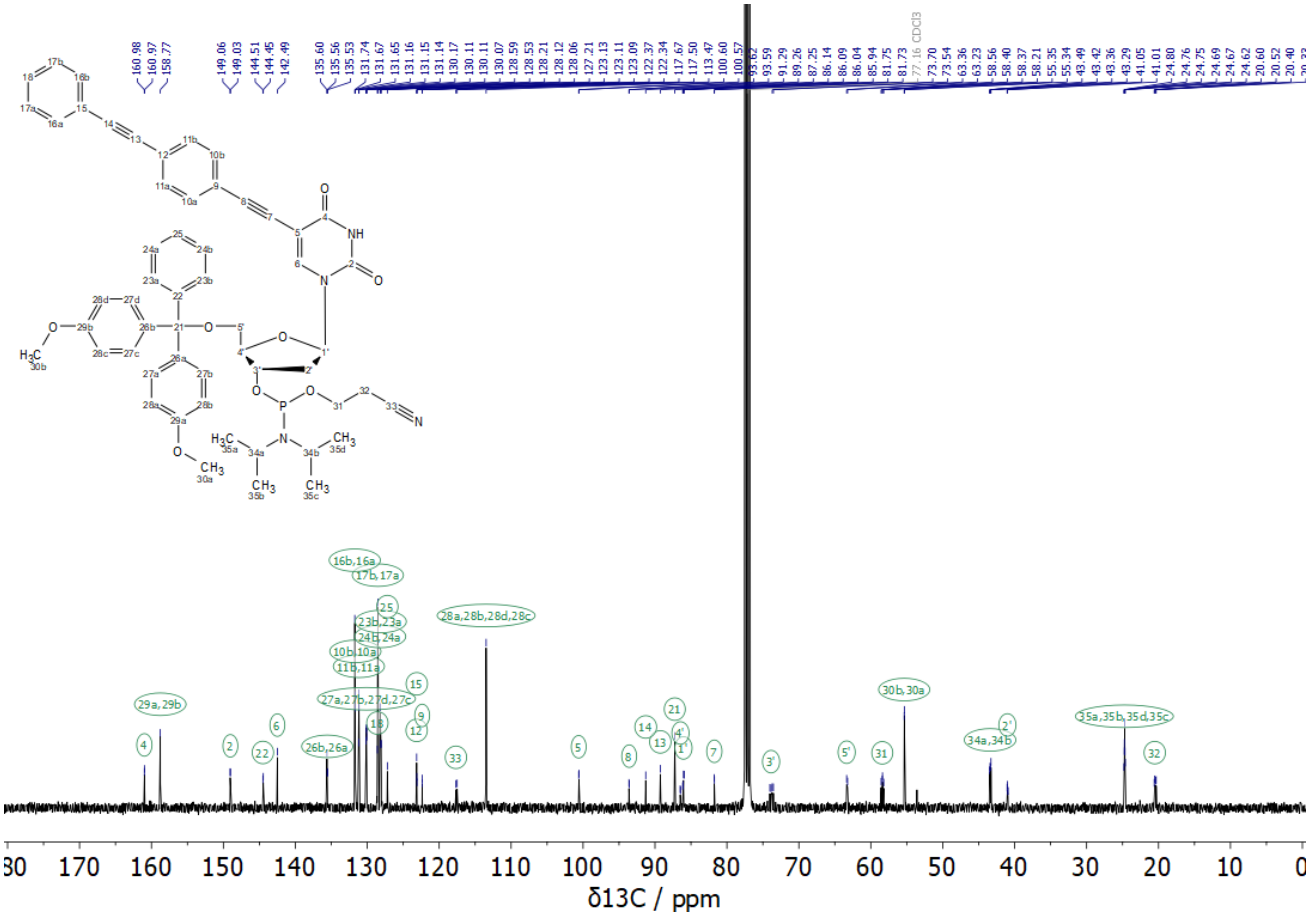
3.90 – 3.44 (m, 22H, 5'-H, 31-H, 34-H, DMT-H), 3.34 – 3.25 (m, 2H, 5'-H), 2.71 – 2.54 (m, 4H, 2'-H, 32-H), 2.50 – 2.31 (m, 4H, 2'-H, 32-H), 1.30 – 0.98 (m, 24H, 35-H);

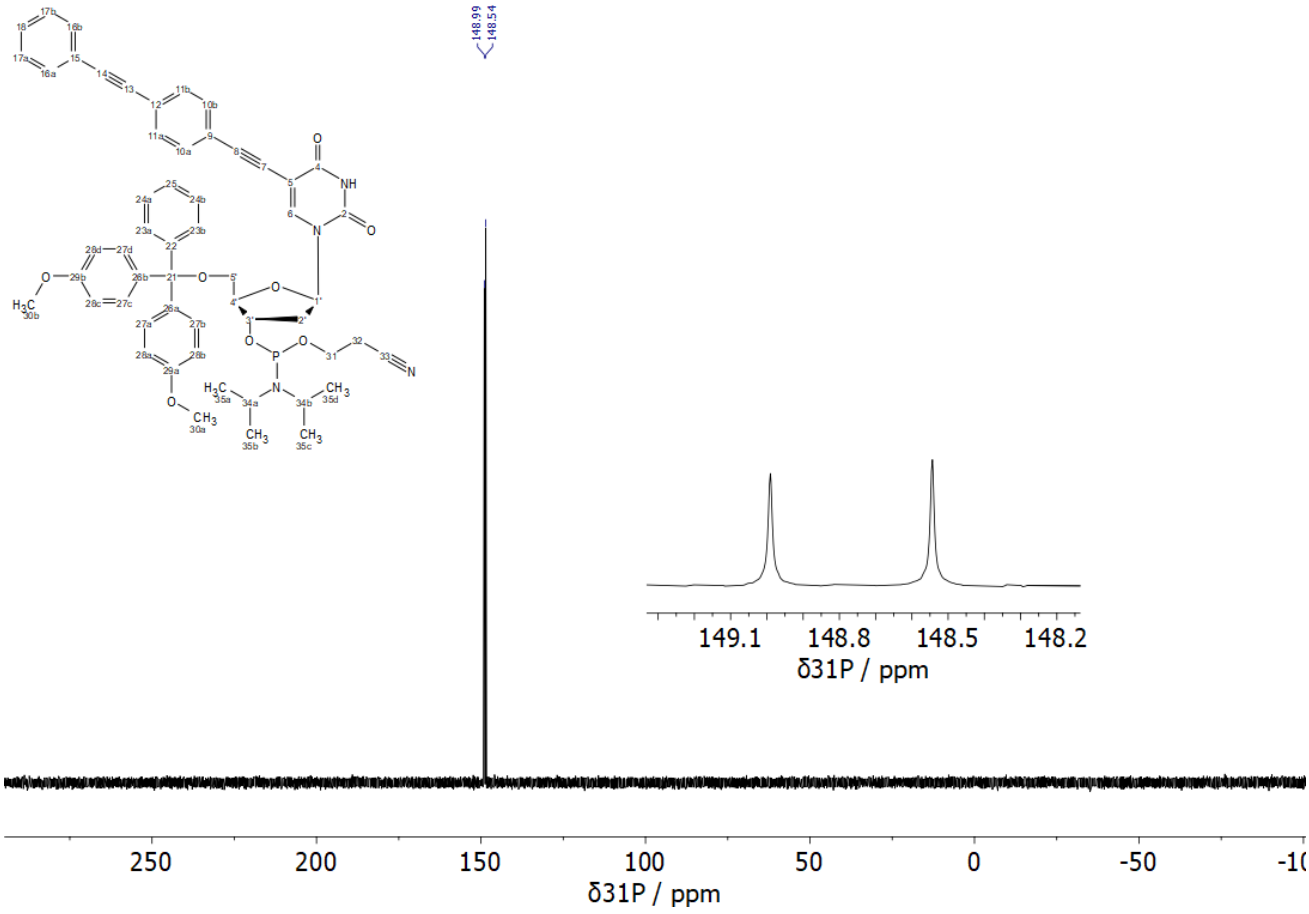
^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 160.98 (4-C), 160.97 (4-C), 158.77 (DMT-C), 149.06 (2-C), 149.03 (2-C), 144.51 (DMT-C), 144.45 (DMT-C), 142.49 (6-C), 135.60 (DMT-C), 135.56 (DMT-C), 135.53 (DMT-C), 131.74 (16-C), 131.67 (10-C), 131.65 (10-C), 131.16 (11-C), 131.14 (11-C), 130.17 (DMT-C), 130.11 (DMT-C), 130.11 (DMT-C), 130.07 (DMT-C), 128.59 (18-C), 128.53 (17-C), 128.21 (DMT-C), 128.12 (DMT-C), 128.06 (DMT-C), 127.21 (DMT-C), 123.13 (12-C), 123.11 (15-C), 123.09 (15-C), 122.37 (9-C), 122.34 (9-C), 117.67 (33-C), 117.50 (33-C), 113.47 (DMT-C), 100.60 (5-C), 100.57 (5-C), 93.62 (8-C), 93.59 (8-C), 91.29 (14-C), 89.26 (13-C), 87.25 (DMT-C), 86.49 (4'-C), 86.46 (4'-C), 86.14 (4'-C), 86.09 (4'-C), 86.04 (1'-C), 85.94 (1'-C), 81.75 (7-C), 81.73 (7-C), 74.03 (3'-C), 73.86 (3'-C), 73.70 (3'-C), 73.54 (3'-C), 63.36 (5'-C), 63.23 (5'-C), 58.56 (31-C), 58.40 (31-C), 58.37 (31-C), 58.21 (31-C), 55.35 (DMT-C), 55.34 (DMT-C), 43.49 (34-C), 43.42 (34-C), 43.36 (34-C), 43.29 (34-C), 41.05 (2'-C), 41.01 (2'-C), 40.96 (2'-C), 40.92 (2'-C), 24.80 (35-C), 24.76 (35-C), 24.75 (35-C), 24.69 (35-C), 24.67 (35-C), 24.62 (35-C), 20.60 (32-C), 20.52 (32-C), 20.40 (32-C), 20.33 (32-C);

^{31}P NMR (162 MHz, $CDCl_3$): δ (ppm) = 148.99, 148.54;

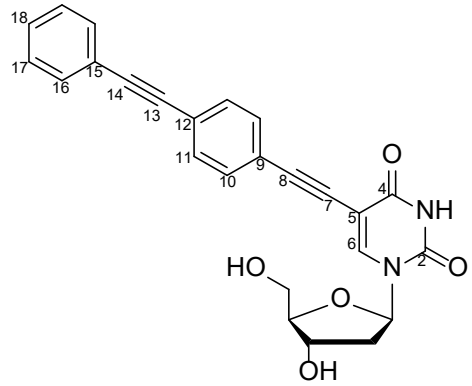
HR-MS (ESI+): *m/z* calc. ($C_{55}H_{55}N_4O_8PNa$, $[M+Na]^+$): 953.36497, found: 953.36499.




