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Recognition of mixed-sequence DNA duplexes: Design guidelines for Invaders based on 2'-O-(pyren-1-yl)methyl-RNA monomers

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Abstract

Development of agents that recognize mixed-sequence double-stranded DNA (dsDNA) is desirable due to their potential as tools for detection, regulation and modification of genes. Despite progress with triplex-forming oligonucleotides, peptide nucleic acids, polyamides and other approaches, recognition of mixed-sequence dsDNA targets remains challenging. Our laboratory studies *Invaders* as an alternative approach toward this end. These double-stranded oligonucleotide probes are activated for recognition of mixed-sequence dsDNA through modification with +1 interstrand zippers of intercalator-functionalized nucleotides such as 2'-O-(pyren-1-yl)methyl-RNA monomers and have been recently shown to recognize linear dsDNA, DNA hairpins and chromosomal DNA. In the present work, we systematically study the role that the nucleobase moieties of the 2'-O-(pyren-1-yl)methyl-RNA monomers have on recognition efficiency of Invader duplexes. Results from thermal denaturation, binding energy, and recognition experiments using Invader duplexes with +1 interstrand zippers of the four canonical 2'-O-(pyren-1-yl)methyl-RNA **A/C/G/U** monomers, show that incorporation of these motifs is a general strategy for activation of probes for recognition of dsDNA. Probe duplexes with interstrand zippers comprised of **C** and/or **U** monomers result in the most efficient recognition of dsDNA. The insight gained from this study will drive the design of effective Invaders for applications in molecular biology, nucleic acid diagnostics and biotechnology.

Introduction

Development of compounds that recognize double-stranded DNA (dsDNA) in a sequence-specific manner is a research area of considerable interest, which is motivated by the prospect for molecular tools that can detect, regulate and modify genes.¹⁻³ Significant advances have been made with triplex-forming oligonucleotides,⁴⁻⁶ peptide nucleic acids (PNAs),^{7,8} polyamides,^{9,10} pseudo-complementary PNA,¹¹⁻¹³ γ -PNA,^{14,15} engineered proteins,^{16,17} and other approaches.¹⁸⁻²⁶ However, development of alternative strategies for specific mixed-sequence recognition of dsDNA at physiological conditions remains a very desirable goal due to the limitations of current probe technologies, which include target sequence restrictions, insufficient binding affinity, need for buffers with low salinity, poor cellular uptake or challenging synthesis.

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Supporting information: General experimental section; MS-data for modified ONs; representative thermal denaturation curves and absorption spectra; steady-state fluorescence emission spectra; absorption maxima for 14-mer probes and duplexes; NMR spectra for nucleosides 2-5. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

Our laboratory is exploring so-called *Invaders* as an alternative strategy for mixed-sequence recognition of dsDNA.²⁷⁻³¹ These double-stranded oligonucleotide probes are activated for dsDNA-recognition through modification with one or more +1 interstrand zipper arrangements of intercalator-modified nucleotide monomers such as 2'-*O*-(pyren-1-yl)methyl-RNA monomers (Fig. 1; for a formal definition of the zipper terminology, see Experimental section). Presumably, the intercalating pyrene moieties are forced into the same region of the duplex core, which causes a violation of the "nearest neighbor exclusion principle",³² resulting in concomitant localized unwinding and destabilization of the duplex (i.e., formation of an 'energetic hotspot', Fig. 1). The two strands that comprise an Invader duplex, on the other hand, display very strong affinity toward complementary single-stranded DNA (ssDNA) as duplex formation results in strongly stabilizing interactions between the pyrene and flanking nucleobases (Fig. 1).²⁷⁻³¹ We have recently demonstrated that the stability difference between Invader probes and probe-target complexes can be used to realize mixed-sequence recognition of: i) linear dsDNA,^{27,28,30} ii) DNA hairpins,^{29,31} and iii) chromosomal DNA.²⁹

In the present article, we systematically study the role that the nucleobase moieties of the 2'-*O*-(pyren-1-yl)methyl-RNA monomers, used to construct hotspots, have on the dsDNA-recognition efficiency of Invader duplexes. It is important to gain this insight as it will guide the future design of Invader duplexes for applications in molecular biology, nucleic acid diagnostics and biotechnology.

Results and Discussion

Synthesis of 2'-*O*-(pyren-1-yl)methyl-RNA phosphoramidites

The corresponding phosphoramidites of monomers **A**,³³ **C**³³ and **U**³⁴ were synthesized as previously described. The novel N2-isobutyryl-protected 2'-*O*-(pyren-1-yl)methyl-guanosine phosphoramidite **5** was obtained from guanosine **1** following the same general strategy that was used by others for the synthesis of the corresponding A^{Bz} and C^{Bz} phosphoramidites (Scheme 1).³³ Thus, unprotected guanosine was treated with 1-chloromethylpyrene in the presence of sodium hydride, which afforded O2'-alkylated nucleoside **2** in a low but consistent yield (24%). The low yield is due to concomitant formation of O3'/O5'/N3'-alkylated products (results not shown). The following N2-isobutyryl protection of the nucleobase to give **3** was accomplished in 74% yield using a transient protection protocol.³⁵ Subsequent O5'-dimethoxytritylation afforded nucleoside **4** in 88% yield, which upon treatment with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite and Hünig's base provided target phosphoramidite **5** in 78% yield. Disappearance of ¹H NMR signals from exchangeable protons upon D₂O addition, along with results from other one- and two-dimensional NMR experiments and high-resolution MALDI-MS, ascertained the proposed structures of nucleosides **2-5**. The modified phosphoramidites were incorporated into oligodeoxyribonucleotides (ONs) using machine-assisted solid-phase DNA synthesis (4,5-dicyanoimidazole as activator, 15 min) in ~98% stepwise coupling yield.

Design of study

In order to systematically study the role that hotspot composition has on the dsDNA-recognition efficiency of Invader duplexes, we designed ten model probes that only vary in the nature of the central hotspot (Table 1). The inherent symmetry of the energetic hotspots, along with the utilized sequence context - i.e., hotspots flanked by A:T pairs on either side - allowed us to study the sixteen possible hotspot compositions using ten probes.

