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Supporting Information

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Supporting Information

for

C5-Functionalized LNA: Unparalleled Hybridization Properties and Enzymatic Stability

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Representative procedure for O3′-phosphitylation (illustrated for 6X).

Alcohol **5X** (0.37g, 0.52 mmol) was dried by the coevaporation with anhydrous 1,2-dichloroethane (2×10 mL) and dissolved in anhydrous CH_2Cl_2 (10 mL). To this was added DIPEA (0.44 mL, 2.6 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.18 mL, 0.78 mmol) and the reaction was stirred under Ar at RT for 2 h. The reaction mixture was then diluted with CH_2Cl_2 (25 mL), and washed with aq. $NaHCO_3$ (2×10 mL), and the combined aqueous phase back-extracted with CH_2Cl_2 (2×10 mL). The combined organic phase was dried (Na_2SO_4), evaporated to dryness, and the resulting crude residue purified by column chromatography (0-2 % MeOH/CH_2Cl_2, *v/v*) and subsequent trituration from CH_2Cl_2 and petroleum ether to provide phosphoramidite **6X** (0.39 g, 80%) as a white solid material.

Synthesis and purification of oligodeoxyribonucleotides: 0.2 µmol scale synthesis of oligodeoxyribonucleotides (ONs) containing LNA or C5-alkynyl functionalized LNA monomers was performed on an Expedite 8909 Synthesizer using succinyl linked LCAA-CPG (long chain alkyl amine controlled pore glass) columns with a pore size of 500 Å . Standard protocols for incorporation of DNA phosphoramidites were used, while extended coupling times (15 min) and oxidations (45 s) were used for incorporation of LNA and C5-alkynyl functionalized derivatives thereof. Cleavage from solid support and removal of protecting groups was accomplished by treatment with 32% aq. ammonia (55 ℃, 24 h). Purification of all modified ONs was performed to minimum 80% purity using either of two methods: *a*) overall synthesis yield >80%: cleavage of DMT using 80% aq. AcOH, followed by precipitation from acetone (-18 ℃ for 12-16 h) and washing with acetone, or *b*) overall synthesis yield <80%: purification of ONs by RP-HPLC as described below (Table S1), followed by detritylation and precipitation as outlined under *a*.

Purification of the crude ONs was performed on a Varian Prostar HPLC system equipped with an XTerra MS C18 pre-column (10 µm, 7.8 x 10 mm) and a XTerra MS C18 column (10 µm, 7.8 x 150 mm) using the representative gradient protocol depicted in Table S1. The composition of all synthesized ONs was verified by MALDI-MS/MS analysis (Table S2) recorded in positive ions mode on a Quadrupole Time-Of-Flight tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K.). Anthranilic acid was used as a matrix, while purity (>80%) was verified by RP-HPLC (Table S1).

Table S1. Representative RP-HPLC gradient protocol.[a]

t [min]	Buffer A [v%]	Buffer B [v%]
0	100	0
2	100	0
50	30	70
64	0	100
69	0	100
71	100	0
80	100	0

[a] Buffer A is 0.05 M TEAA (triethyl ammonium acetate) pH 7.4, while buffer B is 75% MeCN in H_2O *v/v*. A flow rate of 1.2 mL/ min was used.

Table S2. MALDI-MS of synthesized ONs.[a]

ON	Sequence	Found *m*/*z* $[M]^+$	Calcd *m*/*z* $[M]^+$
V1	3′-CGT A**V**A GTG	2780.5	2781.9
V2	5′-GCA TA**V** CAC	2709.5	2710.9
V3	5′-GCA **V**AT CAC	2709.5	2710.9
V4	5′-GCA **V**A**V** CAC	2737.5	2738.9
W1	3′-CGT A**W**A GTG	2791.9	2791.2
W2	5′-GCA TA**W** CAC	2719.5	2720.4
W3	5′-GCA **W**AT CAC	2719.5	2720.4
W4	5′-GCA **W**A**W** CAC	2757.5	2758.4
X1	3′-CGT A**X**A GTG	2820.5	2820.7
X2	5′-GCA TA**X** CAC	2749.5	2749.7
X3	5′-GCA **X**AT CAC	2749.5	2749.7
X4	5′-GCA **X**A**X** CAC	2817.5	2816.8
Y1	3′-CGT A**Y**A GTG	3087.2	3086.5
Y2	5′-GCA TA**Y** CAC	3014.8	3015.8
Y3	5′-GCA **Y**AT CAC	3014.8	3015.9
Y4	5′-GCA **Y**A**Y** CAC	3348.0	3349.1
Z1	3′-CGT A**Z**A GTG	3231.8	3233.1
Z2	5′-GCA TA**Z** CAC	3159.8	3161.5
Z3	5′-GCA **Z**AT CAC	3159.8	3161.9
Z4	5′-GCA **Z**A**Z** CAC	3638.2	3640.8

[a] For structures of monomer **V**-**Z** see Scheme 1 in the main manuscript.

