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Recognition of mixed-sequence DNA using double-stranded probes with interstrand zipper arrangements of O2'-triphenylene- and coronene-functionalized RNA monomers

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Abstract

Development of hybridization-based probes that enable recognition of specific mixed-sequence double-stranded DNA (dsDNA) regions is of considerable interest due to their potential applications in molecular biology, biotechnology, and medicine. We have recently demonstrated that double-stranded probes with +1 interstrand zipper arrangements of intercalator-functionalized nucleotides such as 2'-*O*-(pyren-1-yl)methyl RNA monomers are inherently activated for recognition of mixed-sequence dsDNA targets, including chromosomal DNA. In the present work, we follow up on our previous structure-activity relationship studies and explore if the dsDNA-recognition efficiency of these so-called Invader probes can be improved by using larger intercalators than pyrene. Oligodeoxyribonucleotides modified with 2'-*O*-(triphenylene-2-yl)methyl-uridine monomer **X** and 2'-*O*-(coronen-1-yl)methyl-uridine monomer **Z** form extraordinarily stabilized duplexes with complementary DNA (T_m 's per modification of up to 13 °C and 20 °C, respectively). Invader probes based on **X**- and **Z**-monomers are shown to recognize model dsDNA targets with exceptional binding specificity, but are less efficient than reference probes modified with 2'-*O*-(pyren-1-yl)methyl-uridine monomer **Y**. The insight from this study will inform further optimization of Invader probes.

TOC image

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Electronic supplementary information (ESI) available: General experimental section; MS-data for modified ONs; representative thermal denaturation curves; additional denaturation and thermodynamic data; additional steady-state fluorescence emission spectra; NMR spectra for nucleosides **2X**, **3X** and **4X**. See DOI:

Energetically activated double-stranded probes with interstrand arrangements of intercalator-functionalized nucleotides enable recognition of mixed-sequence DNA hairpins with excellent binding specificity.

INTRODUCTION

Considerable efforts have been devoted over the past 25 years to develop molecular strategies that enable recognition of specific regions of double-stranded DNA (dsDNA) targets to facilitate modulation of gene expression via transcriptional interference, detection of diagnostically relevant sequences, and genome modification. Examples of major advances toward this end include triplex forming oligonucleotides (TFOs),¹ peptide nucleic acids (PNAs),^{2,3} and minor groove binding polyamides,^{4–6} which were followed by optimized TFOs and PNAs that can be applied across a broader range of targets.^{7–16} More recently, engineered proteins such as CRISPR/Cas have gained widespread popularity,¹⁷ despite concerns regarding inadequate binding specificity and cellular delivery.¹⁸ Many DNA targeting applications will benefit from simpler hybridization-based probes that enable rapid, potent, and specific recognition of mixed-sequence dsDNA under physiologic conditions.

Our efforts towards this end have focused on a double-duplex invasion approach that utilizes double-stranded probes with +1 interstrand zipper¹⁹ arrangements of intercalator-functionalized nucleotides (Figure 1 – top panel).²⁰ The two probe strands display very high affinity toward complementary DNA (cDNA), since duplex formation results in strongly stabilizing π - π stacking interactions between the intercalators and nucleobases (Figure 1 – top panel). In contrast, the +1 interstrand zipper motif forces the two intercalators to compete for the same inter-base-pair site in the probe duplex, which results in the formation of an unwound and destabilized region, as the ‘nearest neighbor exclusion principle’ is violated.²¹ The principle asserts that the two sites directly neighboring an occupied intercalation site will strive to remain intercalator-free²² due to limitations in local helix expandability (every intercalation event unwinds the duplex by ~ 3.4 Å) and resistance against disruption of stabilizing stacking interactions between nucleobases and the first intercalator.^{23,24} Double-stranded probes with other interstrand zipper arrangements of intercalator-functionalized nucleotides are not nearly as destabilized, since the intercalators occupy spatially separated base-pair steps and thus do not violate the nearest neighbor exclusion principle.²⁰ The stability difference between the reactants (i.e., the double-stranded probe and DNA target) and products (i.e., the two probe-target duplexes formed as part of a recognition complex) provides the driving force for dsDNA-recognition.

Originally, we used 2'-*N*-(pyren-1-yl)methyl-2'-amino- α -LNA (Locked Nucleic Acid) nucleotides as the key activating components of these so-called Invader probes.²⁵ Subsequent studies revealed that the synthetically more readily available 2'-*O*-(pyren-1-yl)methyl-RNA monomers can be used instead with minimal or no loss in dsDNA-recognition efficiency.²⁰ In fact, we have shown that Invader probes based on the 2'-*O*-(pyren-1-yl)methyl-RNA chemistry can be used for recognition of mixed-sequence chromosomal DNA targets at non-denaturing conditions.²⁶ Recently, we have carried out a series of systematic structure-property studies aiming at improving the dsDNA-recognition

efficiency of Invader probes and have thus examined the impact of different nucleobases,²⁷ relative intercalator orientations,²⁸ intercalator-sugar linkers,²⁰ sugar skeletons,^{20,29} backbones and probe architectures.^{26,30–32}