DNA-hybridization characteristics of ONs modified with 2'-O-(pyren-1-yl)methyl-RNA monomers

Virtually all of the individual Invader strands display strongly increased affinity toward - and increased duplex stability with - complementary single-stranded DNA as compared to unmodified ONs (ΔT_m up to +13.0 °C, see first two ΔT_m columns, Table 1; $\Delta\Delta G^{293}$ values down to -15 kJ/mol, see first two ΔG^{293} two columns, Table 2). Interestingly, the **G** monomer has a considerably less stabilizing effect than the other 2'-O-(pyren-1-yl)methyl-RNA monomers and is even destabilizing in some cases ($\Delta T_m = 3-9$ °C and $\Delta\Delta G^{293} = 3-12$ kJ/mol less favorable than **A/C/U** monomers, Tables 1 and 2; only ONs with identical 3'-flanking nucleotides, such as **ON1**, **ON5**, **ON14**, and **ON13** should be compared (*vide infra*)). Absorbance spectra of single-stranded **ON1-ON20** and their duplexes with ssDNA were recorded to determine if the low stability of **G**-modified duplexes is linked to differences in pyrene binding modes (Fig. S2). Small hybridization-induced bathochromic shifts of pyrene absorption maxima - consistent with increased interactions between pyrene and nucleobases and, hence, pyrene intercalation³⁶ - were generally observed for all ONs ($\Delta\lambda = 0-4$ nm, Table 3). While **G**-modified ONs display slightly smaller bathochromic shifts, the results do not rule out pyrene intercalation as a likely binding mode. We speculate that the destabilizing effect of the **G** monomer, instead, is linked to a weakening of the **G:C** base pair following pyrene intercalation.³⁷

As has been previously observed for **U**-modified ONs,³⁸ the nucleotide flanking a 2'-O-(pyren-1-yl)methyl-RNA monomer on the 3'-side, has a major influence on duplex stability. Thus, the stabilizing effect of 2'-O-(pyren-1-yl)methyl-RNA monomers is greater when flanked by a 3'-purine ($\Delta T_m = 0-12$ °C and $\Delta\Delta G^{293} = 3-11$ kJ/mol more stabilizing than with 3'-flanking pyrimidine; e.g., compare ΔT_m and $\Delta\Delta G^{293}$ for **ON9**, **ON17**, **ON10** and **ON14**, Table 1 and 2). This finding is consistent with the 3'-directed intercalating binding mode that has been proposed for the pyrene moiety based on NMR structures of **U**-modified DNA duplexes,³⁹ as the larger aromatic surface area of a 3'-purine likely facilitates stronger stacking interactions with the pyrene moiety.

Thermostability and thermodynamics of Invader duplexes

The influence on duplex stability upon incorporation of a second monomer can be additive, more-than-additive, or less-than-additive relative to a corresponding singly modified duplex. This is readily quantified in terms of T_m 's by the term 'deviation from additivity' $DA_{\text{ONX:ONY}} \square \Delta T_m (\text{ONX:ONY}) - \Delta T_m (\text{ONX:ssDNA}) - \Delta T_m (\text{ssDNA:ONY})$, or in terms of ΔG^{293} by the term $\Delta G_{\text{rec}}^{293} (\text{ONX:ONY}) = \Delta G^{293} (\text{ONX:ssDNA}) + \Delta G^{293} (\text{ssDNA:ONY}) - \Delta G^{293} (\text{ONX:ONY}) - \Delta G^{293} (\text{dsDNA})$, where **ONX:ONY** is an Invader duplex with a +1 interstrand zipper arrangement of 2'-O-(pyren-1-yl)methyl-RNA monomers. $\Delta G_{\text{rec}}^{293}$ also serves as an estimate for the thermodynamic potential of Invader duplexes for recognition of iso-sequential dsDNA targets (*vide infra*).

In agreement with our preliminary results,²⁹⁻³¹ Invader duplexes are strongly destabilized relative to singly modified duplexes, irrespective of the monomers used to construct the energetic hotspot ($DA \ll 0$ °C, Table 1). In fact, all of the studied Invader duplexes display lower T_m 's than the corresponding unmodified dsDNA (ΔT_m between -17.5 °C and 0.0 °C, see "Invader duplex" column, Table 1). Evidently, the two 2'-O-(pyren-1-yl)methyl-RNA monomers influence each other and destabilize duplexes when placed in +1 interstrand zippers. This is corroborated by steady-state fluorescence emission spectra of Invader duplexes, which generally feature pyrene-pyrene excimer signals at $\lambda_{\text{em}} \sim 490$ nm (Fig. S7), implying a coplanar arrangement of the pyrene moieties with an interplanar separation of ~ 3.4 Å.^{20,27,28,31,40-45}

Similar conclusions are reached on the basis of the thermodynamic data: i) formation of Invader duplexes is 3-15 kJ/mol less favorable than formation of the corresponding reference DNA (see $\Delta\Delta G^{293}$ values, "Invader duplex" column, Table 2); and ii) $\Delta\Delta G^{293}$ values of Invader duplexes are 5-29 kJ/mol less favorable than would be expected for additive contributions from the two 2'-*O*-(pyren-1-yl)methyl-RNA monomers (see ΔG_{rec}^{293} values, Table 2). Invader duplex destabilization is dominated by unfavorable enthalpy ($\Delta\Delta H = 20$ -80 kJ/mol, results not shown), presumably as +1 interstrand monomer arrangements perturb stacking and/or nearby base pairing as a consequence of violating the 'nearest neighbor exclusion principle'.³² Arrangement of monomers in +1 interstrand zippers results in a localized region with one intercalator per base pair (Fig. 1). Local perturbation of the duplex is further corroborated by the broad melting profiles (Fig. S1) and the blue-shifted pyrene absorption maxima observed for Invader duplexes ($\Delta\lambda = 1$ -9 nm relative to probe-target duplexes, Table 3), which is indicative of weaker pyrenenucleobase interactions. Previous modeling and NMR studies of Invader duplexes based on the closely related 2'-*N*-(pyren-1-yl)methyl-2'-amino- α -L-LNA monomers, also strongly suggested localized duplex perturbation.³¹

The specific nature of the energetic hotspot influences the relative magnitude of Invader duplex destabilization. Thus, Invaders with hotspots comprised of **G** monomers are more destabilized relative to the corresponding unmodified dsDNA, than Invader duplexes comprised of other 2'-*O*-(pyren-1-yl)methyl-RNA monomers (see ΔT_m and $\Delta\Delta G^{293}$ of **ON1:ON20**, **ON5:ON16**, **ON7:ON14** and **ON8:ON13**, 'Invader duplex' column, Table 1 and 2).

