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- (71) **Applicant** (for all designated States except US): **UNIVERSITY OF IDAHO** [US/US]; Office of Technology Transfer, P.O. Box 443003, Morrill Hall 414, Moscow, ID 83844-3003 (US).
- (72) **Inventor; and**
- (75) **Inventor/Applicant** (for US only): **HRDLICKA, Patrick, J.** [US/US]; 559 E. Deer Meadow, Moscow, ID 83843 (US).
- (74) **Agent:** **SLATER, Stacey, C.**; Klarquist Sparkman, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).
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(54) **Title:** EMBODIMENTS OF A PROBE AND METHOD FOR TARGETING NUCLEIC ACIDS

(57) **Abstract:** Disclosed embodiments concern a probe comprising one or more pairs of monomers capable of targeting a nucleic acid target. The pair of monomers may be arranged in a manner that promotes thermostability of the probe complex, thus producing a probe capable of locating and/or detecting a target. The probe also may comprise one or more natural or non-natural nucleotides capable of Watson-Crick base pairing with an isosequential nucleic acid target. Particular disclosed embodiments concern a method of using the disclosed probe to target nucleic acids. In particular disclosed embodiments, the probe may be incubated with a target nucleic acid and then be detected.



CROSS REFERENCE TO RELATED APPLICATIONS

10 FIELD

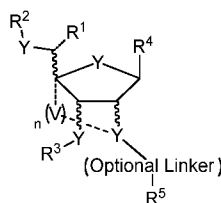
15 **ACKNOWLEDGMENT OF GOVERNMENT SUPPORT**20 **BACKGROUND**

Progress in sequence-selective targeting of double stranded DNA (dsDNA) has been accomplished with minor groove binding polyamides or by DNA triple-helix-based approaches using modified oligonucleotides or helix-invading peptide nucleic acids (PNAs). However, the utility of these methods is limited by the sequence-dependent microstructure of the minor groove of DNA duplexes (polyamides), by target sequence restrictions (triplex-based approaches), or by the necessity of non-physiological salt concentrations (PNAs). An attractive alternative approach was introduced with pseudo-complementary DNA (pcDNA), *i.e.*, DNA duplexes containing modified purine and pyrimidines that do not form stable base pairs with each other, while allowing hybridization to natural complementary DNA. pcDNA is able to strand invade blunt ended duplexes containing terminal mixed sequence target regions and this strategy has been extended into pseudo-complementary PNA (pcPNA), which has been used to target mixed sequence internal target regions of double stranded DNA. Unfortunately, the positively charged lysine residues commonly used to

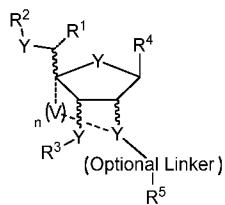
increase pcPNA solubility and binding affinity may lead to self-inhibitory effects of strand invasion at high probe concentrations. The requirement for low salt concentrations during pcPNA-mediated strand invasion of mixed sequence dsDNA is a limitation for all experiments in biological media and for numerous biotechnological applications. Development of alternative strategies for sequence selective recognition of dsDNA at physiologically relevant salt concentrations is therefore highly desirable.

SUMMARY

Particular disclosed embodiments concern a probe, comprising: a pair of monomers comprising a first monomer having a formula



where each Y independently is selected from carbon, oxygen, sulfur, a triazole, and NR^b , wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl; V is selected from carbon, oxygen, sulfur, and NR^b ; n ranges from 0 to 4; R^1 and R^2 are selected from hydrogen, aliphatic, aryl, and a heteroatom-containing moiety; R^3 is a heteroatom-containing functional group; R^4 is selected from any natural or non-natural nucleobase; R^5 is selected from any aromatic moiety suitable for intercalating within a nucleic acid; “optional linker” is selected from alkyl, amide, carbamate, carbonate, urea, and combinations thereof; a second monomer having a formula

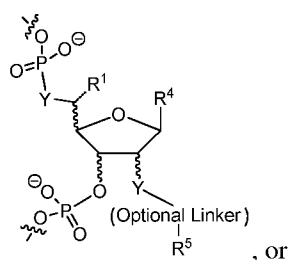
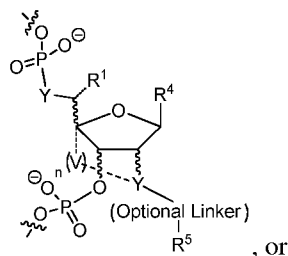


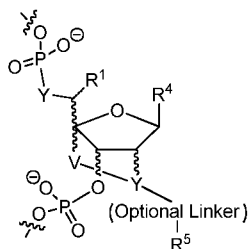
and at least one nucleotide selected from a natural nucleotide, a non-natural nucleotide, and combinations thereof, wherein the at least one nucleotide typically is coupled to the first and/or second monomer at R^2 and/or R^3 by a phosphate group.