In the present study, we set out to explore if the dsDNA-recognition efficiency of Invader probes based on O2'-intercalator-functionalized RNA monomers can be improved through the use of intercalators that are larger than pyrenes, given that the surface area of pyrene (~220 Å²) is smaller than the area occupied by natural base-pairs (~270 Å²).³³ We hypothesized that it is possible to increase the stability of probe-target duplexes by promoting stronger intercalator-nucleobase π - π -stacking interactions, while further destabilizing probe duplexes by inducing a more pronounced violation of the nearest-neighbor exclusion principle, thus resulting in a greater thermodynamic driving force for dsDNA-recognition. Support for this hypothesis is provided by the observation that oligodeoxyribonucleotides (ONs) modified with 2'-*O*-(coronen-1-yl)methyl-uridine monomer **Z** form even more stabilized duplexes with complementary DNA than **Y**-modified ONs, with increases in thermal denaturation temperatures (T_m 's) of up to 20 °C per modification.³⁴ We recently tested a similar hypothesis using the related 2'-*N*-methyl-2'-amino-DNA monomers and found that building blocks featuring larger intercalators result in slightly more efficient dsDNA-recognition.³⁵

Herein, we set out to synthesize and characterize the biophysical properties of single- and double-stranded ONs modified with 2'-*O*-(triphenylen-2-yl)methyl-uridine monomer **X** and 2'-*O*-(coronen-1-yl)methyl-uridine monomer **Z** and compare them to corresponding reference ONs modified with 2'-*O*-(pyren-1-yl)methyl-uridine monomer **Y** (Figure 1 – bottom panel).³⁶

RESULTS AND DISCUSSION

Synthesis of O2'-triphenylene-functionalized uridine phosphoramidite **4X**

Phosphoramidite **4X** was synthesized following our method³⁴ for **4Y** and **4Z** (Scheme 1). Thus, tris(triphenylen-2-yl)methyl borate, which was generated in situ³⁸ through the addition of 2-(hydroxymethyl)triphenylene³⁹ to borane-tetrahydrofuran, was reacted with O2,O2'-anhydrouridine **1**⁴⁰ at elevated temperatures to afford **2X** in a modest but acceptable yield. The disappearance of ¹H NMR signals from exchangeable protons upon D₂O addition, in concert with HRMS and other NMR data (¹H, ¹³C, COSY, HSQC, and DEPT),[†] ascertained the O2'-functionalized nature of nucleoside **2X**. Subsequent standard O5'-dimethoxytritylation afforded nucleoside **3X** in 61% yield, which on treatment with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI reagent) and Hünig's base provided access to target phosphoramidite **4X** in 78% yield. Attempts to obtain the corresponding 2'-*O*-(phenanthren-2-yl-methyl)uridine analogue of **4X** via an equivalent approach were obstructed by the formation of significant amounts (~35%) of inseparable 3- and 9-regioisomers during the synthesis of the prerequisite 2-(hydroxymethyl)phenanthrene (results not shown).

Synthesis of X-modified ONs

Phosphoramidite **4X** was used to incorporate monomer **X** into ONs via machine-assisted solid-phase DNA synthesis using 4,5-dicyanoimidazole as an activator and extended hand-coupling (15 min), which resulted in stepwise coupling yields of ~99%. The identity and purity of the modified ONs were established by MALDI-TOF (Table S1†) and ion-pair reverse phase HPLC (>90% purity), respectively. Monomer **X** was studied in two complementary 9-mer sequences that we have previously used for evaluation of other potential Invader building blocks.³⁴ **Y**- and **Z**-modified ONs were available from previous studies.³⁴ ONs containing a single modification in the 5'-GBG ATA TGC context are denoted **X1**, **Y1**, and **Z1** respectively. Similar conventions apply to the **B2-B6** series (Table 1). Reference DNA and RNA strands are denoted **D1** and **D2**, and **R1** and **R2**, respectively (see footnote, Table 1).

Thermal denaturation properties of duplexes between X-modified ONs and complementary DNA/RNA

Melting temperatures (T_m 's) of duplexes between **B1-B6** and complementary DNA and RNA targets were determined from thermal denaturation experiments conducted in a medium salt phosphate buffer ($[Na^+] = 110$ mM, pH 7). The resulting denaturation curves display the expected monophasic sigmoidal transitions (Figure S1†). Prominent increases in duplex T_m 's are observed upon incorporation of **X** monomers relative to the reference DNA duplex **D1:D2** (T_m per modification (T_m/mod) = +3.0 to +13.0 °C, Table 1). In fact, the observed stabilization trends closely mirror those seen with the corresponding **Y**-modified DNA duplexes, whereas duplexes modified with coronene-functionalized **Z** monomers are even more stable. The DNA duplex stabilizing properties of monomer **X** are strongly sequence dependent, as is also seen with **Y** and **Z** monomers.³⁴ Thus, ONs in which the **X** monomer is flanked by a 3'-purine result in greater duplex stabilization than ONs with 3'-flanking pyrimidines, which is indicative of 3'-intercalative binding modes^{27,41,42} (e.g., compare T_m/mod values for **B2** and **B4**-series, Table 1).

Duplexes between **X**-modified ONs and cRNA are far less stable ($T_m/mod = -4.0$ to +7.0 °C, Table 1). In other words, pronounced DNA-selectivity – defined as T_m/mod (DNA-RNA) = T_m/mod (vs DNA) – T_m/mod (vs RNA) > 0 °C – is observed for **X**-modified ONs ($T_m/mod = +5.0$ to +8.0 °C, Table S2†). The observed DNA-selectivity is another indicator that the triphenylene moiety of monomer **X** is intercalating since intercalators typically favor the less compressed *B*-type helix geometry of DNA:DNA duplexes.^{28,42,43} The trends in RNA affinity and DNA-selectivity among the intercalator-modified monomers studied herein are **Z** > **X** > **Y** and **Z** > **Y** > **X**, respectively (compare T_m/mod and T_m/mod (DNA-RNA) values in Table 1 and Table S2†, respectively).