Binding energy for Invader-mediated dsDNA-recognition

As mentioned above, the binding energy for recognition of iso-sequential dsDNA targets using Invader duplexes (i.e., the process depicted in Fig. 1) is described by ΔG_{rec}^{293} highly negative ΔG_{rec}^{293} values signify that the two resulting probe-target duplexes are much more stable than the Invader and target duplexes. Interestingly, all of the Invader duplexes studied herein display favorable binding energies for recognition of iso-sequential dsDNA targets (ΔG_{rec}^{293} between -29 and -5 kJ/mol, Table 2). Invader duplexes with energetic hotspots comprised exclusively of **C** and/or **U** monomers display the most favorable binding energies (ΔG_{rec}^{293} between -29 and -24 kJ/mol, Table 2), which largely reflects the greater stabilization of probe-target duplexes when 2'-*O*-(pyren-1-yl)methyl-RNA monomers are flanked by 3'-purines. Concomitantly, Invader duplexes with hotspots exclusively comprised of **A** and/or **G** monomers, display the least favorable binding energies (ΔG_{rec}^{293} between -14 and -5 kJ/mol, Table 2). The binding energies of Invaders with other hotspots fall between these two extremes (ΔG_{rec}^{293} between -23 and -14 kJ/mol, Table 2). Importantly, these results indicate that incorporation of +1 interstrand zippers of 2'-*O*-(pyren-1-yl)methyl-RNA monomers is a general strategy to activate probes for dsDNA-recognition.

Invader-mediated recognition of DNA hairpins

The dsDNA-recognition characteristics of four Invader duplexes - selected for their wide range of ΔG_{rec}^{293} and absolute T_m values - were evaluated using the electrophoretic mobility shift assay that we recently introduced and validated (Fig. 2a).^{29,31} Briefly described, digoxigenin (DIG) labeled DNA hairpins (DH) - comprised of 9-mer double-stranded stems, which are linked via T_{10} loops - serve as model dsDNA targets. The unimolecular nature of the hairpins confers extra stability to the stem region, which renders it as a more challenging target than iso-sequential dsDNA targets (compare T_m 's of hairpins (Fig. 2b) and iso-sequential dsDNA targets (Table 1)). Invader-mediated recognition of DNA hairpins is

expected to yield recognition complexes with lower electrophoretic mobilities on non-denaturing PAGE gels than DNA hairpins alone (Fig. 2a).

Indeed, room temperature incubation of Invader duplexes with their respective DNA hairpin targets results in dose-dependent formation of recognition complexes (Fig. 2c-2g). The results indicate that ΔG_{rec}^{293} -values and dsDNA-recognition efficiency are correlated. Thus, the Invader duplex with the greatest recognition potential (**ON9:ON12**,

$\Delta G_{rec}^{293} = -29$ kJ/mol, Table 2) results in ~80% recognition of **DH1** when used in 100-fold excess (Fig. 2g), while **ON8:ON13**, which exhibits the lowest recognition potential

($\Delta G_{rec}^{293} = -5$ kJ/mol, Table 2), hardly results in any recognition of **DH4** (Fig. 2g).

ON6:ON15 and **ON10:ON11**, which were predicted to have intermediate recognition

potential ($\Delta G_{rec}^{293} = -24$ kJ/mol and -19 kJ/mol, respectively, Table 2), display intermediate dsDNA recognition efficiency (Fig. 2g). It is interesting to note that dsDNA-recognition is slightly more efficient with the more strongly activated **ON6:ON15** as compared to **ON10:ON11**, in spite of the significantly greater Invader ($T_m = 41.5$ °C vs 26.5 °C, Table 1) and duplex target thermostability (69.5 °C vs 59.0 °C, Fig. 2b). This indicates that Invader-mediated dsDNA-recognition can occur at experimental temperatures that are considerably lower than the T_m 's of the Invader and/or target duplexes, which is likely to prove important for biological applications, where the experimental temperature is not easily adjustable.

Less than 40% recognition of **DH1** is observed when single-stranded **ON9** or **ON12** are used at 500-fold molar excess (Fig. S8), which demonstrates that both strands of Invader duplexes are needed to ensure efficient recognition of dsDNA. Moreover, incubation of Invader duplexes **ON9:ON12**, **ON6:ON15** or **ON10:ON11** with DNA hairpins that feature fully base-paired but non-isosequential stem regions (i.e. one or two base pair deviations relative to the Invader probes) fails to produce significant amounts of recognition complexes even when the Invaders are used at 500-fold excess, which demonstrates that the recognition process proceeds with excellent fidelity (Fig. 2h-j).

Next, we set out to examine if the design principles inferred from the 9-mer study can be applied to longer and more densely modified Invader duplexes. Toward this end, we designed four 14-mer Invaders, each modified with three energetic hotspots that are dispersed in a similar manner and selected to afford differentially activated probes (Table 4). For example, the three hotspots used in Invader duplex **ON21:ON22** are comprised of C and/or U monomers and expected to strongly promote dsDNA-recognition based on the observed ΔG_{rec}^{293} values in the 9-mer model study (Table 2). Conversely, the three hotspots used in Invader duplex **ON27:ON28** are comprised of A and/or G monomers and therefore expected to result in a less efficient probe. Invaders **ON23:ON24** and **ON25:ON26** should fall between these two extremes.

The thermal denaturation (Table 4) and thermodynamic data (Table 5) exhibit the expected trends with a couple of exceptions. Thus, **ON21** and **ON22** display the highest affinity toward ssDNA targets, while **ON27** and **ON28** display the lowest affinity toward ssDNA targets in this series (first two T_m columns, Table 4). The observed changes in Gibbs free energy upon duplex formation between individual probe strands and ssDNA, corroborate these findings (first two ΔG^{293} columns, Table 5), except that **ON21:ssDNA** is less stable than expected. The Invader duplexes are destabilized relative to reference duplexes; note, **ON21:ON22** is the most thermostable of the evaluated Invaders (see ΔT_m and $\Delta \Delta G^{293}$ values in the "Invader duplex" column, Tables 4 and 5, respectively). As a result, the dsDNA-recognition potential decreases in the order: **ON23:ON24** > **ON21:ON22** > **ON25:ON26** > **ON27:ON28** (see ΔG_{rec}^{293} values, Table 5).