δ13C / ppm


δ31P / ppm

Compound **S4**

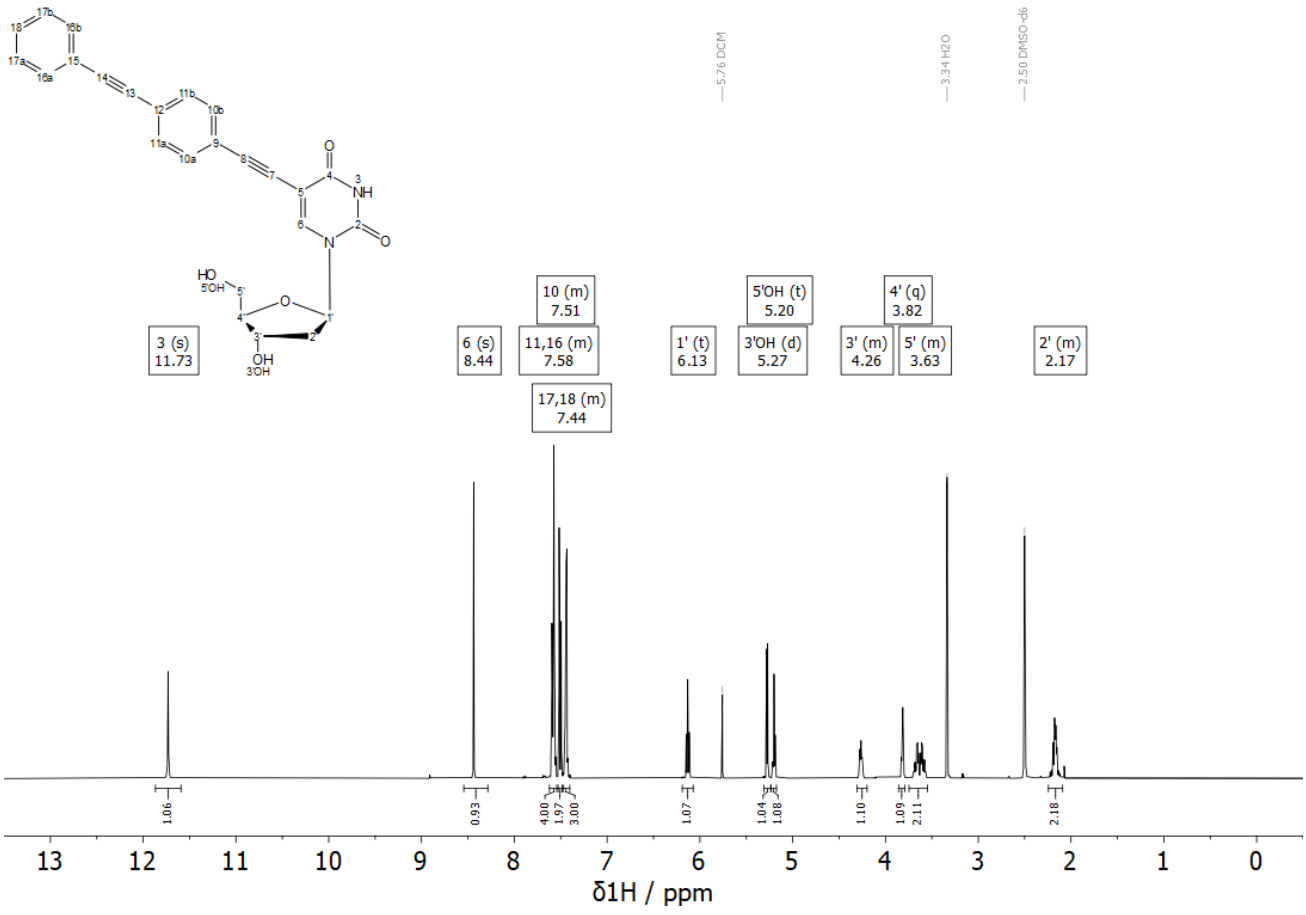


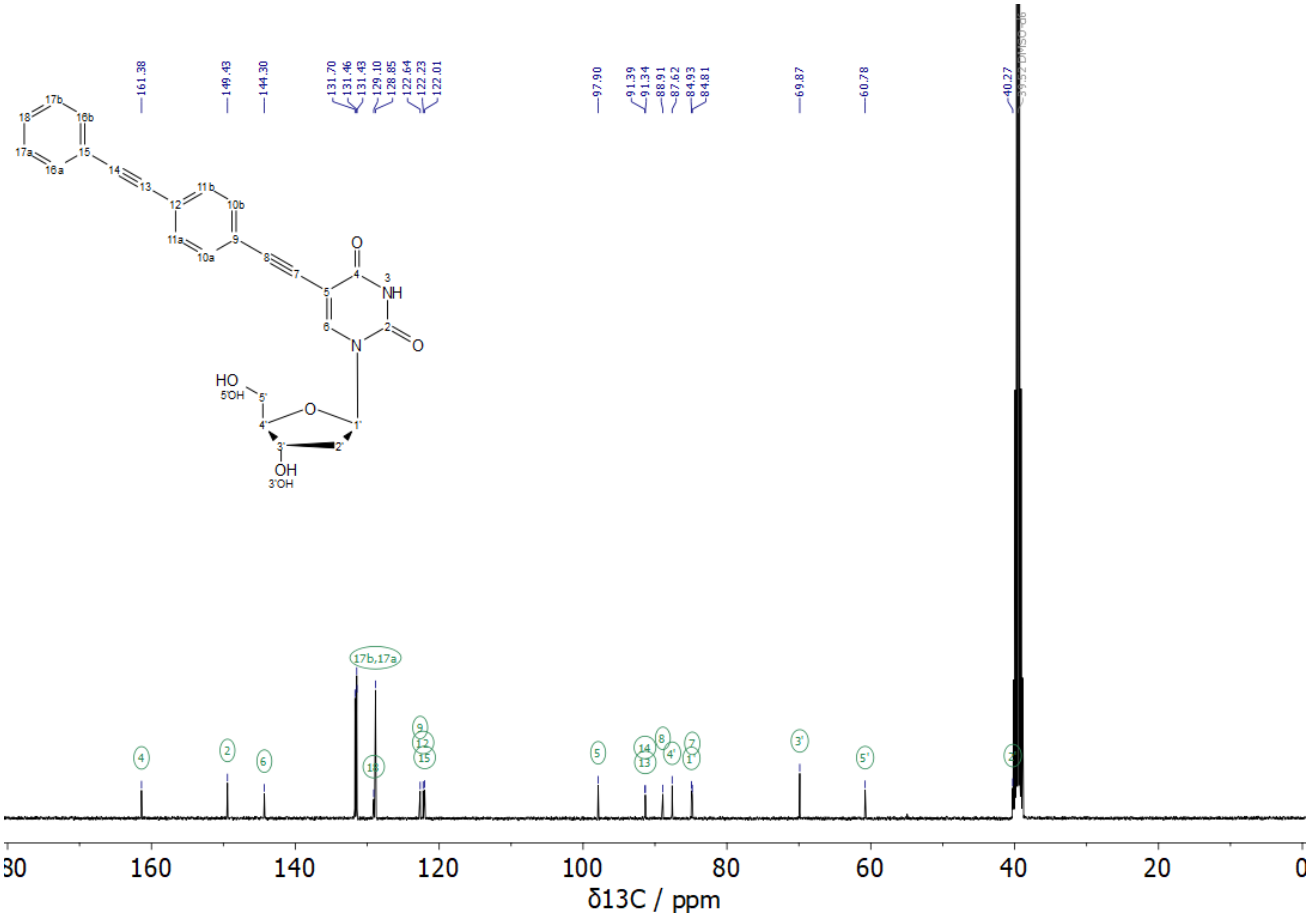
Compound **S2** (200 mg, 274 µmol, 1.00 eq.) was dissolved in DCM (22 mL). TFA (425 µL, 633 mg, 5.55 mmol, 20.3 eq.) was added and the mixture was stirred for 15 min at room temperature. Nitrogen was bubbled through the solution to remove TFA. MeOH (2 mL) was added and removed under reduced pressure four times. The residue was purified by column chromatography (DCM + 2 % MeOH → DCM + 10 % MeOH). Additionally, the residue was washed with MeCN to afford compound **S4** as a yellow solid (65.4 mg, 254 µmol, 24%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 11.73 (s, 1H, 3-H), 8.44 (s, 1H, 6-H), 7.62 – 7.53 (m, 4H, 11-H, 16-H), 7.54 – 7.47 (m, 2H, 10-H), 7.48 – 7.40 (m, 3H, 17-H, 18-H), 6.13 (t, J = 6.5 Hz, 1H, 1'-H), 5.27 (d, J = 4.3 Hz, 1H, 3'-OH), 5.20 (t, J = 4.9 Hz, 1H 5'-OH), 4.30 – 4.20 (m, 1H, 3'H), 3.82 (q, J = 3.4 Hz, 1H, 4'-H), 3.75 – 3.55 (m, 2H, 5'-H), 2.24 – 2.09 (m, 2H, 2'-H);

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 161.38 (4-C), 149.43 (2-C), 144.30 (6-C), 131.70 , 131.46, 131.43, 129.10 (18-C), 128.85 (17-C), 122.64 (9-C), 122.23 (12-C), 122.01 (15-C), 97.90 (5-C), 91.39 (14-C), 91.34 (13-C), 88.91 (8-C), 87.62 (4'-C), 84.93 (1'-C), 84.81 (7-C), 69.87 (3'-C), 60.78 (5'-C), 40.27 (2'-C);

HR-MS (ESI+): *m/z* calc. ($C_{25}H_{20}N_2O_5Na$, $[M+Na]^+$): 451.12644, found: 451.12733.





Oligonucleotide synthesis

Oligonucleotides were synthesized on an Applied Biosystems ABI 392 DNA/RNA synthesizer on a 0.6 µmol scale using standard phosphoramidite chemistry. DMT-dA(bz)-CPG, DMT-dC(ac)-CPG, DMT-dG(dmf)-CPG and DMT-dT-CPG with a pore size of 1000 Å and loading density of 25 – 35 µmol/g were used as solid supports and were obtained from Sigma Aldrich. For 3'- ^{Tol}dU functionalized oligonucleotides a ^{Tol}dU-modified solid support was prepared using native amino lcaa CPG (1000 Å) from ChemGenes Corporation. Compound **S2** was attached with BOP after functionalization with a succinyl ester to a lcaa CPG support. The loading density of the CPG support was determined via UV/Vis absorption of the cleaved DMT cation at 498 nm.[3] DMT-dA(bz), DMT-dC(ac), DMT-dG(dmf) and DMT-dT were purchased from ChemGenes Corporation. Hex-5-yn-1-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite[4] and 3-(4,4'-dimethoxytrityloxy)propyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite[5] were synthesized as described previously. Solutions of standard phosphoramidites (70 mM) and synthesized phosphoramidites (80 mM) were prepared in anhydrous MeCN. The following solutions were used in the synthesis:

Activator: 0.25 M ethylthiotetrazole (ETT) in anhydrous acetonitrile,
Oxidation: 20 mM iodine in THF/water/pyridine (66:12:22, *v*/*v*/*v*)
Cap A: pyridine/acetic anhydride/THF (10/10/80, *v*/*v*/*v*)
Cap B: NMI in THF (84/16, *v*/*v*).
Deprotection: 3% trichloro acetic acid in 1,2-dichloroethane

Cleavage from the solid support and removal of the base labile protecting groups was performed by treatment with concentrated ammonium hydroxide (33% NH_3) at 25 °C or 50 °C for 3' ^{Tol}dU-modified oligonucleotides overnight. The solid support was filtered off and the solvent was removed under reduced pressure. The residue was dissolved in water. The crude product was purified by denaturing PAGE. Gels (0.7x200x300 mm) were prepared using a 15% or 20% acrylamide solution containing 7 M urea in 1x TBE. After polymerization the gels were run in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 2.5 h at a constant power (35 W). Product bands were visualized using a TLC plate under UV illumination. Oligonucleotides were extracted with TEN buffer (10 mM Tris-HCl, 0.1 mM EDTA, 300 mM NaCl, pH 8.0) and recovered after precipitation with ethanol. DNA strands that could not be precipitated with ethanol were desalted by size exclusion chromatography using two HiTRAP desalting columns (5 mL each) from GE Healthcare on an ÄKTAstart purification system. Oligonucleotides were eluted with water (1 mL/min). After lyophilization the oligonucleotides were dissolved in a defined volume of water.