Binding specificity of X-modified ONs

The DNA binding specificities of singly modified ONs (**B2**-series) were evaluated using DNA strands with a mismatched nucleotide opposite of the centrally incorporated intercalator-functionalized monomer (Table 2). **X2** displays similar mismatch discrimination as the reference strand **D1**, except when a thymidine is opposite (T-T mismatch) of the

triphenylene-functionalized uridine monomer **X**, as this results in less efficient discrimination. **X2** displays more efficient mismatch discrimination than the corresponding pyrene- and coronene-functionalized **Y2** and **Z2**. In line with our previous observations,³⁴ **X6** with two triphenylene-functionalized modifications positioned as next-nearest neighbors displays very efficient discrimination of DNA targets with a mismatched nucleotide opposite of the central adenosine (Table 3).⁴⁴ The structural underpinnings for the improved mismatch discrimination are unknown, but we speculate that intercalator-mediated rigidification of the central duplex region increases demands for base-pair geometry which, consequentially, increases binding specificity.

Photophysical characterization of X-modified ONs and duplexes with cDNA/cRNA

UV/Vis absorption and steady-state fluorescence emission spectra of **X**-modified ONs and the corresponding duplexes with cDNA/cRNA were recorded in an attempt to gain additional insight into the binding mode of the triphenylene moiety as, for example, intercalation of aryl moieties is known to result in hybridization-mediated bathochromic shifts of absorption peaks.⁴⁵ However, the overlap between the absorption maxima of the triphenylene and nucleobase moieties in the 250–270 nm region precluded a detailed binding mode analysis by UV/Vis signals (results not shown). Steady-state fluorescence emission spectra of **X**-modified ONs and the corresponding duplexes with cDNA/cRNA ($\lambda_{\text{ex}} = 260$ nm, $T = 5$ °C) exhibit broad and unstructured emission between 350–450 nm (Figure 2 and Figure S2†). The Stokes shift of more than 100 nm is noteworthy. The emission intensity decreases upon hybridization with cDNA/cRNA targets, with slightly more pronounced decreases being observed upon cDNA binding. Similar observations were made with **Y**-modified ONs, where it was subsequently demonstrated that the pyrene moiety is intercalating into the duplex upon cDNA binding but adopts a more pronounced minor groove orientation upon cRNA binding.^{28,42}

Properties of double-stranded probes with interstrand zippers of X/Y/Z-monomers

Next, we studied double-stranded DNA probes with different interstrand zipper configurations¹⁹ of **X** and **Z** monomers to determine their suitability for dsDNA-targeting via the Invader approach, comparing them to the corresponding **Y**-modified reference probes (Table 4). In agreement with our previous observations,^{20,25,26,28–30} probes with +1 interstrand zippers of **X** or **Z** monomers display significantly lower T_m 's than probes with other interstrand monomer arrangements as the nearest neighboring exclusion principle is violated (compare T_m 's for the **B2:B5** probes relative to the **B1:B5**, **B1:B4**, **B2:B4**, **B3:B5**, and **B3:B4** probes, Table 4). The unique characteristics of the **B2:B5** probes are further underscored by their broad thermal denaturation profiles, which are indicative of low cooperativity (Figure S1†). The T_m 's of probes modified with triphenylene-functionalized monomer **X** and pyrene-functionalized monomer **Y** are very similar, whereas the corresponding probes modified with the coronene-functionalized monomer **Z** display markedly higher T_m 's (e.g., compare T_m 's of **B2:B5** probes, Table 4). The relatively low T_m 's of the +1 zipper probes and high cDNA affinity of the individual probe strands suggests that the **B2:B5** probes are activated for dsDNA-recognition. The dsDNA-targeting potential of these probes can be estimated by the term $\Delta G_{\text{rec}}^{293}(\text{ON}_A:\text{ON}_B) = G^{293}$

$(\text{ON}_\text{A}:\text{cDNA}) + G^{293}(\text{cDNA}:\text{ON}_\text{B}) - G^{293}(\text{ON}_\text{A}:\text{ON}_\text{B}) - G^{293}(\text{dsDNA})$, i.e., the free energy difference between the probe-target duplexes, and the probe and target duplexes.⁴⁶ Thermodynamic parameters for duplex formation were derived from thermal denaturation curves via line-fitting.⁴⁷

In general agreement with the aforementioned T_m data, duplexes between individual probe strands and cDNA are much more stable than the unmodified reference duplex **D1:D2**, with **Z**-modified ONs forming the most stable duplexes (compare values in the first two G^{293} columns, Table 4). In further accordance with the T_m data, **B2:B5** probes were found to form the most labile duplexes among the **X**- or **Y**-based probes ($G^{293} = 0$ and 3 kJ/mol, respectively, Table 4). Moreover, **Z2:Z5** is not particularly destabilized relative to the other **Z**-based probe duplexes. We speculate that this is due to very prominent π - π stacking between the two intercalating coronene moieties in **Z2:Z5** resulting in unusual probe duplex stabilization. As a consequence of these trends, the +1 zipper probes are strongly thermodynamically activated for recognition of isosequential dsDNA targets as evidenced by the highly negative $\Delta G^{293}_{\text{rec}}$ values (Table 4). The triphenylene-based +1 zipper probe **X2:X5** is slightly more activated for dsDNA-recognition than **Y2:Y5** or **Z2:Z5**. Probes with other interstrand arrangements of **X/Y/Z**-monomers display significantly lower dsDNA-targeting potential ($\Delta G^{293}_{\text{rec}}$ between -21 and -1 kJ/mol, Table 4).