Incubation of these Invader duplexes with their DNA hairpin target **DH5** results in dose-dependent recognition of the mixed-sequence dsDNA stem region (GC-content ~43%) in all four cases, with **ON21:ON22** as the most efficient probe (50% recognition at ~125-fold excess, Fig. 4). It is noteworthy that even Invader **ON27:ON28**, which features less desirable energetic hotspots, recognizes **DH5** albeit with the lowest efficiency in this probe series. Importantly, this suggests that the effect of suboptimal energetic hotspots can be compensated by beneficial probe architectures.

In summary, the design principles inferred from the 9-mer study can be confidently applied to longer and more densely modified Invader duplexes, but a systematic examination of the interplay between probe architecture (i.e., number and position of energetic hotspots) and dsDNA-recognition efficiency, is necessary to gain a more complete picture of Invader probe design. Studies along these lines are ongoing and will be presented in due course. Nonetheless, the results presented herein will already guide the design of efficient Invaders for recognition of mixed-sequence dsDNA.

Conclusion

The present study demonstrates that incorporation of +1 interstrand zippers of 2'-*O*-(pyren-1-yl)methyl-RNA monomers is a general approach toward activation of double-stranded oligodeoxyribonucleotide probes for recognition of mixed-sequence dsDNA targets. The recognition efficiency of the probes is, however, influenced by the composition of the energetic hotspots. Thus, Invader duplexes with hotspots comprised exclusively of **C** and/or **U** monomers display very favorable dsDNA-binding affinity since: i) the resulting probe-target duplexes are most strongly stabilized when 2'-*O*-(pyren-1-yl)methyl-RNA monomers are flanked by 3'-purines (Table 1 and 2), and ii) probe-target stabilization is more important than Invader duplex destabilization in driving the favorable thermodynamics of dsDNA-recognition (compare relative contributions of probe-target duplexes to ΔG_{rec}^{293} relative to Invader duplexes, Table 2). Invaders with hotspots constructed using one or two **G** monomers (or two **A** monomers) are the least activated constructs for dsDNA-recognition. However, a suboptimal choice of energetic hotspots for construction of Invader duplexes can be compensated by beneficial probe architectures (Figure 3). The insight gained from this study will guide the design of efficient dsDNA-targeting Invaders for enabling applications in molecular biology, nucleic acid diagnostics and biotechnology.

Experimental section

2'-*O*-(Pyren-1-yl)methyl-guanosine (2)

1-Chloromethylpyrene (1.86 g, 7.41 mmol) was added to a suspension of guanosine **1** (3.00 g, 10.6 mmol) and NaH (60% suspension in mineral oil, 0.64 g, 26.5 mmol) in anhydrous DMSO. The reaction mixture was stirred ~14h at room temperature at which point water (100 mL) was added. The resulting solids were filtered off, washed with water (3 × 20 mL), and dried. The filtrate was extracted with EtOAc (3 × 50 mL) and the organic layer was dried (Na₂SO₄). The aqueous layer was partially evaporated (~100 mL remaining) and left overnight at rt, which resulted in an additional crop of solids. The solids and organic layer were combined and purified by silica gel column chromatography (4-8% MeOH in CH₂Cl₂, v/v) to afford an off-white residue, which was precipitated from acetone to afford nucleoside **2** (0.90 g, 24%) as a white solid. *R*_f = 0.3 (10% MeOH in CH₂Cl₂, v/v); MALDIHRMS *m/z* 520.1598 ([M+Na]⁺, C₂₇H₂₃N₅O₅Na⁺, Calc. 520.1591); ¹H NMR (DMSO-*d*₆): δ 10.58 (s, 1H, ex, NH), 8.27-8.32 (m, 3H, Py), 8.01-8.21 (m, 6H, Py), 7.97 (s, 1H, H8), 6.38 (s, 2H, 2ex, NH₂), 5.97 (d, 1H, *J* = 6.7 Hz, H1'), 5.40-5.43 (m, 2H, 1 ex, 3'-OH, CH₂Py), 5.20-5.23 (d, 1H, *J* = 11.4 Hz, CH₂Py), 5.09 (t, 1H, ex, *J* = 5.2 Hz, 5'-OH), 4.61-4.64 (m, 1H, H2'),

4.43-4.47 (m, 1H, H3'), 4.00-4.03 (m, 1H, H4'), 3.63-3.68 (m, 1H, H5'), 3.57-3.61 (m, 1H, H5'); ^{13}C NMR (DMSO- d_6) δ 156.6, 153.6, 151.2, 135.3 (C8), 131.3, 130.65, 130.63, 130.2, 128.7, 127.30 (Py), 127.26 (Py), 127.1 (Py), 126.2 (Py), 125.23 (Py), 125.18 (Py), 124.4 (Py), 123.9, 123.7, 123.5 (Py), 116.7, 86.1 (C4'), 84.6 (C1'), 81.3 (C2'), 69.9 (CH₂Py), 69.0 (C3'), 61.3 (C5').