Purified oligonucleotides were analyzed by anion exchange HPLC using a GE Healthcare ÄKTApurifier with a DNAPac PA 200 column (2x250 mm) from Thermo Scientific at a flow rate of 0.5 mL min^{-1}. Linear gradients of 0–48 % B over 24 min of buffer A (25 mM Tris-HCl, 6 M urea, pH 8.0) and buffer B (25 mM Tris-HCl, 0.5 M $NaClO_4$, 6 M urea, pH 8.0) were used for analysis. The chromatograms were monitored at 260 nm. All analyses were performed at 60 °C. High resolution ESI mass spectra were recorded on a Bruker micrOTOF-Q III spectrometer.

Labeling of 5'-alkyne functionalized oligonucleotides

5'-Alkyne functionalized oligonucleotides were fluorescently labeled using copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). Sulfo-Cy3-azide was obtained by Lumiprobe GmbH. Freeze-dried DNA oligonucleotide (5 nmol) was dissolved in water (5 µL) and mixed with a DMSO/^{t}BuOH mixture (3:1, 3 µL). A solution of azide (0.63 µL, 50 mM) in DMSO/^{t}BuOH (3:1, *v*/*v*) was added. A freshly prepared solution of CuBr (0.63 µL, 100 mM) in DMSO/ ^{t}BuOH (3:1) was combined with a solution of tris(benzyltriazolylmethyl)amine (1.26 µL, 100 mM) in DMSO/ ^{t}BuOH (3:1) and then added to the reaction mixture. After incubation for 3 h in the dark at 37 °C, the reaction mixture was purified by PAGE (20% polyacrylamide).

UV/Vis spectroscopy / Thermal denaturing experiments

UV/Vis spectra were measured in 10 mm quartz cuvettes from Agilent using an Agilent Cary 3500 with following settings:

- Wavelength: 200-750 nm
- Averaging time: 0.02 s
- Data interval: 0.2 nm
- Scan rate: 300 nm min^{-1}
- Spectral bandwidth: 2 nm

For the determination of the extinction coefficient of the ^{Tol}dU nucleoside a stock solution in DMSO (10 mM) was prepared. Spectra measurement for extinction coefficient determination started with a 20 µM dilution in DMSO or MeOH which were serially diluted six times with DMSO or seven times with MeOH in a 1:1 ratio (20.0, 10.0, 5.00, 2.5, 1.25, 0.63, 0.31 µM) to prepare a concentration series. ^{Tol}dU crosslinking unit was measured in pure acetonitrile. Spectra were recorded at 20 °C.

Temperature dependent UV/Vis spectra were recorded every 10 °C in a range of 10-90 °C. 1 µM DNA samples in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) were heated for 1 min at 95 °C and stored at least 30 min at room temperature prior to measurement. The samples in the cuvettes were overlaid with silicone oil.

UV denaturing melting experiments were recorded on Varian Cary100 equipped with a 6x6 Multicell Block Peltier Series II cell changer and a VARIAN CARY Temperature Controller. Absorption was measured at 250, 260 and 280 nm. The absorption was recorded with a spectral bandwidth of 1 nm and the averaging time was set to 2 s. The temperature cycle was programmed as follows: 1. 20 °C to 90 °C; 2. 90 °C to 10 °C; 3.10 °C to 90 °C; 4. 90 °C to 10 °C; 5. 10 °C to 90 °C

The first ramp was performed for annealing and was not considered for further melting temperature analysis. The heating rate was set to 0.5 °C min^{-1}. 500 µL sample in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) with a duplex concentration of 1 µM, 2 µM and 5 µM was measured in 10 mm quartz cuvettes from VARIAN and 300 µL sample in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) with a duplex concentration of 10 µM and 20 µM was measured in 1 mm quartz cuvettes from HELLMA. The samples in the cuvettes were overlaid with silicone oil.

The obtained curves were fitted in a two-state transition model with upper and lower limit to obtain the melting temperature T_m. Absorption of the melted duplexes was used to recalculate c_{total}. For the estimation of the thermodynamic parameters melting curves were analyzed according to Breslauer et al[6]:

$$\frac{\mathbf{1}}{\boldsymbol{T}_{\mathbf{m}}} = \frac{(\mathbf{n}-\mathbf{1})}{\Delta \boldsymbol{H}^{\mathbf{0}}} \ln \boldsymbol{c}_{\mathbf{total}} + \frac{\Delta \boldsymbol{S}^{\mathbf{0}} - (\mathbf{n}-\mathbf{1}) \cdot \boldsymbol{R} \cdot \ln \mathbf{2n}}{\Delta \boldsymbol{H}^{\mathbf{0}}} \quad (1)$$

Assuming a bimolecular association of two non-self-complementary strands leads to a molecularity n = 2:

$$\frac{\mathbf{1}}{\boldsymbol{T}_{\mathbf{m}}} = \frac{\ln \boldsymbol{c}_{\mathbf{total}}}{\Delta \boldsymbol{H}^{\mathbf{0}}} + \frac{\Delta \boldsymbol{S}^{\mathbf{0}} - \boldsymbol{R} \cdot \ln \mathbf{4}}{\Delta \boldsymbol{H}^{\mathbf{0}}} \quad (2)$$

Using van't Hoff analysis enthalpy ΔH^0 could be obtained from the slope and entropy ΔS^0 from the intercept of a linear fit of ln c_{total} vs $1/T_m$. Afterwards the free energy ΔG^{298} was calculated using the Gibbs-Helmholtz equation with T = 298 K:

$$\Delta \boldsymbol{G}^{\mathbf{298}} = \Delta \boldsymbol{H}^{\mathbf{0}} - \boldsymbol{T} \Delta \boldsymbol{S}^{\mathbf{0}} \quad (3)$$

Fluorescence spectra

Fluorescence spectra were recorded on a JASCO FP-8300 spectrofluorometer equipped with an FCT-817S cell changer and a F12 temperature controller device from Julabo. 1 µM samples were measured in a high precision cell from Hellma (made of quartz SUPRASIL, 10x2 mm). Excitation and emission bandwidth were set to 5 nm. Fluorescence of the samples was measured with a PMT voltage of 350 V and a response time of 1 s. Data were recorded with a scan speed of 500 nm/min with a data interval of 0.2 nm. Spectra for the ^{Tol}dU were recorded in DMSO or MeOH (dilution of a 10 mM DMSO stock solution) with an λ_{ex} = 320 nm or λ_{em} = 390 nm and annealed DNA samples in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) or ^{Tol}dU crosslinking unit in pure acetonitrile (1:10 dilution from the UV-Vis sample) with an λ_{ex} = 320 nm or λ_{em} = 405 nm at 20 °C.

Absolute quantum yield

Absolute quantum yield for the ^{Tol}dU nucleosid started with a 50 µM in solution DMSO which was serially diluted in a 1:1 ratio (25.0, 12.5, 6.25, 3.13, 1.56 µM) in a Jasco 6808-H150A liquid cell (1x10x250 mm). Spectra were recorded on a JASCO FP-8300 spectrofluorometer equipped with an ILF-835 integrating sphere with the following settings:

- Ex wavelength: 320 nm
- Ex bandwidth: 5 nm
- Em bandwidth: 5 nm
- Response: 1 s
- PMT voltage: 350 V
- Data interval: 0.2 nm
- Scan speed: 100 nm/min
- Measurement range: 305-600 nm

To obtain the scattering values for the blank (s_0) and the analyte (s_x) the area of scattering peak (λ_{ex}±15 nm) was integrated. For the values of the fluorescence peak of the sample (f_x) and the blank (f_0) the rest of the spectrum (λ_{ex}+15 nm-600nm) was used for integration. The absolute quantum yield was determined according following formula:

$$\boldsymbol{\phi_{abs}} = \frac{\boldsymbol{f_x - f_0}}{\boldsymbol{s_0 - s_x}} \quad (4)$$

Relative quantum yield

Relative quantum yields were determined using a 1'-(4-cyano-1H-indol-1-yl)- β -D-ribofuranose (r4CI, Φ=0.67)[7]. Samples for r4CI (6.25, 12.5 and 25 µM) were prepared in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) using a 10 mM stock solution in DMSO. DNA samples (1, 2 and 5 µM) were annealed in a phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0).

UV/Vis spectra were measured in a10 mm quartz cuvettes from Agilent using an Agilent Cary 3500 with following settings:

- Wavelength: 200-750 nm
- Averaging time: 0.032 s
- Data interval: 0.2 nm
- Scan rate: 375 nm min^{-1}
- Spectral bandwidth: 1 nm

Fluorescence spectra were recorded in a high precision cell from Hellma (made of quartz SUPRASIL, 10x2 mm) with a JASCO FP-8300 spectrofluorometer:

- Ex wavelength: 320 nm
- Ex bandwidth: 2.5 nm
- Em bandwidth: 2.5 nm
- Response: 1 s
- PMT voltage: 350 V
- Data interval: 0.2 nm
- Scan speed: 100 nm/min
- Measurement range: 340-750 nm

The measured absorbance (A) is then converted to an absorption factor (a) according following formula:

$$a = \int_{\lambda_{ex}-\Delta\lambda_{ex}/2}^{\lambda_{ex}+\Delta\lambda_{ex}/2} 1 - 10^{-A}\, d\lambda \quad (5)$$

The emission spectra were integrated between 340-600 nm to get f_x. Comparison with the standard r4CI led to the relative quantum yield:

$$\phi_{rel} = \phi_{r4CI} \cdot \frac{f_x}{a_x} \cdot \frac{a_{r4CI}}{f_{r4CI}} \quad (6)$$

Fluorescence lifetime

Fluorescence lifetimes were determined using a time-correlated single photon counting setup at λ_{em} = 405 nm for a 1 µM DNA sample in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0) on a Horiba DeltaFlex spectrometer with a DeltaDiode excitation source DD-320 (λ_{ex} = 318 nm). Samples were measured after annealing in a in a high precision cell from Hellma (made of quartz SUPRASIL, 10x2 mm). The emission bandwidth has been set for a stop rate close to 2%. The photon arrival time was set to 4096 channels, resulting in a width of 0.026 ns/channel. Data collection continued until 10000 counts had accumulated in the peak channel. The instrument response function was recorded from a buffer sample by setting the emission monochromator to the actual peak wavelength of the excitation source. Fluorescence decay curves were analyzed by iterative reconvolution fitting with the instrument response function using a proprietary algorithm implemented in Horiba DAS6 (version 6.8).

