Synthesis and biophysical properties of C5-functionalized LNA (Locked Nucleic Acid)

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SUPPORTING INFORMATION

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General experimental section. All reagents and solvents were of analytical grade and obtained from commercial suppliers and used without further purification. Petroleum ether of the distillation range 60-80 °C was used. CH$_3$CN was dried through storage over activated 3 Å molecular sieves. THF was dried by distillation over sodium metal and subsequently stored over 4 Å molecular sieves. Anhydrous DMF was used as obtained from commercial suppliers. Dichloromethane, 1,2-dichloroethane, Et$_3$N and DIPEA were dried through storage over 4 Å molecular sieves. The water content of anhydrous solvents was checked by Karl-Fischer apparatus. Reactions were conducted under an atmosphere of argon whenever anhydrous solvents were used. All reactions were monitored by thin layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator (SiO$_2$-60, F-254), which were visualized under UV light and/or by dipping in 5% conc. H$_2$SO$_4$ in absolute ethanol (v/v) followed by heating. Silica gel column chromatography was performed with silica gel 60 (particle size 0.040-0.063 mm) using moderate pressure (pressure ball). Silica gel columns were generally built with an initial starting eluent containing 1% (v/v) of pyridine whenever DMT$_r$-protected nucleosides were purified. Evaporation of solvents was carried out under reduced pressure at a temperature below 50 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried at high vacuum for at least 12 h to give the obtained products in high purity. $^1$H, $^{13}$C, $^{31}$P NMR spectra were recorded at 500 Mhz, 125.5 MHz, 121.5 MHz respectively, unless otherwise stated. Chemical shifts are reported in parts per million (ppm) relative to deuterated solvents or other internal standards (trimethylsilane, 80% phosphoric acid for $^1$H and $^{31}$P NMR). Exchangeable protons were detected by disappearance of peaks upon D$_2$O addition. Assignments of NMR spectra are based on 2D spectra (COSY, HSQC) and follow standard von Baeyer nomenclature. Quaternary carbons in $^{13}$C NMR are not assigned but their presence was verified.
by HSQC and DEPT spectra (absence of signals). The carbon atom of C4'-substituents is numbered as C5". Similar conventions apply for the corresponding hydrogen atoms. Assignments of \(^1^H\) NMR signals of H5'/H5"/CH\(_2\)Ph and the corresponding \(^{13}^C\) NMR signals are generally interchangeable except when the presence of a free 5'-OH allowed unequivocal identification of H5'. FAB-HRMS were recorded in positive ion mode on a mass spectrometer using 3-nitrobenzyl alcohol as a matrix and PEG as a calibration compound. ESI-HRMS were recorded on a Q-Tof mass spectrometer. Compounds were dissolved in a solution of concentrated solution of NaCl in CH\(_3\)CN. MALDI-HRMS were recorded on a Q-Tof mass spectrometer. Compounds were dissolved in CH\(_2\)Cl\(_2\) with 2,5-dihydroxybenzoic acid (DHB) matrix and PEG as an internal calibration standard.

![Scheme S1](image)