2-*N*-isobutyryl-2'-*O*-(pyren-1-yl)methyl-guanosine (3)

Nucleoside **2** (0.26 g, 0.52 mmol) was dried through repeated co-evaporation with anhydrous pyridine and re-dissolved in anhydrous pyridine (6 mL). Trimethylsilyl chloride (0.5 mL, 3.92 mmol) was added and the reaction mixture was stirred for 2h at rt. After cooling the mixture to 0 °C, isobutyryl chloride (0.15 mL, 1.57 mmol) was added over 10 min with continued cooling. This mixture was then stirred for 3h at rt and then cooled to 0 °C. Water (2 mL) was added and the mixture stirred at 0 °C for 10 min and then for another 5 min at rt, at which point aq. NH₃ (28%, 5 mL) was added. The resulting solution was stirred at rt for 15 min and evaporated to near dryness. The residual material was taken up in CH₂Cl₂ (100 mL) and washed with water (2 × 60 mL). The organic phase was dried (Na₂SO₄), evaporated to dryness, and the resulting crude purified by silica gel column chromatography (1-3% MeOH in CH₂Cl₂, v/v) to afford nucleoside **3** (220 mg, 74%) as a pale yellow solid. R_f = 0.6 (10% MeOH in CH₂Cl₂, v/v). MALDI-HRMS m/z 590.2008 ([M+Na]⁺, C₃₁H₂₉N₅O₆Na⁺, Calc. 590.2010); ^1H NMR (DMSO- d_6): δ 11.85 (s, 1H, ex, NH), 11.30 (s, 1H, ex, NH), 8.21-8.28 (m, 3H, Py), 8.19 (s, 1H, H8), 8.01-8.14 (m, 5H, Py), 7.94-7.97 (d, 1H, J = 7.8 Hz, Py), 5.94 (d, 1H, J = 6.7 Hz, H1'), 5.43-5.46 (m, 2H, 1 ex, 3'-OH, CH₂Py), 5.14-5.16 (d, 1H, J = 11.4 Hz, CH₂Py), 5.08 (t, 1H, ex, J = 5.2 Hz, 5'-OH), 4.65-4.68 (m, 1H, H2'), 4.46-4.48 (m, 1H, H3'), 4.01-4.03 (m, 1H, H4'), 3.57-3.67 (m, 2H, H5'), 2.56-2.61 (septet, 1H, J = 6.7 Hz, CHMe₂), 1.04 (d, 3H, J = 6.7 Hz, CH₃), 1.00 (d, 3H, J = 6.7 Hz, CH₃); ^{13}C NMR (DMSO- d_6): δ 179.8, 154.5, 148.5, 147.8, 137.2 (C8), 131.2, 130.62, 130.58, 130.1, 128.7, 127.3 (Py), 127.2 (Py), 127.07 (Py), 127.06 (Py), 126.1 (Py), 125.2 (Py), 125.1 (Py), 124.3 (Py), 123.8, 123.6, 123.5 (Py), 119.9, 86.5 (C4'), 84.7 (C1'), 81.6 (C2'), 70.2 (CH₂Py), 69.2 (C3'), 61.3 (C5'), 34.5 (CHMe₂), 18.7 (CH₃), 18.6 (CH₃). A trace impurity of CH₂Cl₂ was identified in the ^{13}C NMR spectrum of **3**.

5'-*O*-(4,4'-Dimethoxytrityl)-2-*N*-isobutyryl-2'-*O*-(pyren-1-yl)methyl-guanosine (4)

Nucleoside **3** (200 mg, 3.56 mmol) was co-evaporated with anhydrous pyridine (3 × 1.5 mL) and re-dissolved in anhydrous pyridine (3 mL). To this was added 4,4'-dimethoxytrityl chloride (DMTrCl, 300 mg, 0.53 mmol) and *N,N*-dimethyl-4-aminopyridine (DMAP, ~10 mg) and the reaction mixture was stirred at rt for ~14h. The reaction mixture was diluted with CH₂Cl₂ (20 mL) and the organic phase sequentially washed with water (2 × 10 mL) and sat. aq. NaHCO₃ (2 × 10 mL). The organic phase was evaporated to near dryness and the resulting crude co-evaporated with abs. EtOH and toluene (2:1, v/v, 3 × 2 mL) and purified by silica gel column chromatography (0-5%, MeOH in CH₂Cl₂, v/v) to afford nucleoside **4** (270 mg, 88%) as a pale yellow foam. R_f = 0.80 (7 % MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 892.3325 ([M+Na]⁺, C₅₂H₄₇N₅O₈Na⁺, Calc. 892.3317); ^1H NMR (DMSO- d_6): δ 11.90 (s, 1H, ex, NH), 11.34 (s, 1H, ex, NH), 8.25-8.32 (m, 3H, Py), 8.04-8.15 (m, 5H, Py), 8.03 (s, 1H, H8), 7.99-8.01 (d, 1H, J = 7.8 Hz, Py), 7.32-7.34 (m, 2H, DMTr), 7.19-7.25 (m, 7H, DMTr), 6.79-6.82 (m, 4H, DMTr), 5.97 (d, 1H, J = 5.5 Hz, H1'), 5.49-5.51 (d, 1H, J = 11.7 Hz, CH₂Py), 5.47 (d, 1H, ex, J = 5.4 Hz, 3'-OH), 5.22-5.25 (d, 1H, J = 11.7 Hz, CH₂Py), 4.72 (ap t, 1H, J = 5.5 Hz, H2'), 4.45-4.48 (m, 1H, H3'), 4.11-4.14 (m, 1H, H4'), 3.710 (s, 3H, CH₃O), 3.706 (s, 3H, CH₃O), 3.28-3.31 (m, 1H, H5' - overlap with H₂O signal), 3.18-3.22 (m, 1H, H5'), 2.63 (septet, 1H, J = 6.7 Hz, CHMe₂), 1.04-1.07 (2d, 6H, both J = 6.7 Hz, CH₃); ^{13}C NMR (DMSO- d_6): δ 179.8, 158.0, 154.5, 148.5, 147.9, 144.7, 137.1 (C8), 135.4, 135.3, 131.1, 130.7, 130.6, 130.1, 129.6 (DMTr), 128.7, 127.7 (DMTr), 127.6 (DMTr), 127.31 (Py), 127.29 (Py), 127.2 (Py), 127.0 (Py), 126.6 (DMTr),

126.2 (Py), 125.3 (Py), 125.2 (Py), 124.4 (Py), 123.9, 123.7, 123.5 (Py), 120.1, 113.1 (DMTr), 85.6, 85.1 (C1'), 84.2 (C4'), 80.8 (C2'), 70.3 (CH₂Py), 69.4 (C3'), 63.9 (C5'), 54.9 (CH₃O), 34.6 (CHMe₂), 18.8 (CH₃), 18.7 (CH₃).