Invader-mediated recognition of DNA hairpins

Based on the observed T_m and $\Delta G^{293}_{\text{rec}}$ trends, we decided to study the dsDNA-recognition properties of double-stranded probes with +1 zipper arrangements of **X/Y/Z**-monomers. Toward this end, a digoxigenin (DIG) labeled DNA hairpin (DH) – comprised of a 9-mer double-stranded stem that is linked by a T_{10} loop – was used as a model target (Figure 3a). The unimolecular nature of the DNA hairpin greatly stabilizes the stem region and minimizes fraying (compare $T_\text{m} = 57.0$ °C for **DH1**, Figure 3b, with $T_\text{m} = 29.5$ °C for corresponding linear DNA duplex **D1:D2**, Table 1). A varying excess of **B2:B5** probes was incubated with DNA hairpin **DH1** at room temperature for 15 h. Dose-dependent formation of recognition complexes was observed as evidenced by the emergence of bands with lower electrophoretic mobility on non-denaturing PAGE gels (Figure 3d). The use of a 100-fold molar excess of **B2:B5** probes results in ~50-70% recognition (Figure 3c and 3d). **X2:X5** and **Z2:Z5** display similar dsDNA-recognition efficiency whereas reference probe **Y2:Y5** is far more efficient (C_{50} , i.e., the probe concentration that results in 50% recognition of **DH1** = ~3.1, ~0.034, and ~2.4 μM for **X2:X5**, **Y2:Y5**, and **Z2:Z5**, Figure 3c). Interestingly, these observations do not correlate with the trends in $\Delta G^{293}_{\text{rec}}$ values (Table 4), prompting us to speculate that recognition of **DH1** is kinetically limited with **B2:B5** probes displaying different activation barriers for dsDNA-recognition. Toward this end, we note that **Y2:Y5** is the least stable of the three probe duplexes (compare G^{293} values for **B2:B5** probes, Table 4) and, therefore, most likely to partially denature and initially contact the dsDNA target. However, further studies are needed to fully rationalize these complex relationships.

The binding specificity of **X2:X5**, **Y2:Y5** and **Z2:Z5** was evaluated by incubating the double-stranded probes with DNA hairpins **DH2-DH7**, which have stem regions that only

differ in sequence at one position relative to the probes (underlined residues indicate position of sequence deviation, Figure 3b). Remarkably, none of these probes result in detectable recognition of the “singly mismatched” DNA hairpins, even when used at 500-fold molar excess, which highlights the extraordinary specificity of Invader probes (Figure 3e). These results underscore the attractiveness of dsDNA-recognition via a double-duplex invasion approach as binding to mismatched targets would necessitate the formation of two destabilized probe-target duplex regions.⁴⁸

CONCLUSION

Oligodeoxyribonucleotides that are modified with the O2'-triphenylene-functionalized uridine **X** monomer display very high affinity towards cDNA targets (T_m /mod up to 13.0 °C), prominent DNA-selectivity (T_m /mod (DNA-RNA) up to 8.0 °C/mod), and decreased fluorescence emission upon cDNA binding, i.e., observations that are consistent with an intercalative binding mode of the triphenylene moiety. As expected, double-stranded probes with +1 interstrand zippers of **X** monomers are highly thermodynamically activated for dsDNA-recognition ($\Delta G_{rec}^{293} = 33$ kJ/mol). Corresponding probe duplexes based on O2'-coronene-functionalized uridine **Z** monomers are also energetically activated for dsDNA-recognition but display higher absolute T_m values. **X**- and **Z**-modified Invader probes allow for recognition of DNA hairpins with extraordinary mismatch discrimination, but probes based on the O2'-pyrene-functionalized uridine **Y** reference monomer display even more efficient dsDNA-recognition. The results suggest that the pyrene chromophore is a near-optimal intercalator for Invader probes that are based on O2'-intercalator-functionalized RNA monomers. This differs from our observations with Invader probes based on 2'-*N*-methyl-2'-amino-DNA monomers, where perylene- and coronene-functionalized probes were found to result in similar-to-slightly improved recognition of dsDNA targets relative to the corresponding pyrene-functionalized probes.³⁵ Informed by the present structure-activity relationship study, we are currently exploring Invader probes based on RNA monomers that are functionalized at the 2'-position with substituted pyrene moieties, with the goal of developing probes that display even more efficient recognition of mixed-sequence dsDNA.

EXPERIMENTAL SECTION

2'-O-(Triphenylen-2-yl)methyl-uridine (2X)

2-(Hydroxymethyl)triphenylene³⁹ (3.42 g, 13.3 mmol), NaHCO₃ (0.11 g, 1.33 mmol), and borane-tetrahydrofuran (1.0 M, 3.3 mL, 3.30 mmol) were placed in a pressure tube and suspended in anhydrous DMSO (15 mL), and stirred under an argon atmosphere at room temperature until effervescence ceased (~10 min). At this point, O2,O2'-anhydrouridine **1**⁴⁰ (1.50 g, 6.63 mmol) was added and the pressure tube was purged with argon and sealed and the mixture was heated at ~140 °C until analytical TLC indicated full conversion (~3h). The reaction mixture was cooled to room temperature, poured into water (~100 mL), stirred for 30 min, and diluted with EtOAc (~200 mL). The phases were separated and the organic phase was washed with water (4 × 75 mL), evaporated to dryness, and the resulting residue purified by silica gel column chromatography (2-4%, MeOH in CH₂Cl₂, v/v) to afford a crude material, which was further purified through precipitation from refluxing methanol