**Scheme S1.** Preparation of terminal alkynes Ae-Ag used in Sonogashira reactions.
Table S1. MALDI-MS of new ONs.\(^a\)

| ON | Sequence     | Found \(m/z\, [M+H]^+\) | Calc. \(m/z\, [M]^+\) |
|----|--------------|---------------------------|------------------------|
| M1 | 5'-GTG AMA TGC | 2821.2                    | 2820.5                 |
| M2 | 3'-CAC MAT ACG | 2750.2                    | 2749.3                 |
| M3 | 3'-CAC TAM ACG | 2750.2                    | 2749.3                 |
| M4 | 3'-CAC MAM ACG | 2818.1                    | 2817.5                 |
| O1 | 5'-GTG AOa TGC | 2996.6                    | 2995.6                 |
| O2 | 3'-CAC OaT ACG | 2925.6                    | 2924.6                 |
| O3 | 3'-CAC TAO ACG | 2925.6                    | 2924.6                 |
| P1 | 5'-GTG APA TGC | 3002.5                    | 3001.7                 |
| P2 | 3'-CAC PAT ACG | 2931.7                    | 2930.7                 |
| P3 | 3'-CAC TAP ACG | 2931.6                    | 2930.7                 |
| P4 | 3'-CAC PAP ACG | 3180.6                    | 3179.8                 |
| V1 | 5'-GTG AVa TGC | 3048.8                    | 3048.5                 |
| V2 | 3'-CAC VAT ACG | 2977.8                    | 2977.5                 |
| V3 | 3'-CAC TAV ACG | 2977.8                    | 2977.5                 |
| V4 | 3'-CAC VAV ACG | 3272.8                    | 3272.7                 |
| W1 | 5'-GTG AWa TGC | 2991.5                    | 2990.5                 |
| W2 | 3'-CAC WAT ACG | 2920.5                    | 2919.5                 |
| W3 | 3'-CAC TAW ACG | 2920.5                    | 2919.5                 |
| W4 | 3'-CAC WAW ACG | 3158.5                    | 3157.5                 |
| X1 | 5'-GTG AXa TGC | 3042.1                    | 3040.5                 |
| X2 | 3'-CAC XAT ACG | 2970.2                    | 2970.5                 |
| X3 | 3'-CAC TAX ACG | 2970.2                    | 2970.5                 |
| X4 | 3'-CAC XAX ACG | 3258.2                    | 3257.6                 |
| Y1 | 5'-GTG AYa TGC | 3035.1                    | 3033.6                 |
| Y2 | 3'-CAC YAT ACG | 2964.1                    | 2962.6                 |
| Y3 | 3'-CAC TAY ACG | 2964.1                    | 2962.6                 |
| Y4 | 3'-CAC YAY ACG | 3245.1                    | 3243.6                 |
| Z1 | 5'-GTG AZa TGC | 3049.2                    | 3047.6                 |
| Z2 | 3'-CAC ZAT ACG | 2978.2                    | 2976.6                 |
| Z3 | 3'-CAC TAZ ACG | 2978.2                    | 2976.6                 |
| Z4 | 3'-CAC ZAZ ACG | 3273.2                    | 3271.6                 |
| W5 | 5'-CG CAA CWa AAC GC | 4125.7                    | 4125.7                 |
| X5 | 5'-CG CAA CXa AAC GC | 4176.2                    | 4175.7                 |
| Y5 | 5'-CG CAA CYa AAC GC | 4170.3                    | 4168.3                 |
| Z5 | 5'-CG CAA CZa AAC GC | 4182.3                    | 4182.1                 |
| Z6 | 5'-CG CAA AZa AAC GC | 4230.2                    | 4229.8                 |
| Z7 | 5'-CG CAA GZa AAC GC | 4262.3                    | 4261.8                 |
| Z8 | 5'-CG CAA TZa AAC GC | 4212.3                    | 4211.8                 |

\(^a\) For structures of monomer M-Z see Figure 1 in the main manuscript.
Table S2. Thermal denaturation temperatures of DNA duplexes with +1 and -1 interstrand arrangements of select C5-functionalized LNA monomers.\(^a\)

| ON | Sequence | \(T_m (\Delta T_m/\text{mod})\) [°C] | Schematic illustration |
|----|----------|----------------------------------|------------------------|
| K1 | 5'-GTG A\(\text{K}A\) TGC | 40.0 (+ 5.5) | |
| K2 | 3'-CAC K\(\text{A}T\) ACG | | |
| N1 | 5'-GTG A\(\text{N}A\) TGC | 42.0 (+ 6.5) | |
| N2 | 3'-CAC N\(\text{A}T\) ACG | | |
| Q1 | 5'-GTG A\(\text{Q}A\) TGC | 37.5 (+ 4.0) | |
| Q2 | 3'-CAC Q\(\text{A}T\) ACG | | |
| K1 | 5'-GTG A\(\text{K}A\) TGC | 44.0 (+ 7.5) | |
| K3 | 3'-CAC T\(\text{A}K\) ACG | | |
| N1 | 5'-GTG A\(\text{N}A\) TGC | 47.0 (+ 9.0) | |
| N3 | 3'-CAC T\(\text{A}N\) ACG | | |
| Q1 | 5'-GTG A\(\text{Q}A\) TGC | 43.0 (+ 7.0) | |
| Q3 | 3'-CAC T\(\text{A}Q\) ACG | | |