DNA-Crosslinking procedures

For analytic analysis, a 1 µM DNA duplex solution was prepared in phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0). The duplex was annealed in the dark by heating to 95 °C for 1 min and cooling down on ice for 60 min.

Irradiation with a Xe-lamp (150 W) was performed on a JASCO FP-8300 spectrofluorometer equipped with an FCT-817S cell changer and a F12 temperature controller device from Julabo. All measurements were performed in an FMM-200 5 mm quartz fluorescence microcell from JASCO with a magnetic stir bar using an FMH-802 5 mm microcell jacket from JASCO as cell adapter. The temperature was set at 10 °C or 15 °C if the duplex contains a Cy3 label. After 5 min equilibration in the spectrometer the reaction mixture (300 µL) was irradiated with the following settings:

- Ex wavelength: 320 nm
- Em wavelength: 410 nm
- Ex bandwidth: 5 nm
- Em bandwidth: 5 nm
- Response: 1 s
- PMT voltage 350 V
- Data interval: 1 s

Aliquots (5 µL) were taken at different time points (0, 1, 3, 5, 10, 20 and 30 min for ICL and hairpin CL; 0, 5, 10, 15, 20, 25 and 30 min for ligation CL) and quenched with loading buffer (5 µL).

Fluorescence increase at 410 nm and anion exchange HPLC were measured for oligonucleotides without Cy3 label.

The kinetics were analyzed by denaturing PAGE (0.4x200x200 mm, 20% acrylamide/ bisacrylamide 19:1, 7 M urea) with 1×TBE and run at 25 W power for 1 hour. The gels were imaged on a Chemidoc device, and the crosslink yield was determined from the band intensities of the Cy3 fluorescence quantified by ImageLab software. Kinetic data were obtained by fitting the resulting curve to the following equation:

$$Y = Y_{max} \cdot (1 - e^{-k_{obs} \cdot t}) + y0 \quad (7)$$

For a preparative crosslinking reaction, a concentration of 20 µM was used in 300 µL phosphate buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.0). After the annealing step the sample was irradiated with a Xe-lamp (150 W) at 350 nm in the fluorescence spectrometer for 30 min in the case of ICL and hairpin CL and 60 min for ligation CL. The reaction mixture was lyophilized and purified by PAGE (20%).

Enzymatic Digestion and LC-MS analysis

For LC-MS characterization PAGE purified and lyophilized crosslinked DNA duplex (500 pmol) was dissolved in antarctic phosphatase buffer (10 µL, 50 mM Bis-Tris-Propane-HCl,1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6, New England Biolabs) and Nuclease P1 (0.25 µL, 25 U, New England Biolabs), Phosphodiesterase I (0.5 µL, 0.05 U, Sigma Aldrich) and antarctic phosphatase (0.2 µL, 1 U, New England Biolabs) were added and the mixture was kept at 37 °C overnight. The digested product was diluted with water (40 µL), extracted with chloroform (2x25 µL) and analyzed by LC-MS using a RP-18 column (Synergi, 4-µm Fusion-RP C18 80 Å, 250x2 mm) from Phenomenex at 25 °C with aqueous mobile phase A (10mM NH_4OAc, pH 5.3) and organic mobile phase B (100% acetonitrile). The flow rate was 0.2 mL/min with a gradient of 0–80% B in 40 min.The micrOTOF-Q III with an ESI ion source was operated in positive-ion mode, with a capillary voltage of 4.5 kV, an end plate offset of 500 V, a nitrogen nebulizer pressure of 1.4 bar, a dry gas flow of 9 L/min and dry temperature of 200 °C. Data were analyzed using Data Analysis software DA 4.2 (Bruker Daltonics).

The chloroform phases were combined in the reaction vessel of the digestion and the solvent was carefully removed under reduced pressure. Water (100 µL) was added, and the suspension was heated to 65 °C for 5 min to denature remaining proteins. After cooling down to room temperature MeCN (100 µL) was added, and the mixture was centrifuged for 3 min. The supernatant was transferred to another reaction vessel. The precipitate was discarded. After lyophilization of the supernatant the residue was resuspended in water (100 µL) and centrifuged for 30 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in MeCN/10mM NH_4OAc buffer (2/3, *v*/*v*). The organic phase was analyzed by LC-MS using a gradient of 40–90 % B in 40 min.

For NMR analysis of the digested crosslink unit the enzymatic digestion was upscaled. PAGE purified and lyophilized crosslinked DNA duplex (11 nmol) was dissolved in Antarctic phosphatase buffer (210 µL, 71.4 mM bis-tris-propane-HCl,1.4 mM $MgCl_2$, 0.14 mM $ZnCl_2$, pH 6, New England Biolabs) and Nuclease P1 (6 µL, 600 U, New England Biolabs), phosphodiesterase I (12 µL, 1.2 U, Sigma Aldrich) and Antarctic phosphatase (5 µL, 25 U, New England Biolabs) were added and the mixture was kept at 37 °C overnight. The digested product was diluted with water (800 µL) and extracted with chloroform (2x200 µL). The chloroform phases were combined in the reaction vessel of the digestion and the solvent was carefully removed under reduced pressure. Water (200 µL) was added, and the suspension was heated to 65 °C for 5 min to denature remaining proteins. After cooling down to room temperature MeCN (200 µL) was added, and the mixture was centrifuged for 3 min. The supernatant was transferred to another reaction vessel. The precipitate was discarded. After lyophilization of the supernatant the residue was dissolved in MeCN/H_2O (500 µL, 2/3, *v*/*v*).

Purification was performed on a HPLC system (ÄKTAmicro) from GE Healthcare using a NUCLEOSIL reversed-phase column (C18, 250x4.6 mm, 100 Å, 5 µm) from Machery Nagel at a flow rate of 1 mL min^{-1}. Linear gradients of 40–80 % B over 35 min of buffer A (water) and buffer B (MeCN) were used for purification. Chromatograms were monitored at 260 nm and 320 nm. The purified crosslink units were lyophilized and dissolved in MeCN-d_3 and analyzed on a Bruker Avance III 600 NMR spectrometer equipped with a DCH ^{13}C / ^{1}H cryoprobe. NMR spectra were evaluated using MestReNova v12.0.4 or Topspin 3.2 (Bruker BioSpin).

Hybridization chain reaction (HCR)

For hybridization chain reaction hairpin (3 µM) and initiator (1 µM) stock solutions in 5xSSC buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0) were prepared. The DNA was annealed in the dark by heating to 95 °C for 2 min and cooling down on ice for 30 min. To start the HCR both hairpins (500 nM) and the initiator (500, 250, 125, 50, 10, 2.5 nM) were combined in a reaction volume of 12 µL in 5xSCC buffer. The reaction incubated 2 h in the dark and were afterwards loaded on a 1% agarose gel containing 0.01% SybrGold for staining (90 V for 45 min or 60 V for 70 min).

Initiator dependent crosslink experiments were irradiated 1 min on a transilluminator device from HEROLAB GmbH with an irradiation maximum of 312 nm. For time dependent crosslink experiments, a constant initiator concentration of 50 nM was used, and the samples were irradiated 0.5, 1, 2, 3 and 5 min. The crosslinked product was analyzed via denaturing PAGE (5%, 200 V for 30 min).

For fluorescence measurements 280 µL of a solution containing H1- ^{Tol}dU-HCR and H2- ^{Tol}dU-HCR (final concentration 100 nM) in 5xSSC buffer was prepared. Fluorescence measurements were performed like described in “DNA-Crosslinking procedures” for 15 min at 20 °C. After 1 min irradiation 20 µL of initiator solution in 5xSCC were used to start the HCR. Fluorescence increase was measured for final initiator concentration of 50, 20, 10, 5 and 2 nM in 300 µL reaction volume. For the negative control the oligonucleotide Lig-Template with a concentration of 50 nM in 300 µL reaction volume was used. Results were normalized using following equation:

$$\boldsymbol{F_{\mathrm{norm}} = \frac{F}{F_0}} \tag{6}$$

With: F_{norm} = normalized fluorescence, F = measured fluorescence, F_0 = starting fluorescence