3'-O-(2-Cyanoethoxy(diisopropylamino)phosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyryl-2'-O-(pyren-1-yl)methyl-guanosine (**5**)

Nucleoside **4** (260 mg, 0.29 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2 × 2 mL) and re-dissolved in anhydrous CH₂Cl₂ (7 mL). To this was added anhydrous *N,N*-diisopropylethylamine (DIPEA, 213 μL, 1.19 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI-reagent, 133 μL, 0.59 mmol) and the reaction mixture was stirred at rt for ~3h, whereupon abs. EtOH (1 mL) and CH₂Cl₂ (20 mL) were sequentially added. The organic phase was washed with sat. aq. NaHCO₃ (10 mL), evaporated to near dryness, and the resulting residue purified by silica gel column chromatography (40-70% EtOAc in petroleum ether, v/v) to afford the desired phosphoramidite **5** (250 mg, 78%) as a white foam. *R*_f = 0.8 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1092.4389 ([M+Na]⁺, C₆₁H₆₄N₇O₉P·Na⁺, Calc. 1092.4395); ³¹P NMR (CDCl₃): δ 150.39, 150.35.

Synthesis and purification of modified ONs

The corresponding phosphoramidites of monomers **A**, **C** and **U** (A^{Bz}, C^{Bz}, U) were obtained as previously described.^{33,34} The phosphoramidite of monomer **G** was obtained as described above. Modified ONs were synthesized on an automated DNA synthesizer (0.2 μmol scale; succinyl linked LCAA-CPG support) using a ~50-fold molar excess of 2'-O-(pyren-1-yl)methyl-RNA phosphoramidites in anhydrous acetonitrile (at 0.02 M) and extended coupling (4,5-dicyanoimidazole as activator, 15 min, ~98% coupling yield) and oxidation (45s). Standard protocols for incorporation of DNA phosphoramidites were used. Cleavage from solid support and removal of protecting groups was accomplished using 32% aq. ammonia (55 °C, 12 h). ONs were purified via ion-pair reverse phase HPLC using a triethylammonium acetate buffer - water/acetonitrile (v/v) gradient, detritylated (80% aq. AcOH), and precipitated from acetone (-18 °C for 12-16h). The identity of all modified ONs was verified through MALDI-MS/MS analysis recorded in positive ion mode on a quadrupole time-of-flight tandem mass spectrometer equipped with a MALDI source using anthranilic acid as a matrix (Table S1). Purity (>90%) was verified by ion-pair reverse phase HPLC running in analytical mode.

Thermal denaturation experiments

ON concentrations were estimated using the following extinction coefficients (OD/μmol) at 260 nm: dA (15.2), dC (7.05), dG (12.0), T (8.4), and pyrene (22.4). Quartz optical cells with a path length of 1.0 cm were used. Strands were mixed and denatured through heating to ~70 °C, followed by cooling to the starting temperature of the experiment. Thermal denaturation curves (1.0 μM final concentration of each strand) were recorded on a Peltier-controlled UV/VIS spectrophotometer using a medium salt buffer (100 mM NaCl, 0.1 mM EDTA, and pH 7.0 adjusted with 10 mM Na₂HPO₄ and 5 mM Na₂HPO₄). A temperature ramp of 0.5 °C/min was used in all experiments. Thermal denaturation temperatures (*T*_m's) were determined as the maximum of the first derivative of denaturation curves. The experimental temperatures ranged from at least 15 °C below *T*_m (although not below 3 °C) to at least 20 °C above *T*_m. Reported *T*_m-values are averages of at least two experiments within ± 1.0 °C unless otherwise mentioned.

Determination of thermodynamic parameters

Thermodynamic parameters for duplex formation were determined through baseline fitting of denaturation curves using the software provided with the UV/VIS spectrometer. Bimolecular reactions, two-state melting behavior, and a heat capacity change of $\Delta C_p = 0$ upon hybridization were assumed.⁴⁶ 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 listed.

Absorption spectra

UV-vis absorption spectra (range: 300-400 nm) were recorded at 5 °C using the same samples and instrumentation as in the thermal denaturation experiments.

Steady-state fluorescence emission spectra

Steady-state fluorescence emission spectra were recorded on a Peltier-controlled fluorimeter using the same samples as in the thermal denaturation experiments (i.e., in non-deoxygenated T_m buffer). Spectra were obtained as an average of five scans using an excitation wavelength of $\lambda_{ex} = 350$ nm, excitation slit = 5.0 nm, emission slit = 2.5 nm, and a scan speed of 600 nm/min. Spectra were recorded at 5 °C to ascertain maximal duplex formation.

Recognition of DNA hairpins in cell-free assay

This assay, which was chosen *in lieu* of footprinting experiments to avoid the use of ^{32}P -labeled targets, was performed in a similar manner as previously described.^{29,31} Thus, unmodified **DH1-DH5** were obtained from commercial sources and used without further purification. **DH1-DH5** were 3'-DIG-labeled using the 2nd generation DIG Gel Shift Kit (Roche Applied Bioscience) as recommended. The DIG-labeled ONs were diluted and used in the recognition experiments without further purification. Pre-annealed Invader duplexes (85 °C for 2 min, cooled to room temperature over 15 min) and DIG-labeled dsDNA targets (34.4 nM) were mixed and incubated in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl_2 , 10% sucrose, 1.44 mM spermine tetrahydrochloride, pH 7.2) for 15h at room temperature. The reaction mixtures were diluted with 6x DNA loading dye (Fermentas) and loaded onto a 12 or 16% non-denaturing polyacrylamide gel. Electrophoresis was performed using constant voltage (70 V) for 2h at ~4 °C using 0.5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) as a running buffer. Gels were blotted onto a positively charged nylon membrane (Roche Applied Bioscience) using constant voltage (100V, ~4 °C). The membranes were exposed to anti-digoxigenin-AP F_{ab} fragments as recommended 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-ray film developer. The resulting bands were quantified using a multiimager equipped with appropriate software. Recognition efficiency was determined as the intensity ratio between the recognition complex band and the total lane. An average of three independent experiments is reported along with standard deviations. Non-linear regression was used to fit data points from dose-response experiments.

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 own 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 own strand relative to a second reference monomer on the other strand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Monomer **A** B = adenin-9-yl
Monomer **C** B = cytosin-1-yl
Monomer **G** B = guanin-9-yl
Monomer **U** B = uracil-1-yl

Figure 1. Illustration of the Invader concept for recognition of mixed-sequence dsDNA (droplets denote intercalating pyrene moieties) and structures of monomers used in this study.