Thermal denaturation studies: Concentrations of ONs were estimated using the following extinctions coefficients for DNA (OD/µmol): G (12.01), A (15.20), T (8.40), C (7.05); for RNA (OD/µmol): G (13.70), A (15.40), U (10.00), C (9.00). ONs (1.0 µmol of each strand) were thoroughly mixed, denatured by heating and subsequently cooled to the starting temperature of the experiment. Quartz optical cells with a path-length of 1.0 cm were used. Thermal denaturation temperatures (T_m values [℃]) were measured on a Cary 100 UV/VIS spectrophotometer equipped with a 12-cell Peltier temperature controller and determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs. temperature) recorded in medium salt buffer (T_m buffer: 100 mM NaCl, 0.1 mM EDTA, and pH 7.0 adjusted with 10 mM Na_2HPO_4/ 5 mM Na_2HPO_4). The temperature of the denaturation experiments ranged from at least 15 ℃ below T_m to 20 ℃ above T_m (although not below 1 ℃). A temperature ramp of 0.5 ℃/min was used in all experiment . Reported thermal denaturation temperatures are an average of two experiments within ±1.0 ℃.

Exonuclease studies: SVPDE (snake venom phosphordiesterase) was purchased from Worthington Biochemical Corporation. The change in absorbance at 260 nm as a function of time was monitored for a solution of ONs (3.3 µM) in magnesium buffer (600 µL, 50 mM Tris·HCl, 10 mM $MgCl_2$, pH 9.0) at 37 ℃, to which SVPDE dissolved in H_2O was added (12 µL, 0.52 µg, 0.03 U).

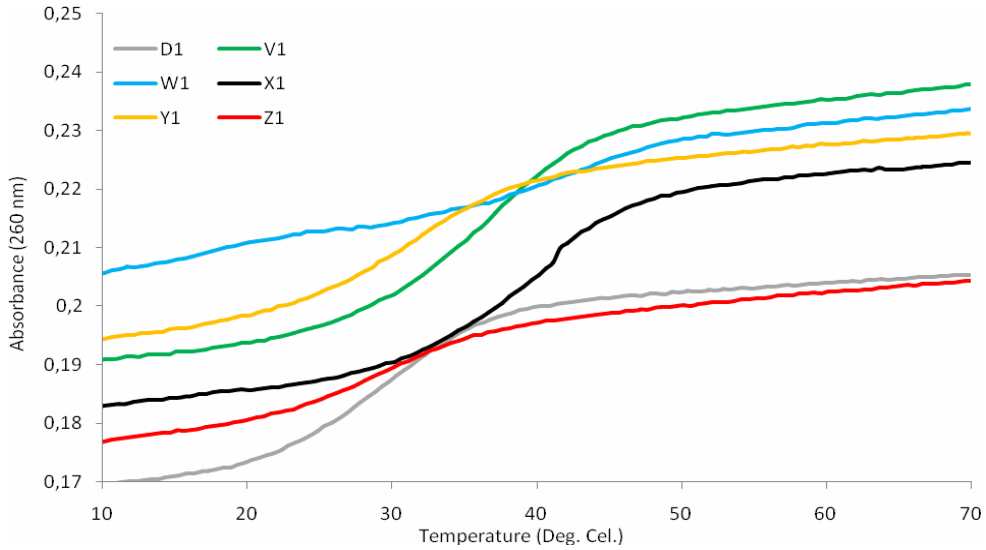
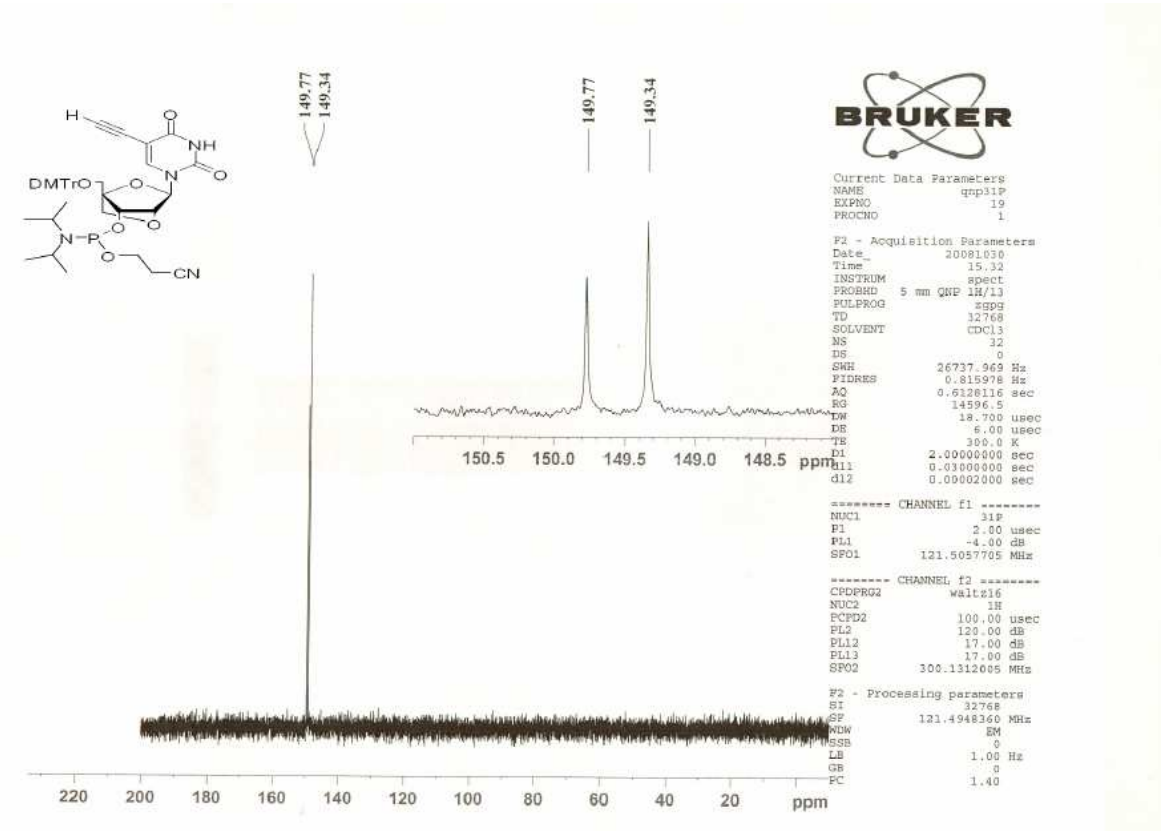