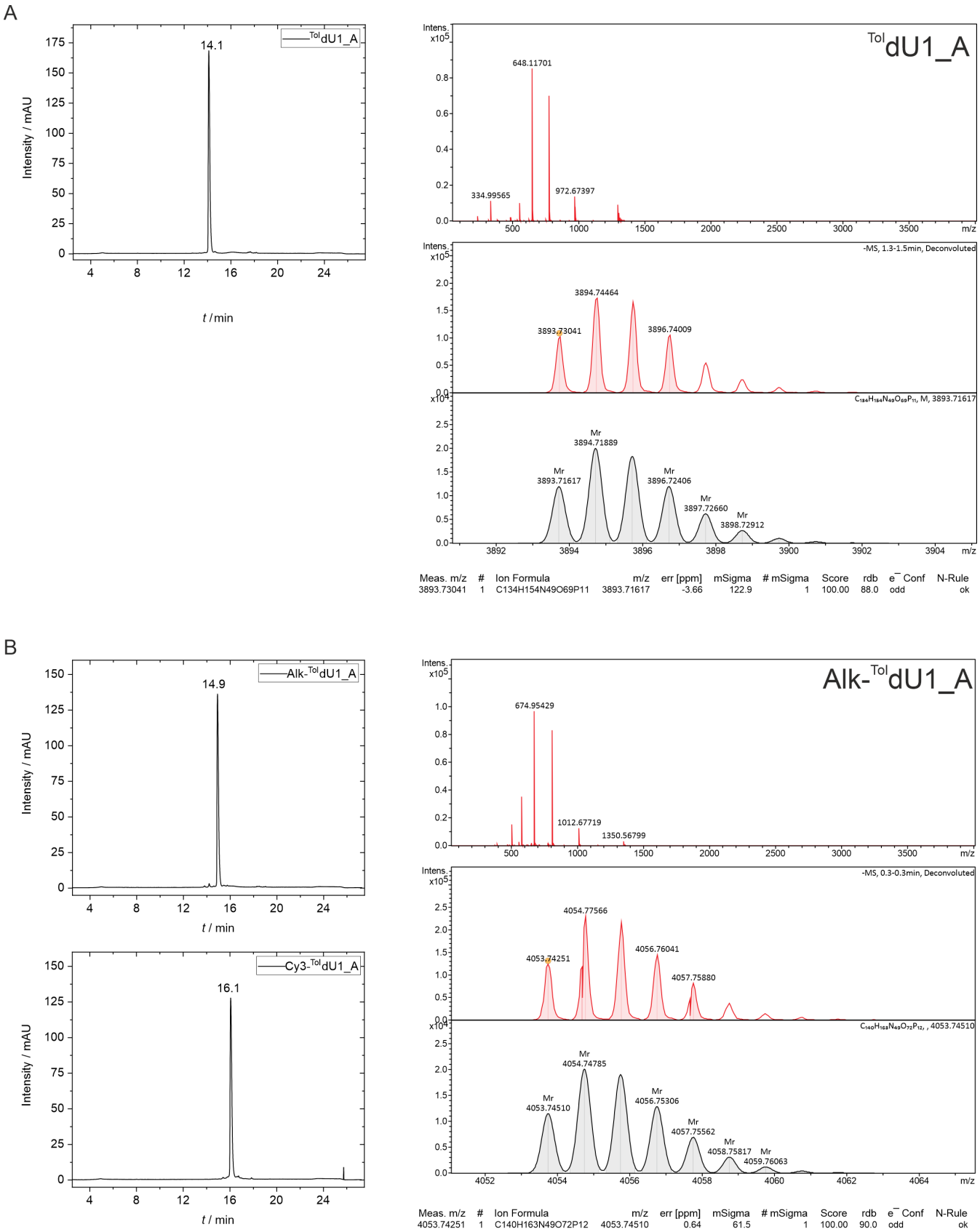
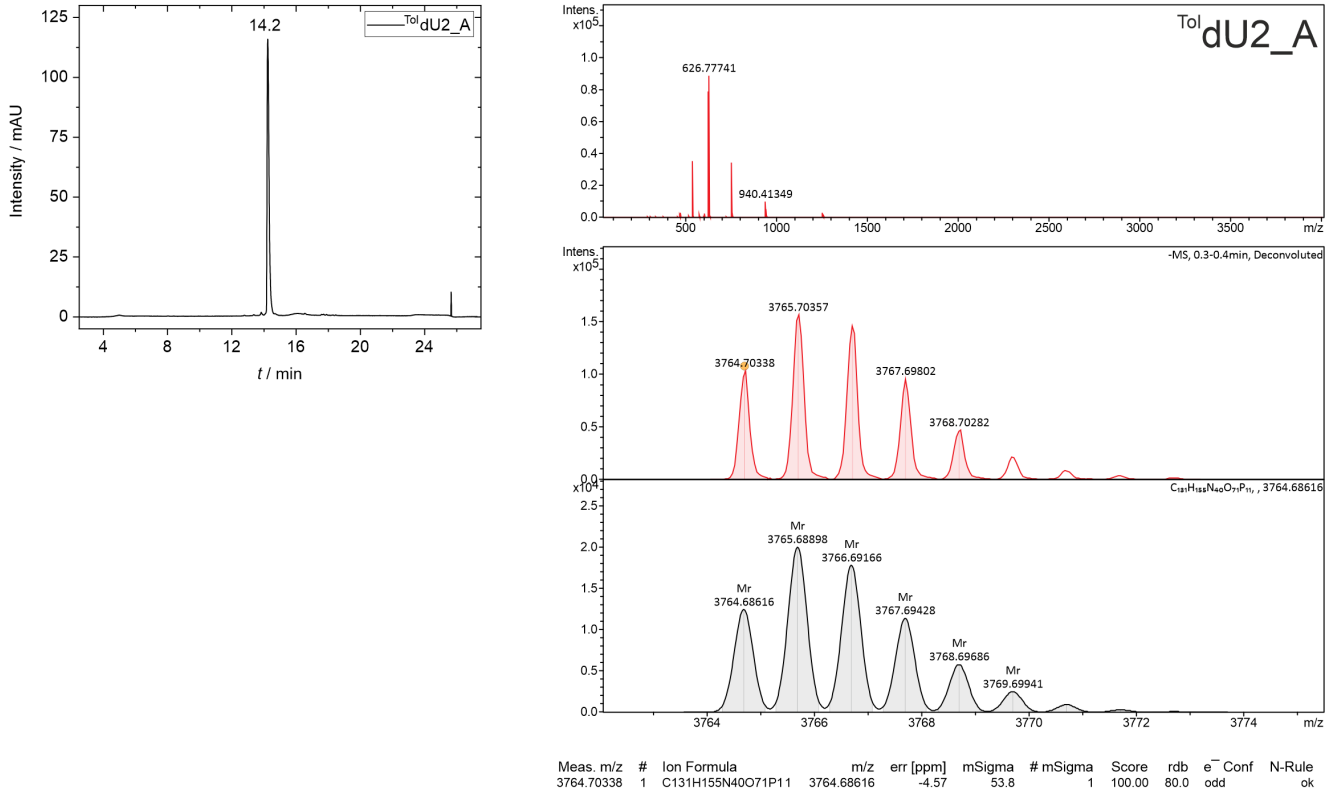


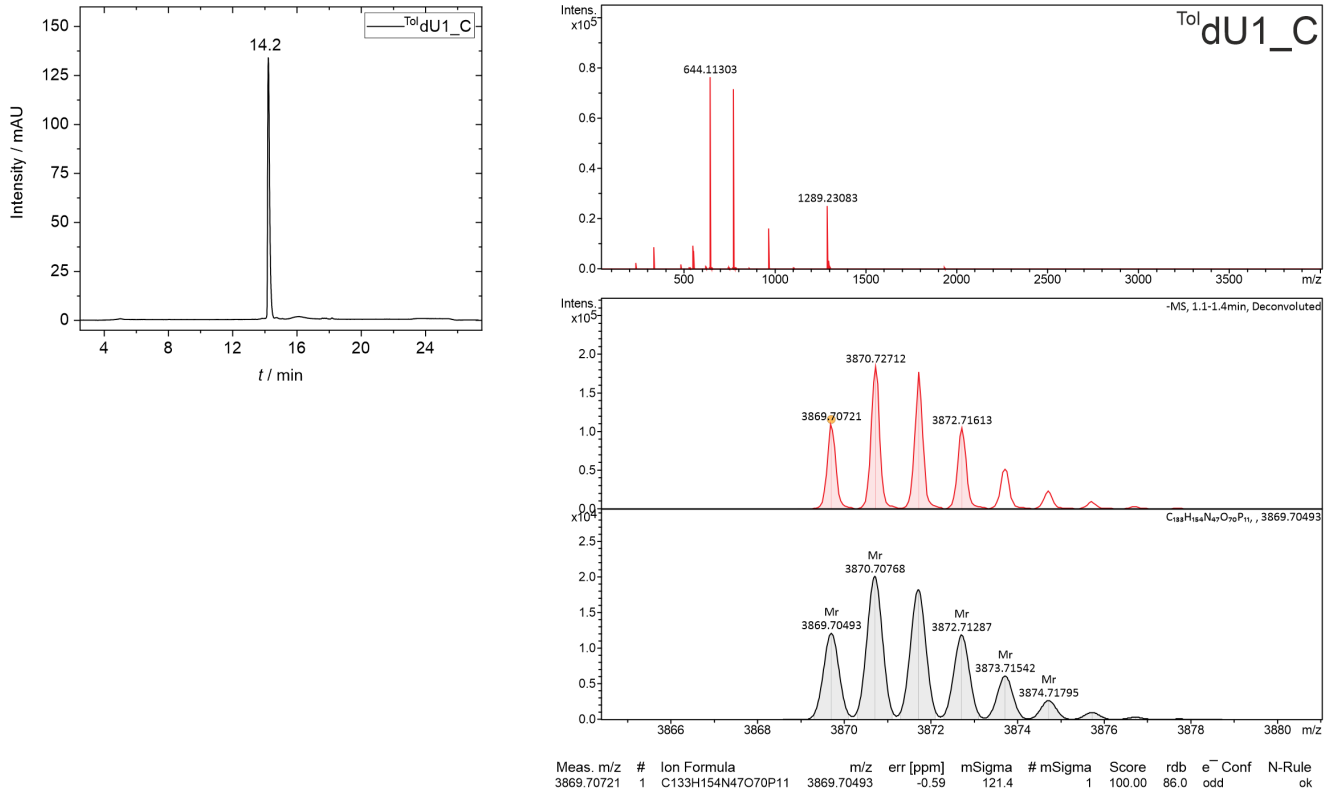
Figure S19 Analytical anion exchange HPLC chromatograms (monitored at 260 nm) and ESI-MS (top to bottom: raw m/z trace, deconvoluted and simulated spectra) of the DNA oligonucleotides ^{Tol}dU1_A (A) and Alk-^{Tol}dU1_A/Cy3-^{Tol}dU1_A (B).

A



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
3764.70338	1	C131H155N40O71P11	3764.68616	-4.57	53.8	1	100.00	80.0	odd	ok

B



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
3869.70721	1	C133H154N47O70P11	3869.70493	-0.59	121.4	1	100.00	86.0	odd	ok

Figure S20 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides ^{Tol}dU2_A (A) and ^{Tol}dU1_C (B).

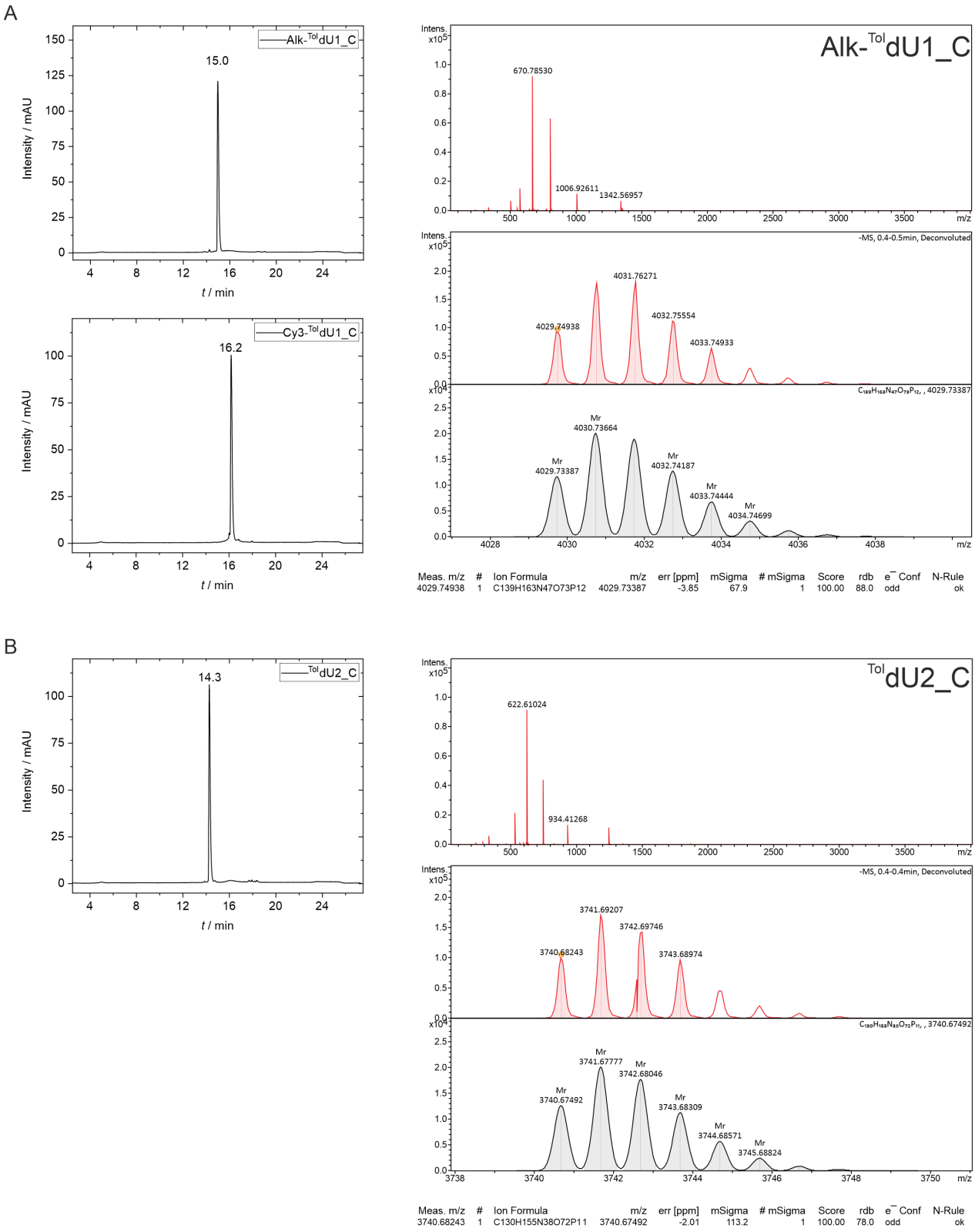


Figure S21 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides Alk-^{Tol}dU1_C/ Cy3-^{Tol}dU1_C (A) and Alk-^{Tol}dU2_C (B).