Scheme 1.

Synthesis of 2'-O-(pyren-1-yl)methyl-RNA-GⁱBu phosphoramidite **5**. Py = pyren-1-yl; TMSCl = trimethylsilyl chloride; DMTrCl = 4,4'-dimethoxytrityl chloride; PCl = 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite.

Figure 3.

Invader-mediated recognition of DNA hairpins with 14-mer iso-sequential stem regions. Gel electropherograms illustrating: dose-response experiments between (c) **DH1** and **ON9:ON12**, (d) **DH2** and **ON6:ON15**, (e) **DH3** and **ON10:ON11**, and (f) **DH4** and **ON8:ON13**; (h-j) gel electropherograms illustrating incubation of **DH1-DH3** with 500-fold excess of **ON9:ON12**, **ON6:ON15** or **ON10:ON11**, respectively; (e) sequence and T_m 's of DNA hairpins (conditions of thermal denaturation experiments, see Table 1); (f) dose-response curves. See Figure 2 for experimental conditions.

Table 1

Thermal denaturation temperatures (T_m) and deviation of additivity (DA) values of 9-mer DNA duplexes modified with 2'-*O*-(pyren-1-yl)methyl-RNA monomers.^a

ON	Sequence	$T_m [\Delta T_m] (^{\circ}\text{C})$			dsDNA	DA ($^{\circ}\text{C}$)
		upper strand vs ssDNA	lower strand vs ssDNA	Invader duplex		
ON1 ON20	5'-GTGA <u>AC</u> TGC 3'-CACT <u>TG</u> ACG	42.0 [+6.5]	34.5 [-1.0]	24.0 [-11.5]	35.5	-17.0
ON2 ON19	5'-GTGA <u>AG</u> TGC 3'-CACT <u>TC</u> ACG	46.0 [+8.5]	42.5 [+5.0]	32.5 [-5.0]	37.5	-18.5
ON3 ON18	5'-GTGA <u>AT</u> TGC 3'-CACT <u>TA</u> ACG	40.0 [+8.5]	38.5 [+7.0]	25.0 [-6.5]	31.5	-22.0
ON4 ON17	5'-GTGA <u>CA</u> TGC 3'-CACT <u>GU</u> ACG	48.5 [+13.0]	45.5 [+10.0]	35.5 [$\pm$ 0.0]	35.5	-23.0
ON5 ON16	5'-GTGA <u>CC</u> TGC 3'-CACT <u>GG</u> ACG	46.0 [+6.5]	45.0 [+5.5]	33.0 [-6.5]	39.5	-18.5
ON6 ON15	5'-GTGA <u>CG</u> TGC 3'-CACT <u>GC</u> ACG	54.5 [+11.0]	53.5 [+10.0]	41.5 [-2.0]	43.5	-23.0
ON7 ON14	5'-GTGA <u>GA</u> TGC 3'-CACT <u>CU</u> ACG	42.0 [+6.5]	39.0 [+3.5]	26.0 [-9.5]	35.5	-20.0
ON8 ON13	5'-GTGA <u>GC</u> TGC 3'-CACT <u>CG</u> ACG	40.0 [-3.5]	38.0 [-5.5]	26.0 [-17.5]	43.5	-8.5
ON9 ON12	5'-GTGA <u>UA</u> TGC 3'-CACT <u>AU</u> ACG	42.5 [+13.0]	42.5 [+13.0]	26.5 [-3.0]	29.5	-29.0
ON10 ON11	5'-GTGA <u>UT</u> TGC 3'-CACT <u>AA</u> ACG	37.5 [+6.0]	42.5 [+11.0]	26.5 [-5.0]	31.5	-22.0

^a ΔT_m = change in T_m values relative to corresponding unmodified reference duplex. T_m values were determined as the first derivative maximum of thermal denaturation curves (A_{260} vs T) recorded in medium salt buffer ($[\text{Na}^+] = 110 \text{ mM}$, $[\text{Cl}^-] = 100 \text{ mM}$, pH 7.0 ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$)), using 1.0 μM of each strand. $DA = \Delta T_m$ (Invader duplex) - ΔT_m (upper strand vs ssDNA) - ΔT_m (lower strand vs ssDNA). Example of DA calculation: DA (ON10:ON11) = ΔT_m (ON10:ON11) - ΔT_m (ON10:ssDNA) - ΔT_m (ssDNA:ON11) = $-5.0^{\circ}\text{C} - 6.0^{\circ}\text{C} - 11.0^{\circ}\text{C} = -22.0^{\circ}\text{C}$; A/C/G/T = adenine-9-yl/cytosine-1-yl/guanine-9-yl/thymine-1-yl DNA monomers. For structures of A/C/G/U, see Fig. 1.

Table 2

Change in Gibbs free energy upon duplex formation (ΔG^{293}) and binding energy (ΔG_{rec}^{293}) of 9-mer Invaders at 293K.^a

ON	Sequence	$\Delta G^{293}[\Delta \Delta G^{293}]$ (kJ/mol)				ΔG_{rec}^{293} (kJ/mol)
		upper strand vs ssDNA	lower strand vs ssDNA	Invader duplex	dsDNA	
ON1 ON20	5'-GTGA <u>AC</u> TGC 3'-CACT <u>TG</u> ACG	-51±1 [-5]	-46±0 [±0]	-37±0 [+9]	-46±0	-14
ON2 ON19	5'-GTGA <u>AG</u> TGC 3'-CACT <u>TC</u> ACG	-62±1 [-15]	-50±1 [-3]	-42±0 [+5]	-47±0	-23
ON3 ON18	5'-GTGA <u>AT</u> TGC 3'-CACT <u>TA</u> ACG	-48±0 [-4]	-48±0 [-4]	-39±0 [+5]	-44±0	-13
ON4 ON17	5'-GTGA <u>CA</u> TGC 3'-CACT <u>GU</u> ACG	-58±0 [-12]	-56±0 [-10]	-42±0 [+4]	-46±0	-26
ON5 ON16	5'-GTGA <u>CC</u> TGC 3'-CACT <u>GG</u> ACG	-56±1 [-5]	-54±0 [-3]	-41±0 [+10]	-51±1	-18
ON6 ON15	5'-GTGA <u>CG</u> TGC 3'-CACT <u>GC</u> ACG	-63±1 [-8]	-64±1 [-9]	-48±1 [+7]	-55±0	-24
ON7 ON14	5'-GTGA <u>GA</u> TGC 3'-CACT <u>CU</u> ACG	-50±0 [-4]	-49±1 [-3]	-39±0 [+7]	-46±0	-14
ON8 ON13	5'-GTGA <u>GC</u> TGC 3'-CACT <u>CG</u> ACG	-50±1 [+4]	-48±0 [+6]	-39±1 [+15]	-54±0	-5
ON9 ON12	5'-GTGA <u>UA</u> TGC 3'-CACT <u>AU</u> ACG	-55±1 [-14]	-53±1 [-12]	-38±1 [+3]	-41±1	-29
ON10 ON11	5'-GTGA <u>UT</u> TGC 3'-CACT <u>AA</u> ACG	-46±1 [-3]	-54±0 [-11]	-38±1 [+5]	-43±1	-19