(~30 min) to afford nucleoside **2X** (0.81 g, 25 %) as a white solid material. $R_f = 0.4$ (10 % MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 507.1528 ($[\text{M}+\text{Na}]^+$, $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_6\cdot\text{Na}^+$, Calc. 507.1527); ^1H NMR ($\text{DMSO}-d_6$): δ 11.37 (s, ex, 1H, NH), 8.74-8.83 (m, 6H, Trip),⁴⁹ 7.92 (d, 1H, $J = 8.3$ Hz, H6), 7.66-7.74 (m, 5H, Trip), 6.06 (d, 1H, $J = 5.0$ Hz, H1'), 5.56 (d, 1H, $J = 8.3$ Hz, H5), 5.37 (d, ex, 1H, $J = 6.0$ Hz, 3'-OH), 5.14 (t, ex, 1H, $J = 5.0$ Hz, 5'-OH), 4.98-5.01 (d, 1H, $J = 12.7$ Hz, CH_2Trip), 4.87-4.90 (d, 1H, $J = 12.7$ Hz, CH_2Trip), 4.21-4.25 (m, 1H, H3'), 4.12-4.15 (m, 1H, H2'), 3.97-4.01 (m, 1H, H4'), 3.67-3.72 (m, 1H, H5'), 3.58-3.64 (m, 1H, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 163.0, 150.6, 140.2 (C6), 137.5, 129.2, 129.05, 129.03, 128.9, 128.4, 127.58 (Trip), 127.56 (Trip), 127.48 (Trip), 126.8 (Trip), 123.6 (Trip), 123.53 (Trip), 123.50 (Trip), 123.4 (Trip), 122.0 (Trip), 101.7 (C5), 86.4 (C1'), 85.2 (C4'), 80.9 (C2'), 70.9 (CH_2Trip), 68.4 (C3'), 60.4 (C5').

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(triphenylen-2-yl)methyl-uridine (**3X**)

Nucleoside **2X** (0.65 g, 1.34 mmol) was co-evaporated twice with anhydrous pyridine and re-dissolved in anhydrous pyridine (20 mL). To this was added 4,4'-dimethoxytritylchloride (DMTrCl) (0.78 g, 2.01 mmol) and catalytic DMAP (~4 mg), and the reaction mixture was stirred under an argon atmosphere at rt for ~14h. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and the organic phase was washed sequentially with water (2×30 mL) and sat. aq. NaHCO_3 (2×50 mL). The organic phase was evaporated to near dryness and the resulting residue was co-evaporated with abs. EtOH and toluene (2:1, v/v, 3×3 mL) and purified by silica gel column chromatography (0-5%, MeOH in CH_2Cl_2 , v/v) to afford nucleoside **3X** (0.78 g, 74 %) as a pale yellow foam. $R_f = 0.6$ (5%, MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 809.2841 ($[\text{M}+\text{Na}]^+$, $\text{C}_{49}\text{H}_{42}\text{N}_2\text{O}_8\cdot\text{Na}^+$, Calc. 809.2833); ^1H NMR ($\text{DMSO}-d_6$) δ 11.42 (br s, ex, 1H, NH), 8.74-8.82 (m, 6H, Trip),⁴⁹ 7.69-7.74 (m, 6H, H6, Trip), 7.17-7.35 (m, 9H, DMTr), 6.82-6.86 (m, 4H, DMTr), 6.05 (d, 1H, $J = 3.9$ Hz, H1'), 5.46 (d, ex, 1H, $J = 7.0$ Hz, 3'-OH), 5.20 (dd, 1H, $J = 6.0$ Hz, 2.2 Hz, H5), 5.01-5.05 (d, 1H, $J = 12.9$ Hz, CH_2Trip), 4.97-5.01 (d, 1H, $J = 12.9$ Hz, CH_2Trip), 4.31-4.36 (m, 1H, H3'), 4.17-4.19 (m, 1H, H2'), 4.09-4.13 (m, 1H, H4'), 3.69 (s, 3H, CH_3O), 3.68 (s, 3H, CH_3O), 3.30-3.33 (dd, 1H, $J = 11.0$ Hz, 4.3 Hz, H5'), 3.25-3.28 (m, 1H, $J = 11.0$ Hz, 2.5 Hz, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 162.9, 158.08, 158.07, 150.4, 144.4, 140.1 (C6), 137.4, 135.3, 135.0, 129.72 (DMTr), 129.65 (DMTr), 129.2, 129.08, 129.06, 129.05, 129.01, 128.5, 127.8 (DMTr), 127.7 (DMTr), 127.6 (Trip), 127.5 (Trip), 126.8 (Trip), 126.7 (DMTr), 123.7 (Trip), 123.55 (Trip), 123.53, 123.51 (Trip), 123.46 (Trip), 122.0 (Trip), 113.19 (DMTr), 113.18 (DMTr), 101.5 (C5), 87.2 (C1'), 85.9, 82.9 (C4'), 80.5 (C2'), 71.0 (CH_2Trip), 68.7 (C3'), 62.7 (C5'), 55.0 (CH_3O).

3'-O-(*N,N*-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2'-O-(triphenylen-2-yl)methyl-uridine (**4X**)

Nucleoside **3X** (0.51 g, 0.65 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (3 mL) and re-dissolved in anhydrous CH_2Cl_2 (20 mL). To this was added anhydrous *N,N*-diisopropylethylamine (DIPEA) (0.46 mL, 2.59 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI-reagent) (0.29 mL, 1.29 mmol). The reaction mixture was stirred under an argon atmosphere at rt for ~3h, whereupon abs. EtOH (1 mL) and CH_2Cl_2 (12 mL) were added sequentially. The organic phase was washed with sat. aq. NaHCO_3 (6 mL), evaporated to near dryness, and the resulting residue was purified by silica

gel column chromatography (40-70% EtOAc in petroleum ether, v/v) to afford phosphoramidite **4X** (0.50 g, 78 %) as a white foam. $R_f = 0.8$ (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 1009.3916 ($[\text{M}+\text{Na}]^+$, $\text{C}_{58}\text{H}_{59}\text{N}_4\text{O}_9\text{P}\cdot\text{Na}^+$, Calc. 1009.3912); ^{31}P NMR (CDCl_3) δ 149.5, 148.9.