Figure S1. Representative thermal denaturation curves of duplexes between **V1**-**Z1** or reference **D1** and complementary RNA **R2**. The low hyperchromicity of **W1**:**R2** was only observed in this particular sequence.



BRUKER

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FIDRES 0.815978 Hz
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RG 14596.5
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DE 6.00 usec
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d11 0.03000000 sec
d12 0.00002000 sec

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PL1 -4.00 dB
SFO1 121.5057705 MHz

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NUC2 1H
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PL12 17.00 dB
PL13 17.00 dB
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F2 - Processing parameters
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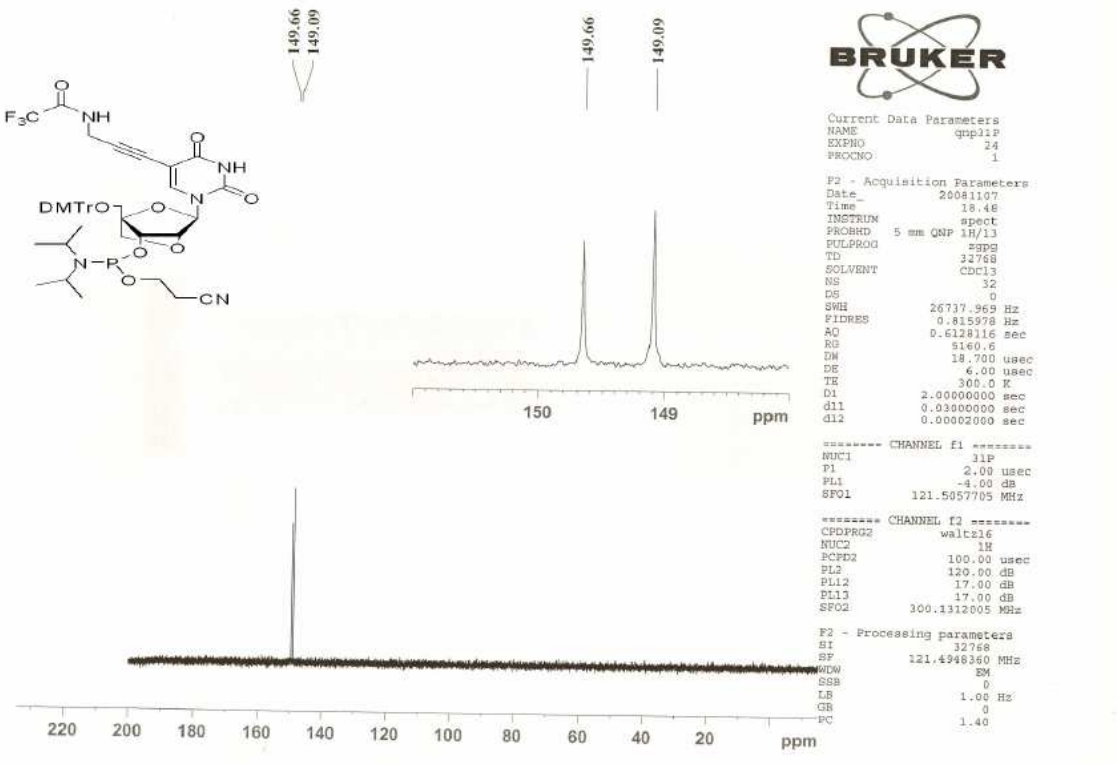