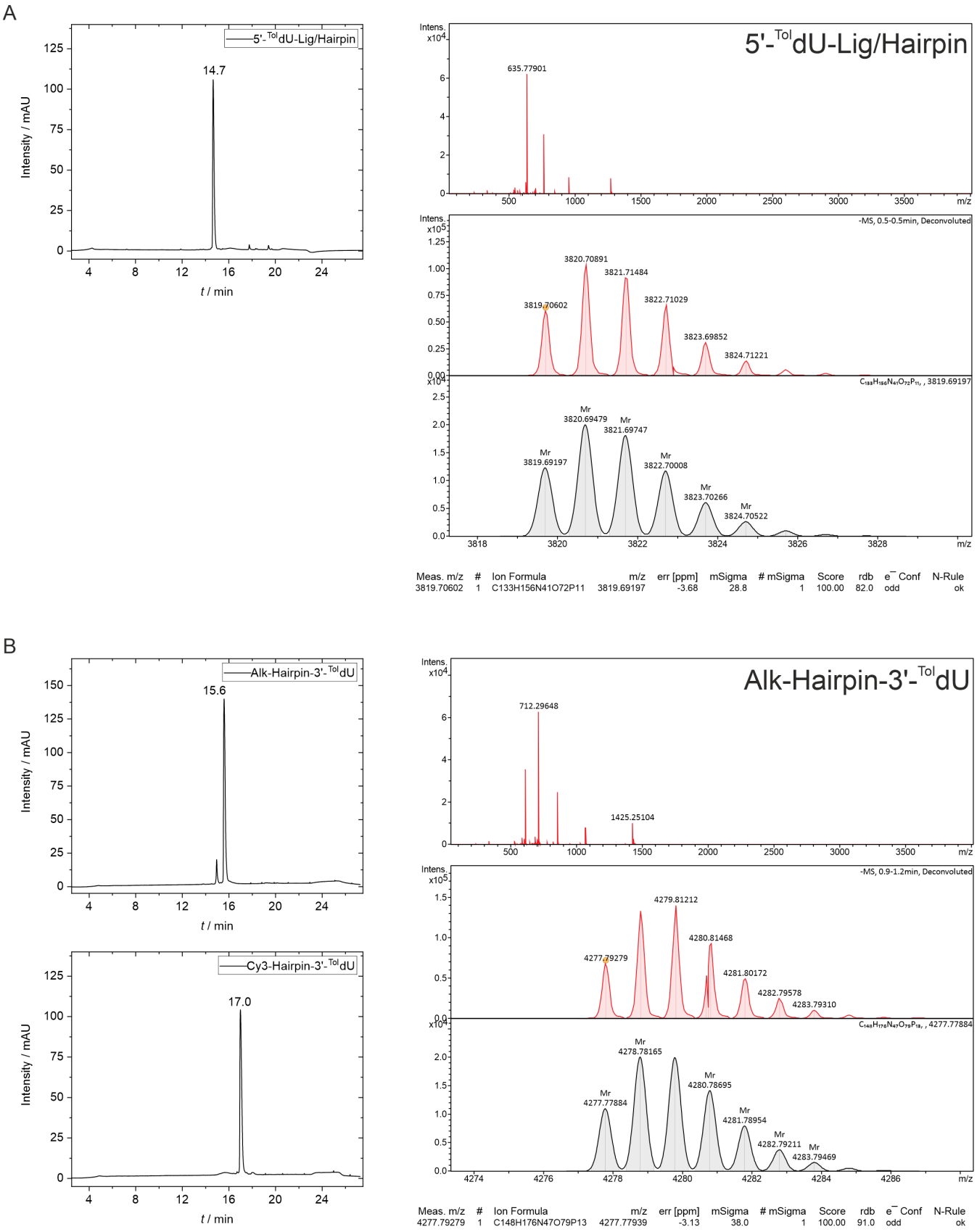


Figure S22 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides 5'-^{Tol}dU1-Lig/Hairpin (A) and Alk-Hairpin-3'-^{Tol}dU/Cy3-Hairpin-3'-^{Tol}dU (B).

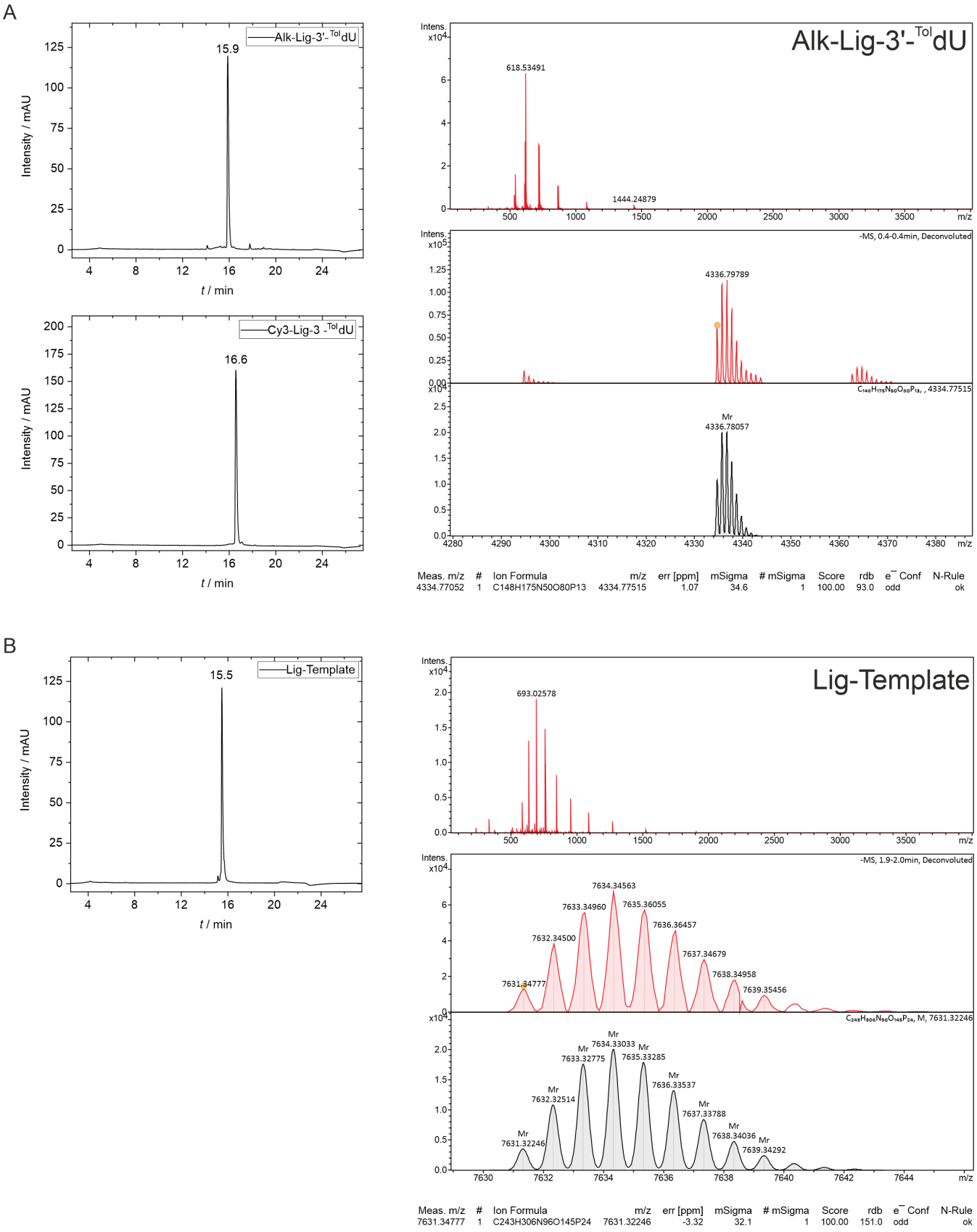


Figure S23 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides Alk-Lig-3'-^{Tol}dU/Cy3-Lig-3'-^{Tol}dU (A) and Lig-Template (B).