Synthesis and purification of ONs

X-modified ONs were synthesized on a 0.2 μmol scale using a computer-controlled DNA synthesizer and 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. A ~50-fold molar excess of **4X** in anhydrous acetonitrile (at 0.05 M), along with extended oxidation (45 s) and coupling times (activator: 0.01 M 4,5-dicyanoimidazole, 15 min), was used during hand-couplings which resulted in stepwise coupling yields of monomer **X** of ~99%. Cleavage from solid support and removal of protecting groups was accomplished by treatment with 32% aq. ammonia (55 °C, 24 h). The crude ONs were purified via ion-pair reverse phase HPLC (XTerra MS C18 column) using a 0.05 M triethylammonium acetate - water/acetonitrile gradient, followed by detritylation (80% aq. AcOH), and precipitation ($\text{NaOAc}/\text{NaClO}_4/\text{acetone}$, -18 °C for 12–16 h). The identity of synthesized ONs was established through MALDI-MS which was recorded in positive ion mode on a Quadrupole Time-Of-Flight Tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K) using anthranilic acid as a matrix, while purity (>90%) was verified by ion-pair reverse phase HPLC running in analytical mode.

Thermal denaturation experiments

Concentrations of ONs were estimated using the following extinction 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); and for triphenylene 17.0 OD/ μmol .⁵⁰ Strands were mixed and denatured by heating to 70–85 °C, followed by cooling 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 's) of duplexes (1.0 μM final concentration of each strand) 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. T) 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 and 5 mM Na_2HPO_4). The temperature of the denaturation experiments ranged from at least 15 °C below T_m to 20 °C above T_m (although not below 3 °C). A temperature ramp of 0.5 °C/min was used in all experiments. Reported T_m -values were determined as the average of at least two independent experiments yielding T_m 's within ± 1.0 °C (i.e., measurements were performed on at least two independently assembled samples).

Determination of thermodynamic parameters

Thermodynamic parameters for duplex formation were determined through fitting of baselines of denaturation curves (van't Hoff analysis) using software provided with the UV/vis spectrometer. Bimolecular reactions, two-state melting behavior, and a heat capacity

change of $C_p = 0$ upon hybridization were assumed.⁴⁷ A minimum of two experimental denaturation curves were each analyzed at least three times to minimize errors arising from baseline choice. Averages and standard deviations are reported.

Steady-state fluorescence emission spectra

Steady-state fluorescence emission spectra of X-modified ONs and the corresponding duplexes with cDNA/cRNA, were recorded in non-deoxygenated thermal denaturation buffer (each strand at 1.0 μ M concentration) and obtained as an average of five scans using an excitation wavelength of $\lambda_{ex} = 260$ nm, excitation slit 5.0 nm, emission slit 2.5 nm, and a scan speed of 600 nm/min. Experiments were determined at 5 °C to ascertain maximal hybridization of probes to targets.

Recognition of DNA hairpins

Unmodified 9-mer DNA hairpins DH1-DH7 were obtained from commercial sources and used without further purification. The DNA hairpins were 3'-DIG-labeled using the 2nd generation DIG Gel Shift Kit (Roche Applied Bioscience) as recommended by the manufacturer. The DIG-labeled hairpins were then diluted and used directly in the recognition experiments. Pre-annealed probes (90 °C for 10 min, cooled to room temperature over 15 min) and the DIG-labeled DNA hairpin (34.4 nM) were mixed and incubated in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 10% sucrose, 1.44 mM spermine tetrahydrochloride, pH 7.2) for 15 h at room temperature. The reaction mixtures were diluted with 6 $\times$ DNA loading dye (Fermentas) and loaded onto a 12% non-denaturing polyacrylamide gel. Electrophoresis was performed using constant voltage (70 V) for 3 h at $\sim$ 4 °C (internally circulating ice-cold water) using 0.5 $\times$ TBE as a running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Gels were blotted onto a positively charged nylon membrane (Roche Applied Bioscience) using constant voltage with external cooling (100 V, $\sim$ 4 °C). The membranes were exposed to anti-digoxigenin-AP F_{ab} fragments as recommend by the manufacturer of the DIG Gel Shift Kit, transferred to a hybridization jacket, and incubated with the substrate (CSPD) in detection buffer for 10 min at 37 °C. The chemiluminescence of the formed product was captured on X-ray film, which was developed using an X-Omatic 1000A X-ray film developer (Kodak). The resulting bands were quantified using a Fluor-S MultiImager (Bio-Rad, Hercules, CA) equipped with Quantity One software. Invasion efficiency was determined as the signal intensity ratio between the recognition complex band and the total lane. An average of three independent experiments is reported along with standard deviations. The best-fit line was calculated using non-linear regression analysis via an iterative least squares fitting routine written for the "Solver" module in Microsoft Office Excel.⁵¹

Definition of interstrand zipper arrangement

The following nomenclature describes the relative arrangement between two monomers positioned on opposing strands in a duplex. The number n describes the distance measured in number of base-pairs and has a positive value if a monomer is shifted toward the 5'-side of its strand relative to a second reference monomer on the other strand. Conversely, n has a negative value if a monomer is shifted toward the 3'-side of its strand relative to a second reference monomer on the other strand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
 Top panel: Principle of sequence-unrestricted targeting of dsDNA using Invader probes (droplets denote intercalators). Bottom panel: Structures of monomers used in this study.

Figure 2.