\(^a\) For condition of thermal denaturation experiments, see Table 1. Gray droplets denote C5 substituent.
**Binding specificity of B4 ONs.** The binding specificities of ONs with two next-nearest neighbor modifications (B4-series) were determined using DNA/RNA targets with a mismatched nucleotide opposite of the central 2'-deoxyriboadenosine (Table S3). Conventional LNA L4 generally discriminates the mismatched DNA/RNA targets more efficiently than unmodified reference strand D2; the challenging A:G mismatch is discriminated particularly efficiently (compare ΔT_m's for L4 and D1, Table S3). Contrary to observations in the B1-series (Table 3), B4-series ONs modified with K/M/P monomers do not display additional improvements in binding specificity relative to their conventional LNA counterpart (compare ΔT_m's for K4/M4/P4 and L4, Table S3). On the other hand, the strongly stabilizing N4, which is modified with the C5-aminopropynyl conjugated LNA monomer, exhibits exceptional target specificity (Table S3). The structural underpinnings accounting for the differences in mismatch discrimination between N4 and K4/M4/P4 are not clear.

Pyrene-functionalized V4 and Z4 display very different behavior. While V4 displays excellent mismatch discrimination, Z4 hardly discriminates between matched and mismatched targets at all. The absolute T_m's of mismatched duplexes involving W4 were too low to allow conclusions regarding specificity. The presence of pyrene-pyrene excimer signals in the fluorescence emission spectra of V4/Z4-modified duplexes (Figure S2) is indicative of pyrene-pyrene stacking in the major groove of fully base-paired duplexes. We speculate that the presence of a mismatched base pair causes greater disruption of the pyrene-pyrene stacking interactions in V4:DNA than in Z4:DNA, leading to greater duplex destabilization and mismatch discrimination. Similar trends have been reported for related chromophore array-forming ONs.\(^\text{51}\) However, the fluorescence properties of mismatched duplexes involving V4 were not studied due to their low thermostability.
Table S3. Discrimination of singly mismatched DNA/RNA by select doubly modified C5-functionalized LNA (B4-series).a

| ON  | Sequence              | DNA: 5'-GTG ABA TGC | RNA: 5'-GUG ABA UGC |
|-----|-----------------------|----------------------|---------------------|
|     |                       | Tm       | ΔTm  | Tm       | ΔTm   |
|     |                       | T  | A | C | G   | U  | A | C | G   |
| D2  | 3'-CAC TAT ACG        | 29.5 | -19.5 | -16.5 | -7.5 | 27.0 | -16.0 | -16.0 | -11.0 |
| L4  | 3'-CAC LAL ACG        | 40.0 | -17.0 | -15.5 | -19.5 | 43.0 | -20.0 | -15.5 | -16.0 |
| K4  | 3'-CAC KAK ACG        | 40.5 | -12.5 | -14.0 | -12.5 | 44.0 | -15.5 | -15.0 | -15.0 |
| M4  | 3'-CAC MAM ACG        | 40.5 | -15.5 | -15.0 | -15.0 | 43.0 | -18.0 | -14.0 | -15.5 |
| N4  | 3'-CAC NAN ACG        | 45.5 | <35.5 | <35.5 | <35.5 | 49.0 | -23.0 | -19.0 | -22.0 |
| P4  | 3'-CAC PAP ACG        | 35.5 | -15.5 | -14.0 | -15.5 | 35.5 | -13.0 | -11.5 | -13.0 |
| V4  | 3'-CAC VAV ACG        | 30.5 | -20.5 | -20.0 | <20.5 | 30.5 | -19.5 | <20.5 | -20.0 |
| W4  | 3'-CAC WAW ACG        | 17.0 | <7.0  | <7.0  | <7.0  | 16.0 | <6.0  | <6.0  | <6.0  |
| Z4  | 3'-CAC ZAZ ACG        | 25.0 | -1.0  | -2.5  | -3.0  | 26.0 | -4.0  | -7.0  | -6.0  |

*a For experimental conditions and sequences see Table 1. ΔTm = change in Tm value relative to fully matched duplexes.