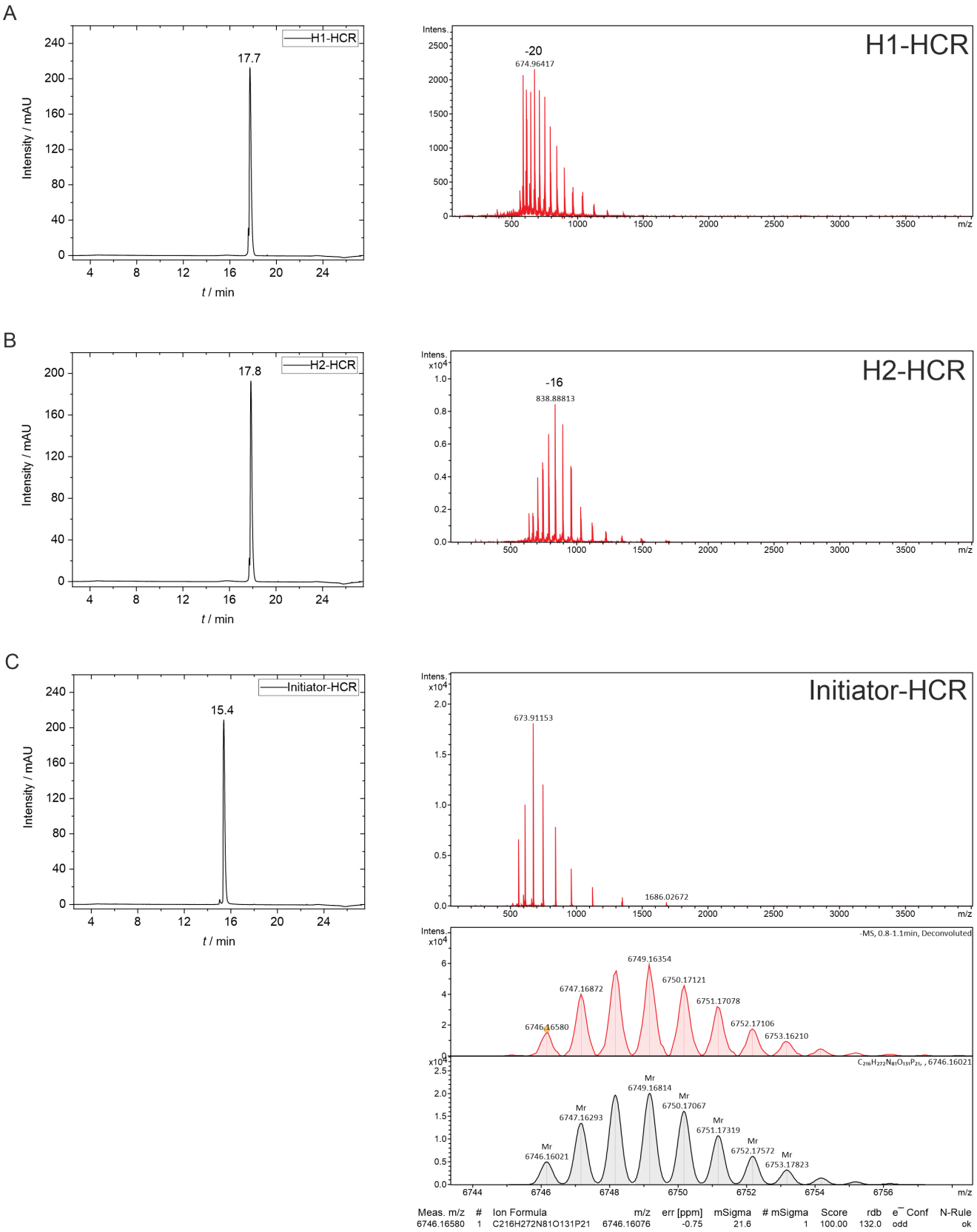


Figure S24 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides H1-HCR (A), H2-HCR (B) and Initiator-HCR (C).

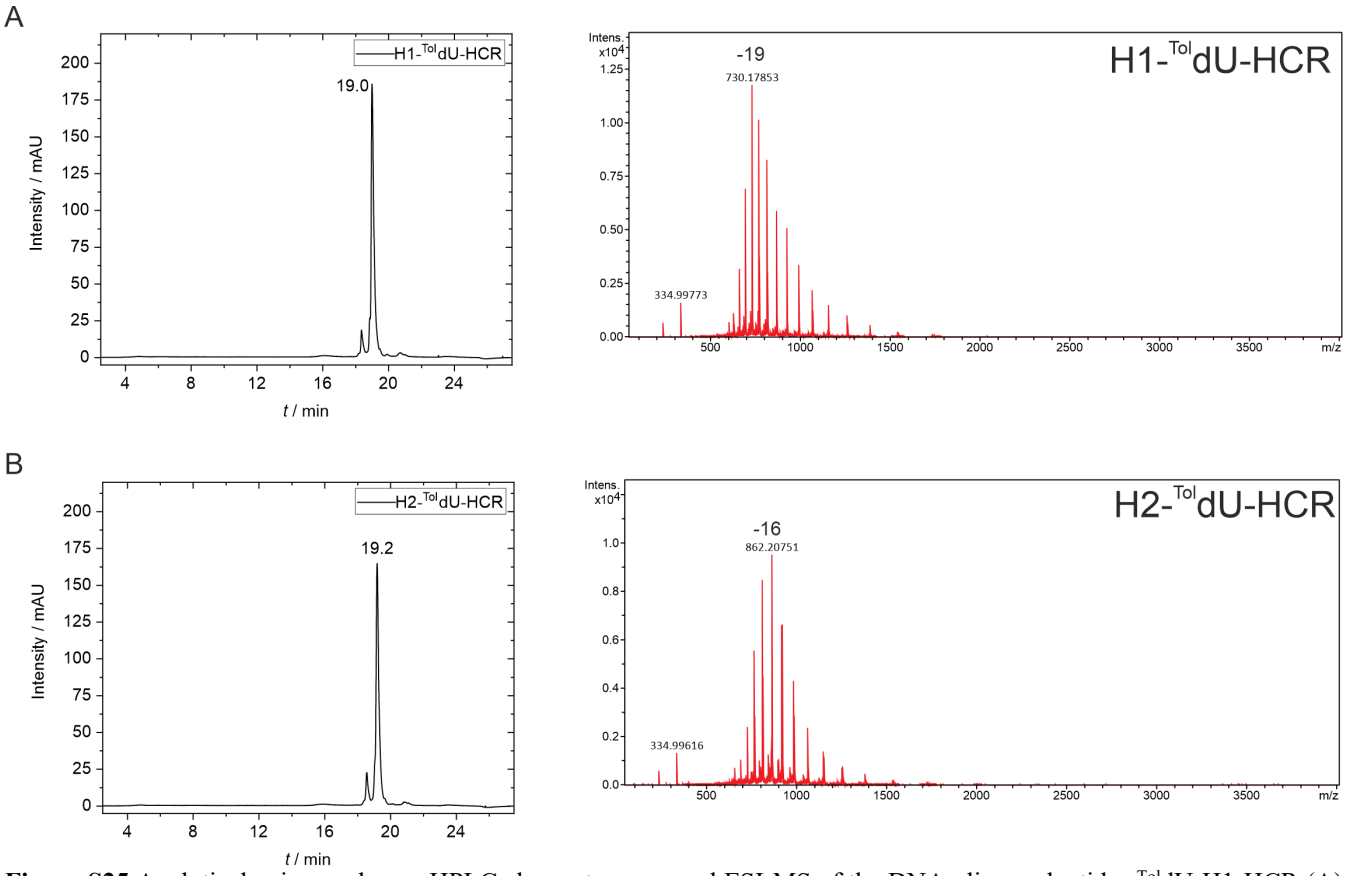


Figure S25 Analytical anion exchange HPLC chromatograms and ESI-MS of the DNA oligonucleotides ^{Tol}dU-H1-HCR (A) and ^{Tol}dU-H2-HCR (B).

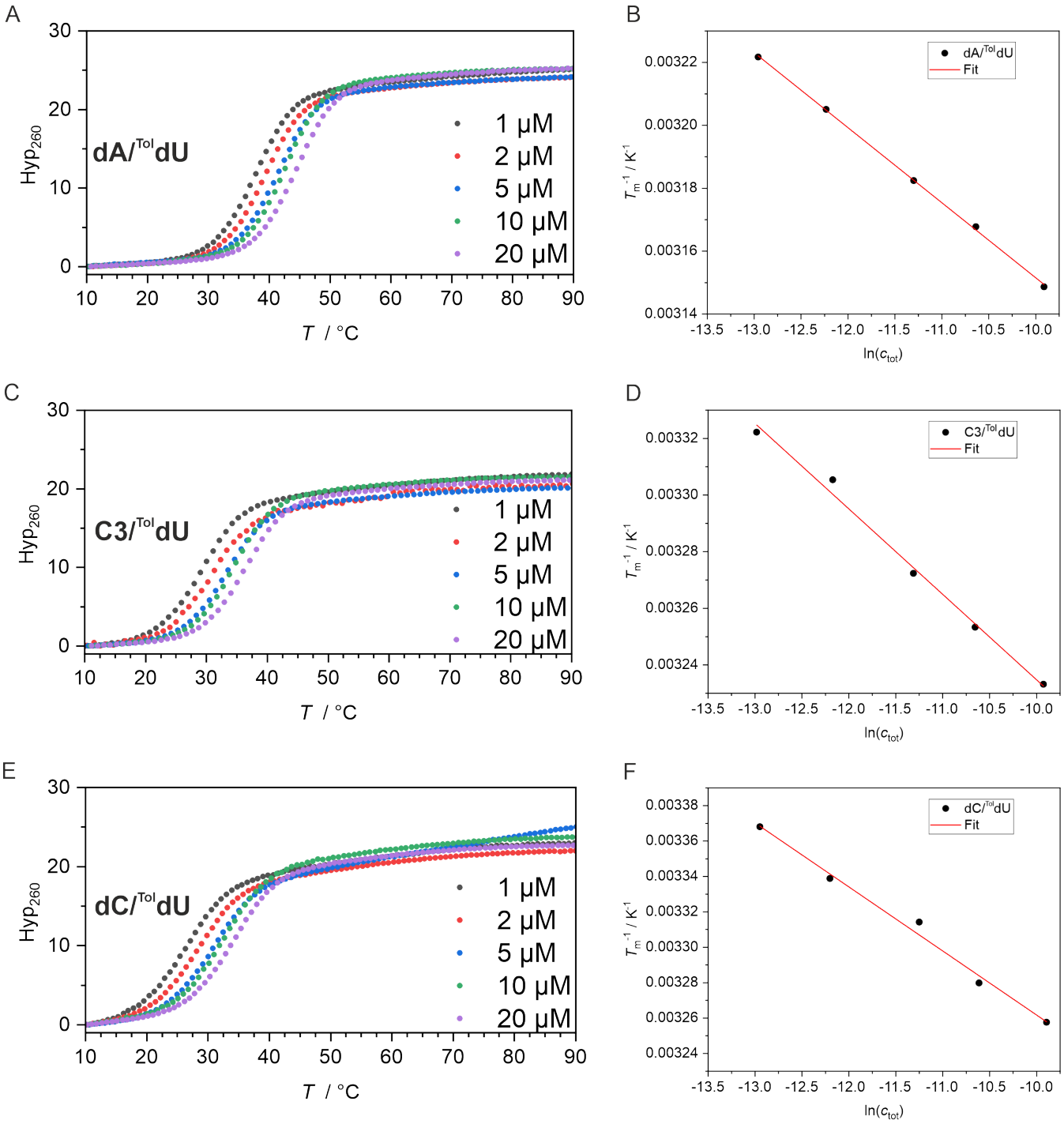


Figure S26 UV-thermal denaturation curves at different duplex concentrations in phosphate buffer (100 mM NaCl, 10 mM phosphate, pH7.0) and the corresponding van't Hoff plot for dA/^{Tol}dU (A and B), C3/^{Tol}dU (C and D) and dC/^{Tol}dU (E and F).