Steady-state fluorescence emission spectra of select X-modified ONs and the corresponding duplexes with cDNA/cRNA. Spectra were recorded at $T = 5\text{ }^{\circ}\text{C}$ using $\lambda_{\text{ex}} = 260\text{ nm}$ and each strand at $1.0\text{ }\mu\text{M}$ concentration in T_{m} buffer. SSP = single-stranded probe. For additional spectra, see Figure S2†.

Figure 3.

Invader-mediated recognition of DNA hairpins. (a) Illustration of recognition process; (b) sequences and T_m 's of DNA hairpins with isosequential (DH1) or non-isosequential stem regions (DH2-DH7) relative to B2:B5 probes (for conditions of thermal denaturation experiments, see Table 1); (c) dose-response curves of DH1 recognition using B2:B5 probes (average of three independent experiments; error bars represent standard deviation); (d) representative gel electropherograms visualizing recognition of DH1 using 5- to 500-fold molar excess of X2:X5, Y2:Y5, Z2:Z5; (e) and gel electropherograms visualizing outcome of recognition experiments between DH1-DH7 and 500-fold molar excess of X2:X5, Y2:Y5 or Z2:Z5. Experimental conditions for electrophoretic mobility shift assay: separately pre-annealed targets (34.4 nM) and probes were incubated for in 1 $\times$ HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 10% sucrose, 1.4 mM spermine tetrahydrochloride, pH 7.2) for 15 h at room temperature. Reaction mixtures were resolved on 12% non-denaturing PAGE (performed at 70 V, 3 h, ~4 °C) using 0.5 $\times$ TBE as a running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA); DIG: digoxigenin.

Scheme 1.

Synthesis of triphenylene-functionalized phosphoramidite 4X. $DMTr = 4,4'$ -dimethoxytrityl; PCI reagent = 2-cyanoethyl- N,N' -diisopropylchlorophosphoramidite; $Trip$ = triphenylen-2-yl.

Table 1

Thermal denaturation temperatures (T_m 's) for duplexes between B1-B6 and DNA or RNA complements (cDNA/cRNA).^a

ON	Sequence	B =	T_m (T_m /mod) [°C]					
			+ cDNA			+ cRNA		
			X	Y ^b	Z ^b	X	Y ^b	Z ^b
B1	5'-G <u>B</u> G ATA TGC		32.5 [+3.0]	34.5 [+5.0]	34.0 [+4.5]	24.5 [-2.0]	24.5 [-2.0]	21.5 [-5.0]
B2	5'-GTG A <u>B</u> A TGC		42.5 [+13.0]	42.5 [+13.0]	49.5 [+20.0]	32.0 [+5.5]	30.5 [+4.0]	37.5 [+11.0]
B3	5'-GTG ATA <u>B</u> GC		37.5 [+8.0]	37.5 [+8.0]	40.5 [+11.0]	26.5 [±0.0]	26.5 [±0.0]	30.5 [+4.0]
B4	3'-CAC <u>B</u> AT ACG		32.5 [+3.0]	33.0 [+3.5]	36.0 [+6.5]	20.5 [-4.0]	20.0 [-4.5]	22.5 [-2.0]
B5	3'-CAC TA <u>B</u> ACG		42.5 [+13.0]	42.5 [+13.0]	45.5 [+16.0]	31.5 [+7.0]	27.0 [+2.5]	30.5 [+6.0]
B6	3'-CAC <u>B</u> A <u>B</u> ACG		44.5 [+7.5]	43.5 [+7.0]	47.0 [+8.8]	25.5 [+0.5]	24.0 [-0.3]	11.0 [-6.8]

^a T_m = change in T_m relative to reference duplexes **D1:D2** (T_m = 29.5 °C), **D1:R2** (T_m = 26.5 °C), where **D1**: 5'-[DNA]-GTGATATGC, **D2**: 3'-[DNA]-CACTATACG, **R1**: 5'-[RNA]-GUGAUUGC and **R2**: 3'-[RNA]-CACUAUACG; T_m 's are determined as the maximum of the first derivative of denaturation curves (A_{260} vs T) recorded in medium salt phosphate buffer ([Na⁺] = 110 mM, [Cl⁻] = 100 mM, pH 7.0 (NaH₂PO₄/Na₂HPO₄)), using 1.0 μM of each strand. T_m 's were determined as an average of at least two independent experiments within 1.0 °C; A = adenine-9-yl DNA monomer, C = cytosine-1-yl DNA monomer, G = guanine-9-yl DNA monomer, T = thymine-1-yl DNA monomer. For structures of monomers **X**, **Y** and **Z** see Figure 1.

^b Data from reference³⁴ are included to facilitate comparison.

Table 2Discrimination of mismatched DNA targets by X2/Y2/Z2 and reference strands.^a

DNA: 3'-CAC.TBT.ACG						
ON	Sequence	B =	T_m [°C]		T_m [°C]	
			A	C	G	T
D1	5'-GTG ATA TGC		29.5	-16.5	-9.5	-17.0
X2	5'-GTG AXA TGC		42.5	-17.0	-8.0	-9.5
Y2 ^b	5'-GTG AYA TGC		42.5	-13.0	-5.0	-6.5
Z2 ^b	5'-GTG AZA TGC		49.5	-13.5	-6.0	-7.0

^aFor conditions of thermal denaturation experiments, see Table 1. T_m 's of fully matched duplexes are shown in bold. T_m = change in T_m relative to fully matched DNA duplex.^bData from reference³⁴ are included to facilitate comparison.