Figure S1. Additional 3'-exonuclease degradation experiments. For conditions, see Figure 2.
**Fluorescence properties of B1 ONs.** The fluorescence intensities of pyrene-functionalized ONs V1/Y1/Z1 increase significantly upon hybridization with complementary DNA (2.0, 1.5 and 6.1 fold increases for V1, Y1 and Z1, respectively, Figure S2), whereas much smaller increases are observed with mismatched DNA targets. The low absolute T_m's of some of the mismatched duplexes (Table 3) relative to the experimental temperature of the fluorescence experiments (T = 5 °C), renders it difficult to determine if the observed discrimination of mismatched duplexes is due to different positioning of the pyrene moiety (groove vs intercalation), lack of duplex formation, or a combination thereof. Results with modified 13-mer ONs, which display higher absolute T_m's, suggest the former (see main manuscript).

Hybridization of W1 with complementary or mismatched DNA targets is accompanied by a change in the emission profile and decrease in fluorescence intensity (Figure S2), which differs from the observations with W5 (hybridization with complementary DNA resulted in increased emission, Figure 3). This indicates that the fluorescence properties of W-modified ONs are highly dependent on the sequence context.

The fluorescence intensity of perylene-functionalized X1 does not change significantly upon hybridization with complementary DNA, but drops 50-60% upon binding with mismatched targets, presumably due to nucleobase-mediated quenching of intercalating perylene units (Figure S2).
**Figure S2.** Steady-state fluorescence emission spectra of single stranded W1–Z1 (5'-GTG ABA TGC) and corresponding duplexes with complementary or mismatched DNA strands (mismatched nucleotide opposite to modification listed in parenthesis). $\lambda_{\text{ex}} = 344$ nm (V1/Y1/Z1), $\lambda_{\text{ex}} = 375$ nm (W1) or $\lambda_{\text{ex}} = 448$ nm (X1), $T = 5 \, ^{\circ} \text{C}$. Note different axis scales are used.
Fluorescence properties of B4 ONs. In addition to pyrene monomer emission, fluorescence emission spectra of duplexes between V4/Y4/Z4 and complementary DNA/RNA exhibit a broad unstructured peak centered around 485-505 nm (Figure S3), which is consistent with pyrene-pyrene excimer emission and pyrene-pyrene stacking interactions.\textsuperscript{52} As previously discussed, these duplexes are surprisingly thermostable, presumably due to energetically favorable stacking interactions between the pyrene moieties in the major groove (Table 1). Single-stranded Z4 - and to a lesser degree Y4 - also display significant excimer emission, whereas V4 does not (Figure S3); this indicates that the presence of the triazole unit promotes excimer formation in single-stranded probes. As a result, significant increases in excimer emission are observed upon hybridization of V4 and Y4 with DNA/RNA.

Hybridization of 1-ethynylpyrene-functionalized W4 with complementary DNA/RNA results in moderately increased fluorescence emission, which is centered around ~475 nm (Figure S3). The emission intensity of perylene-functionalized X4 increases only slightly upon hybridization with complementary DNA or RNA (Figure S3).
Figure S3. Steady-state fluorescence emission spectra of V4-Z4 and corresponding duplexes with complementary DNA/RNA. $\lambda_{ex} = 344$ nm ($V4/Y4/Z4$), $375$ nm ($W4$) or $448$ nm ($X4$); $T = 5^\circ C$. Note different axis scales are used.
Table S4. Thermal denaturation temperatures of singly modified 13-mer C5-fluorophore-functionalized LNAs against complementary or singly mismatched DNA/RNA targets.ª