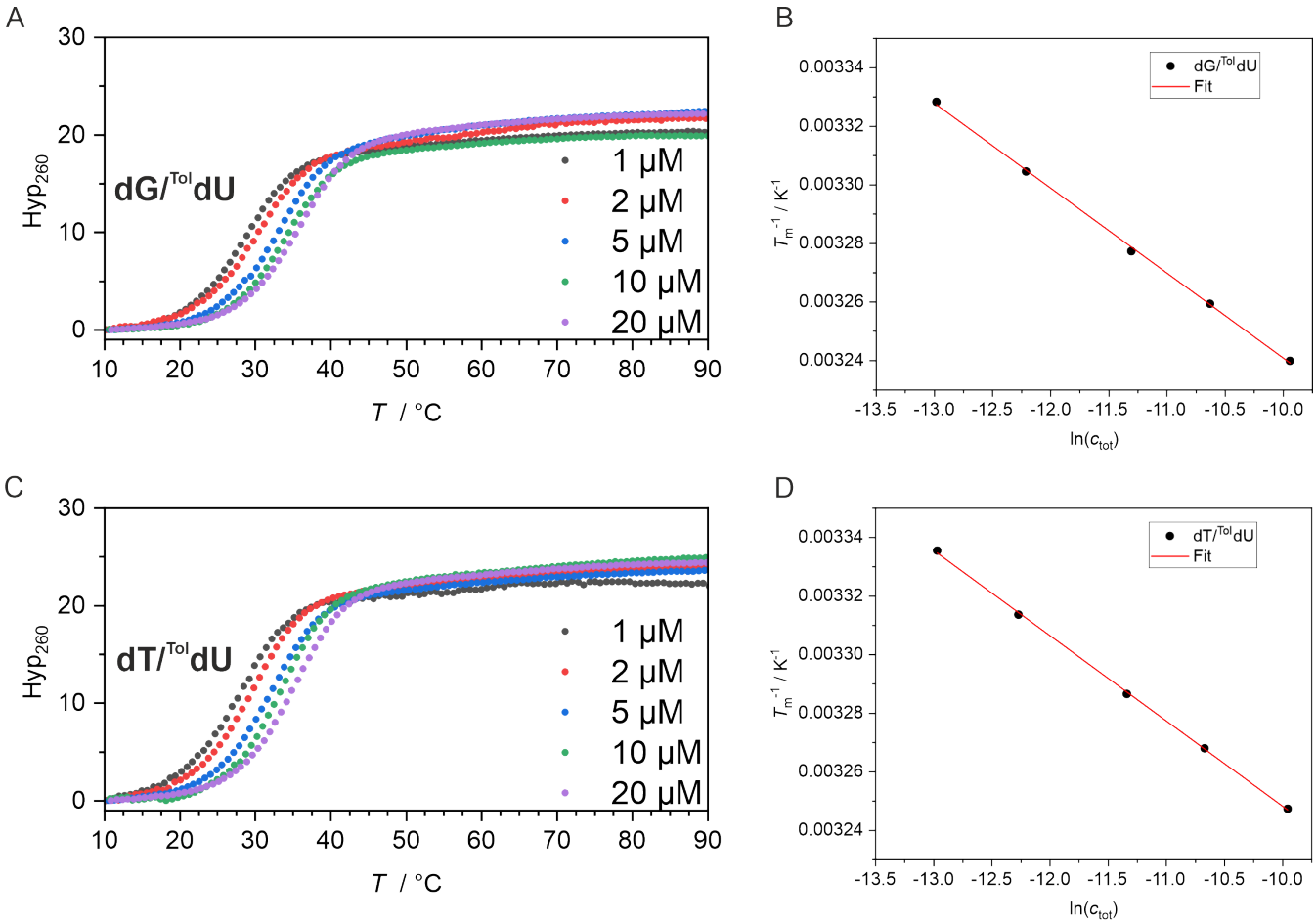


Figure S27 UV-thermal denaturation curves at different duplex concentrations in phosphate buffer (100 mM NaCl, 10 mM phosphate, pH7.0) and the corresponding van't Hoff plot for dG/^{Tol}dU (A and B) and dT/^{Tol}dU (C and D).

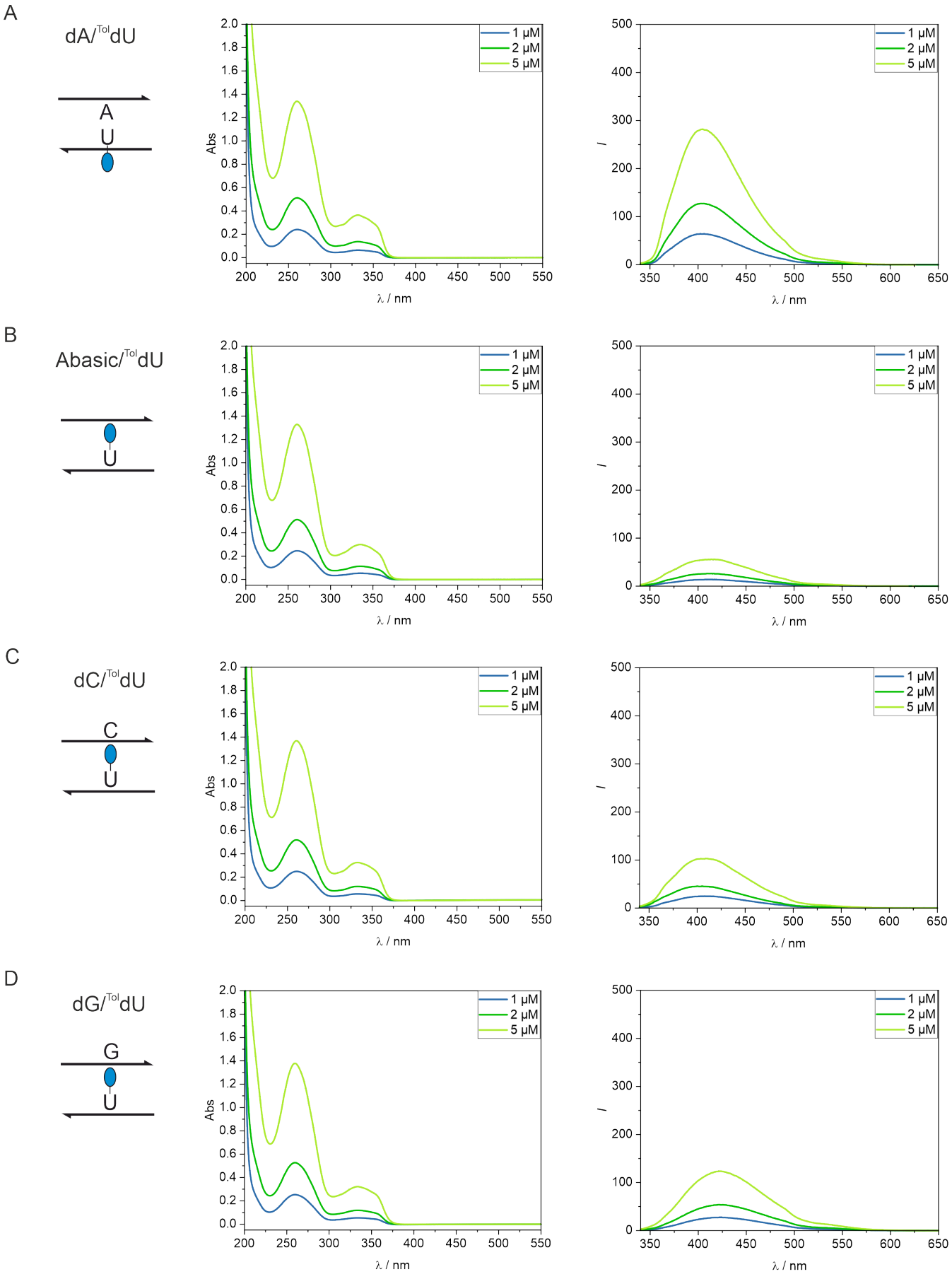


Figure S28 UV/Vis and fluorescence spectra for determination of the relative quantum yield for dA/^{Tol}dU (A), Abasic/^{Tol}dU (B), dC/^{Tol}dU (C) and dG/^{Tol}dU (D) in phosphate buffer (100 mM NaCl, 10 mM phosphate, pH7.0).

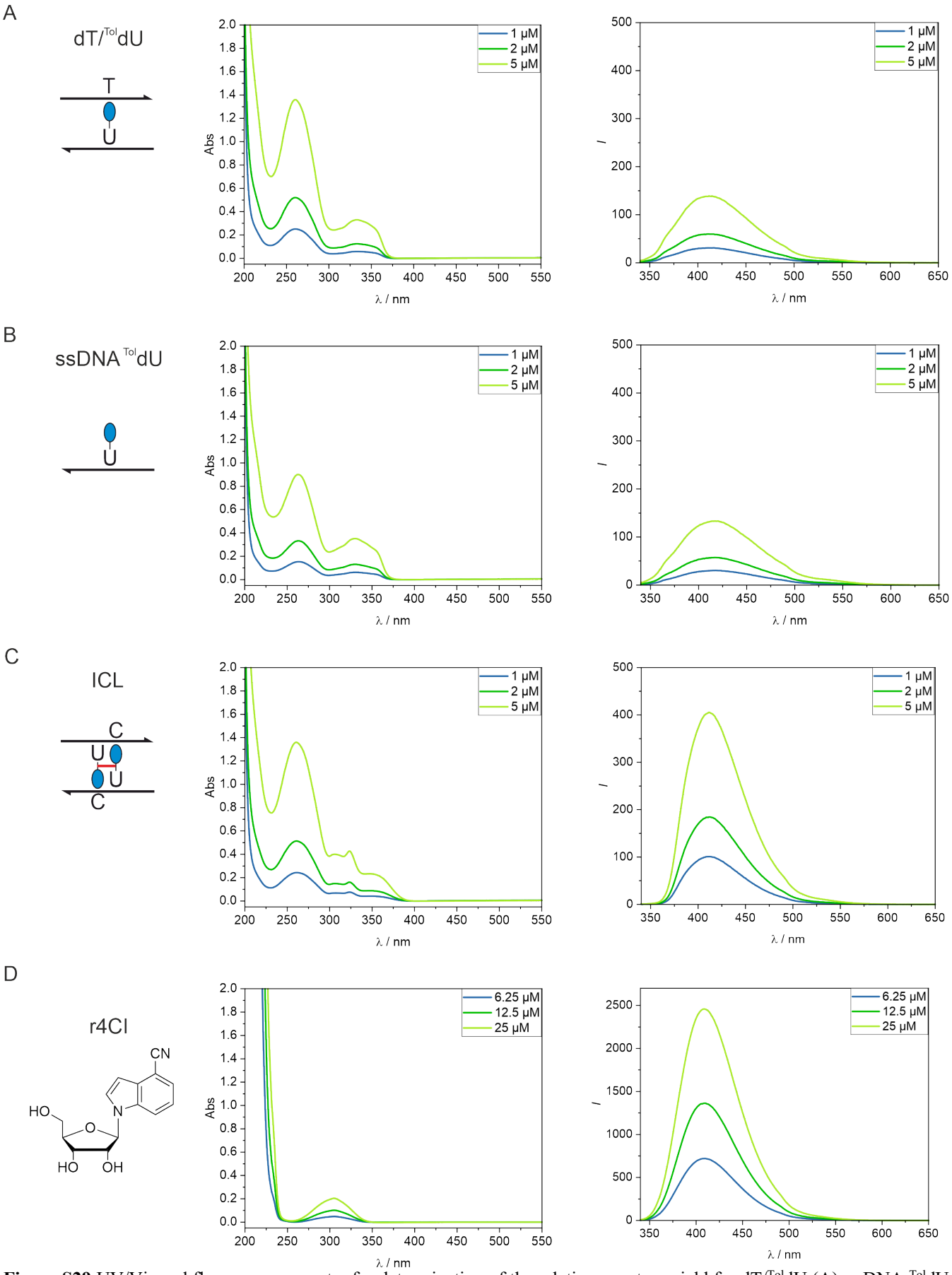


Figure S29 UV/Vis and fluorescence spectra for determination of the relative quantum yield for dT/^{Tol}dU (A), ssDNA ^{Tol}dU (B), ICL (C) and r4CI(D) in phosphate buffer (100 mM NaCl, 10 mM phosphate, pH7.0).

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