Table 3Discrimination of mismatched DNA targets by X6/Y6/Z6 and reference ONs.^a

ON	Sequence	<u>B</u> =	DNA: 5'-GTG ABA ACG			
			<u>T_m</u> [°C]	T	A	C G
D2	3'-CAC TAT ACG		29.5	-17.0	-15.5	-9.0
X6	3'-CAC <u>XAX</u> ACG		44.5	-22.0	-14.0	-12.0
Y6^b	3'-CAC <u>YAY</u> ACG		43.5	-24.0	-17.0	-14.0
Z6^b	3'-CAC <u>ZAZ</u> ACG		47.0	-19.5	-13.0	-11.0

^aFor conditions of thermal denaturation experiments, see Table 1. *T_m*'s of fully matched duplexes are shown in bold. *T_m* = change in *T_m* relative to fully matched DNA duplex.^bData from reference³⁴ are included to facilitate comparison.

Table 4

Properties of **X/Y/Z**-modified probe duplexes.^a

ON	ZP	Sequence	T_m (°C)	T_A (°C)	G^{293} [(kJ/mol)			ΔG_{TC}^{293} (kJ/mol)
					upper ON vs cDNA	lower ON vs cDNA	probe duplex	
X1	+4	5'-GXG ATA TGC	45.0	-0.5	-46±1 [-5]	-58±0 [-17]	-54±1 [-13]	-9
X5		3'-CAC TAX ACG						
X1	+2	5'-GXG ATA TGC	33.5	-2.0	-46±1 [-5]	-46±1 [-5]	-45±1 [-4]	-6
X4		3'-CAC XAT ACG						
X2	+1	5'-GTG AXA TGC	27.5	-28.0	-59±1 [-18]	-58±0 [-17]	-41±1 [±0]	-35
X5		3'-CAC TAX ACG						
X2	-1	5'-GTG AXA TGC	41.5	-4.0	-59±1 [-18]	-46±1 [-5]	-52±1 [-11]	-12
X4		3'-CAC XAT ACG						
X3	-1	5'-GTG ATA XGC	45.5	-5.0	-48±0 [-7]	-58±0 [-17]	-51±1 [-10]	-14
X5		3'-CAC TAX ACG						
X3	-3	5'-GTG ATA XGC	40.5	±0.0	-48±0 [-7]	-46±1 [-5]	-52±1 [-11]	-1
X4		3'-CAC XAT ACG						
^bY1	+4	5'-GYG ATA TGC	47.5	±0.0	-46±0 [-5]	-52±0 [-11]	-55±1 [-14]	-2
Y5		3'-CAC TAY ACG						
^bY1	+2	5'-GYG ATA TGC	31.5	-6.5	-46±0 [-5]	-46±0 [-5]	-45±1 [-4]	-6
Y4		3'-CAC YAT ACG						
^bY2	+1	5'-GTG AYA TGC	26.5	-29.0	-55±1 [-14]	-53±1 [-12]	-38±1 [+3]	-29
Y5		3'-CAC TAY ACG						
^bY2	-1	5'-GTG AYA TGC	39.5	-6.5	-54±0 [-13]	-46±0 [-5]	-51±1 [-10]	-8
Y4		3'-CAC YAT ACG						
^bY3	-1	5'-GTG ATA YGC	45.5	-5.0	-50±0 [-9]	-52±0 [-11]	-55±1 [-14]	-6
Y5		3'-CAC TAY ACG						
^bY3	-3	5'-GTG ATA YGC	40.5	-0.5	-50±0 [-9]	-46±0 [-5]	-53±1 [-12]	-2
Y4		3'-CAC YAT ACG						
Z1	+4	5'-GZG ATA TGC	55.0	5.0	-45±1 [-4]	-59±1 [-18]	-52±1 [-11]	-11

G^{293} [G^{293}] (kJ/mol)						
ON	ZP	Sequence	T_m (°C)	T_A (°C)	upper ON vs cDNA	lower ON vs cDNA
					probe duplex	ΔG_{rec}^{293} (kJ/mol)
Z5		3'-CAC TAZ ACG				
Z1	+2	5'-GZG ATA TGC	48.0	7.5	-45±1 [-4]	-51±1 [-10]
Z4		3'-CAC ZAT ACG				-4
Z2	+1	5'-GTG AZA TGC	42.5	-23.0	-61±0 [-20]	-59±1 [-18]
Z5		3'-CAC TAZ ACG				-28
Z2	-1	5'-GTG AZA TGC	55.5	-0.5	-61±0 [-20]	-51±1 [-10]
Z4		3'-CAC ZAT ACG				-14
Z3	-1	5'-GTG ATA ZGC	58.5	2.0	-53±0 [-12]	-59±1 [-18]
Z5		3'-CAC TAZ ACG				-21
Z3	-3	5'-GTG ATA ZGC	51.0	4.0	-53±0 [-12]	-51±1 [-10]
Z4		3'-CAC ZAT ACG				-9

^aZP = zipper. For conditions of thermal denaturation experiments, see Table 1. T_A (ONA:ONB) = T_m (ONA:ONB) + T_m (dsDNA) - T_m (ONA:cDNA) - T_m (cDNA:ONB). Probes with strongly negative T_A values are activated for recognition of isosequential dsDNA targets since the products of the recognition process (i.e., probe-target duplexes) display higher T_m 's than the reactants (i.e., probe duplexes and target duplexes). G^{293} is measured relative to G_{rec}^{293} for D1:D2 = -41 kJ/mol. ΔG_{rec}^{293} (ONA:ONB) = G^{293} (ONA:cDNA) + G^{293} (cDNA:ONB) - G^{293} (ONA:ONB) - G^{293} (dsDNA). „+„ denotes standard deviation. Data from reference²⁸ are included to facilitate comparison.