| ON | Sequences | DNA: 3'-GC GTT GBB TTG CG | RNA: 3'-GC GUU GBG UUG CG |
|----|-----------|---------------------------|---------------------------|
|    |           | $T_m$ A | $\Delta T_m$ C G T | $T_m$ A | $\Delta T_m$ C G U |
| D5 | 5'-CG CAA CTC AAC GC | 55.5 | -13.5 -7.0 -9.0 | 51.5 | -15.5 -3.0 -13.5 |
| D6 | 5'-CG CAA ATA AAC GC | 48.5 | -10.0 -5.0 -9.0 | - | - | - |
| D7 | 5'-CG CAA GTG AAC GC | 55.0 | -13.0 -9.5 -10.0 | - | - | - |
| D8 | 5'-CG CAA TTT AAC GC | 48.5 | -11.0 -9.0 -11.0 | - | - | - |
| V5 | 5'-CG CAA CVC AAC GC | 53.5 | -9.0 -4.5 -7.0 | 49.5 | -14.0 -5.5 -11.5 |
| W5 | 5'-CG CAA CWC AAC GC | 49.0 | +2.0 +3.0 +0.5 | 47.0 | -12.5 -4.5 -11.0 |
| X5 | 5'-CG CAA CXC AAC GC | 44.5 | -1.5 +1.5 +0.5 | 38.0 | -9.0 -7.0 -8.5 |
| Y5 | 5'-CG CAA CVC AAC GC | 46.5 | -1.0 +0.5 ±0.0 | 43.0 | -11.5 -6.0 -9.5 |
| Z5 | 5'-CG CAA CZC AAC GC | 57.0 | -13.5 -9.5 -7.5 | 53.5 | -16.0 -7.5 -15.0 |
| Z6 | 5'-CG CAA AZA AAC GC | 44.5 | -4.5 -5.0 -4.0 | - | - | - |
| Z7 | 5'-CG CAA GZG AAC GC | 51.5 | -1.5 ±0.0 -1.5 | - | - | - |
| Z8 | 5'-CG CAA TZT AAC GC | 44.0 | -7.5 -5.5 -9.0 | - | - | - |

ª For experimental conditions and sequences see Table 1. $\Delta T_m$ = change in $T_m$ value relative to fully matched ON:DNA or ON:RNA duplex (B=A). “-“ = not determined. The data for D5-D8 and V5 have been previously reported in reference S3.

º The DNA targets for B6-B8 are 3'-GC GTT TBT TTG CG, 3'-GC GTT CBC TTG CG and 3'-GC GTT ABA TTG CG, respectively.

Figure S4. Structure of the DNA analogues of monomers V/Y/Z studied herein.
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PK−I−25a HSQC

3
PK- II−48  DQF−COSY

DMTrO−
ppm
7.50 7.48 7.46 7.44 7.42 7.40 7.38 7.36 7.34 7.32 7.30 7.28 7.26 7.24 7.22
125.5 126.0 126.5 127.0 127.5 128.0 128.5 129.0 129.5 130.0 130.5 131.0

F₃C

DMTrO

4d

S54
| ppm | 123.272 | 123.463 | 124.491 | 124.785 | 125.638 | 125.716 | 126.582 | 126.628 | 127.139 | 127.679 | 127.829 | 128.076 | 128.227 | 128.844 | 129.504 | 129.589 | 130.368 | 130.584 | 130.689 | 130.756 | 135.406 | 141.397 | 144.422 | 149.056 | 149.547 | 157.951 | 158.017 | 161.741 |
|-----|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|     | 120.658 | 120.756 | 123.086 | 123.463 | 124.056 | 124.491 | 125.638 | 125.716 | 126.582 | 126.628 | 127.139 | 127.679 | 127.829 | 128.076 | 128.227 | 128.844 | 129.504 | 129.589 | 130.368 | 130.584 | 130.689 | 130.756 | 135.406 | 141.397 | 144.422 | 149.056 | 149.547 | 157.951 | 158.017 | 161.741 |

**Diagram:**

- The diagram shows a spectrum with peaks at various ppm values, indicating chemical shifts for different elements.
- The chemical structure at the bottom left suggests a specific molecule or compound.
- The peaks are labeled with their respective ppm values, providing a detailed analysis of the spectral data.
HSQC  Pk−10−13

ppm

ppm

54 55 56

57 58

140 150

8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0

S87
Current Data Parameters

NAME qnp31P
EXPNO 24
PROCNO 1

F2 - Acquisition Parameters
Date 20081107
Time 1B.48
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zgpg
TD 32768
SOLVENT CDCl3
NS 32DS 0SWH 26737.969 HzFIDRES 0.815978 HzAQ 0.6128116 seeRG 5160.6
DW 18.700 useeDE 6.00 useeTE 300.0

K1 2.00000000 see
dll 0.03000000 see
ppm

======== CHANNEL f1 ========
NUC1 31PPI 2.00 useePL1 -4.00 dB
SF01 121.5057705 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usee
PL2 120.00 dBPL12 17.00 dBPL13 17.00 dBSF02 300.1312005 MHz

F2 - Processing parameters
SI 3276B
SF 121.4948360 MHz
WDW EMSSB 0
LB 1.00 Hz
GB 0
PC 1.40

220 200 180 160 140 120 100 80 60 40 20 ppm

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