^a $\Delta \Delta G^{293}$ is measured relative to ΔG^{293} for unmodified DNA duplexes (dsDNA). ΔG^{293} values were determined via baseline fitting of thermal denaturation curves. ΔG^{293} (upper strand vs ssDNA) + ΔG^{293} (lower strand vs ssDNA) - ΔG^{293} (Invader duplex) - ΔG^{293} (dsDNA). "±" denotes standard deviation.

Table 3

Absorption maxima in the 300-400 nm region for single stranded **ON1-ON20**, the corresponding duplexes with ssDNA, and Invaders comprised of these ONs.^a

ON	Sequence	$\lambda_{\max}$ [$\Delta\lambda$] (nm)				Invader duplex
		upper strand	lower strand	upper strand vs ssDNA	lower strand vs ssDNA	
ON1	5'-GTGA <u>AC</u> TGC	349	351	352 [+3]	352 [+1]	349
ON20	3'-CACT <u>TG</u> ACG					
ON2	5'-GTGA <u>AG</u> TGC	349	349	352 [+3]	352 [+3]	350
ON19	3'-CACT <u>TC</u> ACG					
ON3	5'-GTGA <u>AT</u> TGC	349	351	352 [+3]	352 [+1]	349
ON18	3'-CACT <u>TA</u> ACG					
ON4	5'-GTGA <u>CA</u> TGC	352	352	352 [$\pm$ 0]	353 [+1]	344
ON17	3'-CACT <u>GU</u> ACG					
ON5	5'-GTGA <u>CC</u> TGC	349	350	352 [+3]	352 [+2]	344
ON16	3'-CACT <u>GG</u> ACG					
ON6	5'-GTGA <u>CG</u> TGC	349	348	352 [+3]	352 [+4]	345
ON15	3'-CACT <u>GC</u> ACG					
ON7	5'-GTGA <u>GA</u> TGC	349	349	351 [+2]	352 [+3]	344
ON14	3'-CACT <u>CU</u> ACG					
ON8	5'-GTGA <u>GC</u> TGC	349	351	351 [+2]	352 [+1]	343
ON13	3'-CACT <u>CG</u> ACG					
ON9	5'-GTGA <u>UA</u> TGC	348	349	352 [+4]	352 [+3]	345
ON12	3'-CACT <u>AU</u> ACG					
ON10	5'-GTGA <u>UT</u> TGC	348	349	351 [+3]	352 [+2]	345
ON11	3'-CACT <u>AA</u> ACG					

^a conditions as described in footnote of Table 1. $T = 5\text{ }^{\circ}\text{C}$, $1.0\text{ }\mu\text{M}$ concentration of each strand.

Table 4

Thermal denaturation temperatures (T_m) and deviation of additivity (DA) values of 14-mer DNA duplexes modified with 2'-*O*-(pyren-1-yl)methyl-RNA monomers.^a

ON	Invader	T_m [ΔT_m] (°C)			dsDNA target	DA (°C)
		upper strand vs ssDNA	lower strand vs ssDNA	Invader duplex		
ON21	5'-TUA CTC ACG AUG CT	71.0	68.0	54.0	51.0	-34.0
ON22	3'-AAU GAG TGC TAC GA	[+20.0]	[+17.0]	[+3.0]		
ON23	5'-TUA CTC ACG ATG CT	67.5	63.5	40.5	51.0	-39.5
ON24	3'-AAU GAG UGC TAC GA	[+16.5]	[+12.5]	[-10.5]		
ON25	5'-TTA CTC ACG ATG CT	63.5	61.0	47.5	51.0	-26.0
ON26	3'-AAT GAG TGC TAC GA	[+12.5]	[+10.0]	[-3.5]		
ON27	5'-TTA CTC ACG ATG CT	56.0	54.0	38.0	51.0	-21.0
ON28	3'-AAT GAG TGC TAC GA	[+5.0]	[+3.0]	[-13.0]		

^aSee Table 1 for experimental conditions and definitions.

Table 5

Change in Gibbs free energy upon duplex formation (ΔG^{293}) and thermodynamic dsDNA-targeting potential (ΔG_{rec}^{293}) of 14-mer Invaders at 293K.^a

ON	Invader	ΔG^{293} [$\Delta \Delta G^{293}$] kJ/mol				ΔG_{rec}^{293} kJ/mol
		upper strand vs ssDNA	lower strand vs ssDNA	Invader duplex	dsDNA target	
ON21	5'-TUA CTC ACG AUG CT					
ON22	3'-AAU GAG TGC TAC GA	-74±1 [-8]	-91±0 [-25]	-58±1 [+8]	-66±1	-41
ON23	5'-TUA CTC ACG ATG CT					
ON24	3'-AAU GAG UGC TAC GA	-83±1 [-17]	-73±1 [-7]	-45±1 [+21]	-66±1	-45
ON25	5'-TTA CTC ACG ATG CT					
ON26	3'-AAT GAG TGC TAC GA	-78±1 [-12]	-74±1 [-8]	-48±1 [+18]	-66±1	-38
ON27	5'-TTA CTC ACG ATG CT					
ON28	3'-AAT GAG TGC TAC GA	-68±1 [-2]	-75±1 [-9]	-47±0 [+19]	-66±1	-30

^aSee Table 1 for experimental conditions and definitions.