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PL1 -4.00 dB
SFO1 121.5057705 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 120.00 dB
PL12 17.00 dB
PL13 17.00 dB
SFO2 300.1312005 MHz

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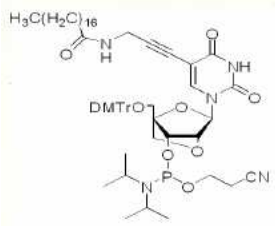

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NS                       32
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FIDRES            0.815978 Hz
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RG                    13004
DW                  18.700 usec
DE                   6.00 usec
TE                  300.0 K
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d12               0.00002000 sec

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P1                    2.00 usec
PL1                  -4.00 dB
SFO1          121.5057705 MHz

======== CHANNEL f2 ========
CPDPRG2              waltz16
NUC2                     1H
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PL12                  17.00 dB
PL13                  17.00 dB
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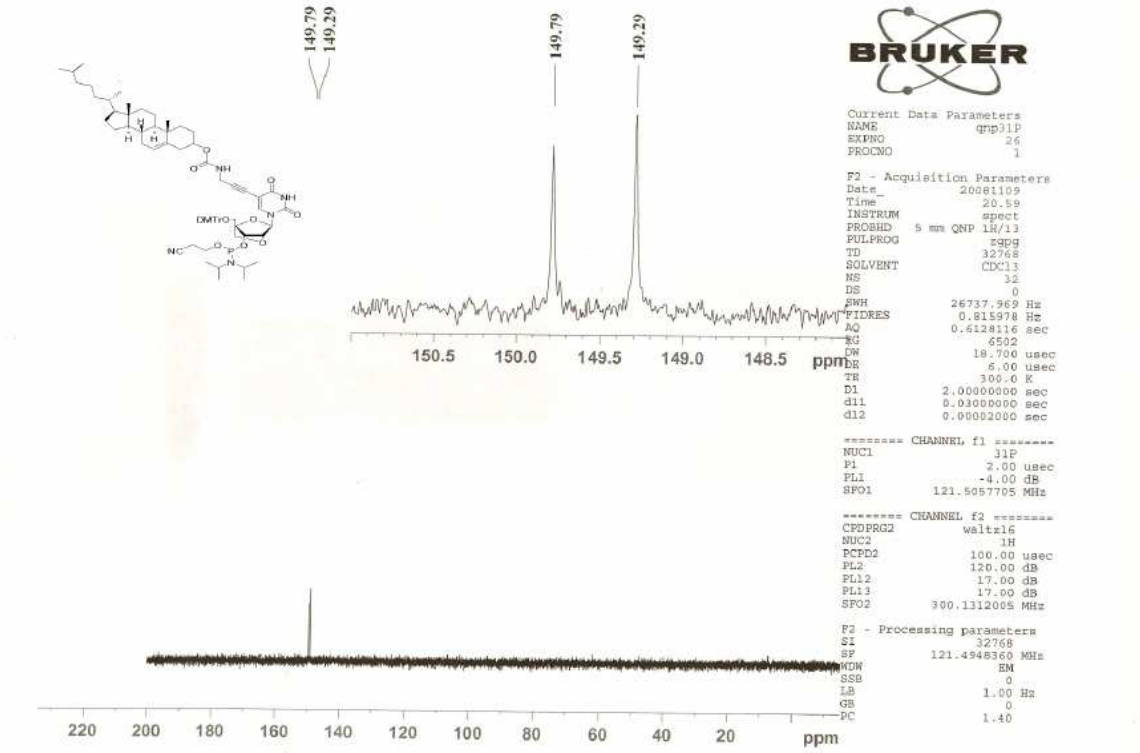

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FIDRES            0.815978 Hz
AQ                0.6128116 sec
RG                     6502
DW                  18.700 usec
DE                   6.00 usec
TE                  300.0 K
D1                2.00000000 sec
d11               0.03000000 sec
d12               0.00002000 sec

======== CHANNEL f1 ========
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P1                    2.00 usec
PL1                  -4.00 dB
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======== CHANNEL f2 ========
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NUC2                     1H
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PL12                  17.00 dB
PL13                  17.00 dB
SFO2          300.1312005 MHz

F2 - Processing parameters
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