In particular disclosed embodiments, the heteroatom-containing functional group is selected from ether (R^aOR^b), hydroxyl (R^aOH), silyl ether ($\text{R}^a\text{R}^b\text{R}^c\text{SiOR}^d$), phosphine ($\text{PR}^a\text{R}^b\text{R}^c$), thiol (R^aSH), thioether/sulfide (R^aSR^b), disulfide (R^aSSR^b), isothiocyanate (R^aNCS), isocyanate (R^aNCO), amine (NH_2 , NHR^a , NR^aR^b), amide ($\text{R}^a\text{NR}^b\text{C(O)R}^c$), ester ($\text{R}^a\text{OC(O)R}^b$), halogen (I, Br, Cl, F), carbonate ($\text{R}^a\text{OC(O)OR}^b$), carboxyl ($\text{R}^a\text{C(O)OH}$), carboxylate (R^aCOO^-), ester ($\text{R}^a\text{C(O)OR}^b$), ketone ($\text{R}^a\text{C(O)R}^b$), phosphate ($\text{R}^a\text{OP(O)OH}_2$), phosphoryl ($\text{R}^a\text{P(O)(OH)}_2$), sulfinyl ($\text{R}^a\text{S(O)R}^b$), sulfonyl ($\text{R}^a\text{SO}_2\text{R}^b$), carbonothioyl ($\text{R}^a\text{C(S)R}^b$ or $\text{R}^a\text{C(S)H}$), sulfinio ($\text{R}^a\text{S(O)OH}$), sulfo ($\text{R}^a\text{SO}_3\text{H}$), amide

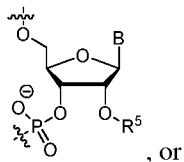
($R^aC(O)NR^bR^c$), azide (N_3), nitrile (R^aCN), isonitrile ($R^aN^+C^-$), and nitro (R^aNO_2); R^a represents the remaining monomer structure, which is attached to the abovementioned functional groups at the position indicated; and R^b , R^c , and R^d independently are hydrogen, aliphatic, aryl, heteroaliphatic, heteroaryl, and any combination thereof. In certain embodiments, R^2 and R^3 independently are selected from a heteroatom functional group comprising phosphorous, sulfur, nitrogen, oxygen, selenium, and/or a metal, more typically R^2 and R^3 independently are selected from a phosphate group of the natural nucleotide, non-natural nucleotide, synthetic nucleotides, or combinations thereof. In particular disclosed embodiments, R^4 is selected from adenine, guanine, cytosine, uracil, thymine, or any derivative thereof and the intercalator is an aromatic hydrocarbon or an aromatic heterocycle. In particular disclosed embodiments, the intercalator is a hydrocarbon selected from pyrene, coronene, perylene, anthracene, naphthalene and functionalized derivatives thereof; or may be an aromatic heterocycle selected from a porphyrin, nucleobase, metal chelator, azapyrene, thiazole orange, indole, pyrrole, and derivatives thereof.

Certain disclosed embodiments concern a probe having at least one monomer, and typically two or more monomers. The monomers are generally incorporated within oligonucleotide strands, and can be located anywhere in the oligonucleotide strand, including the first position, the last position, and anywhere in between, and preferably are arranged in a final structure in an interstrand zipper arrangement, such as a +1 zipper arrangement, as discussed in more detail below. Certain disclosed embodiments concern probes wherein either the first monomer or the second monomer has a formula

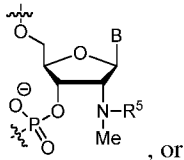




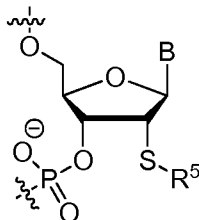
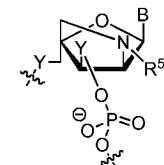
In certain disclosed embodiments, either the first monomer or the second monomer has a formula



, or

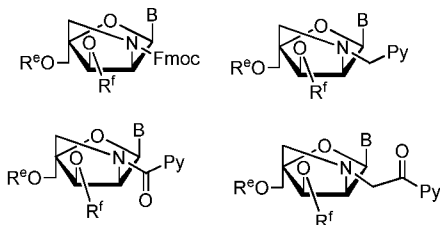


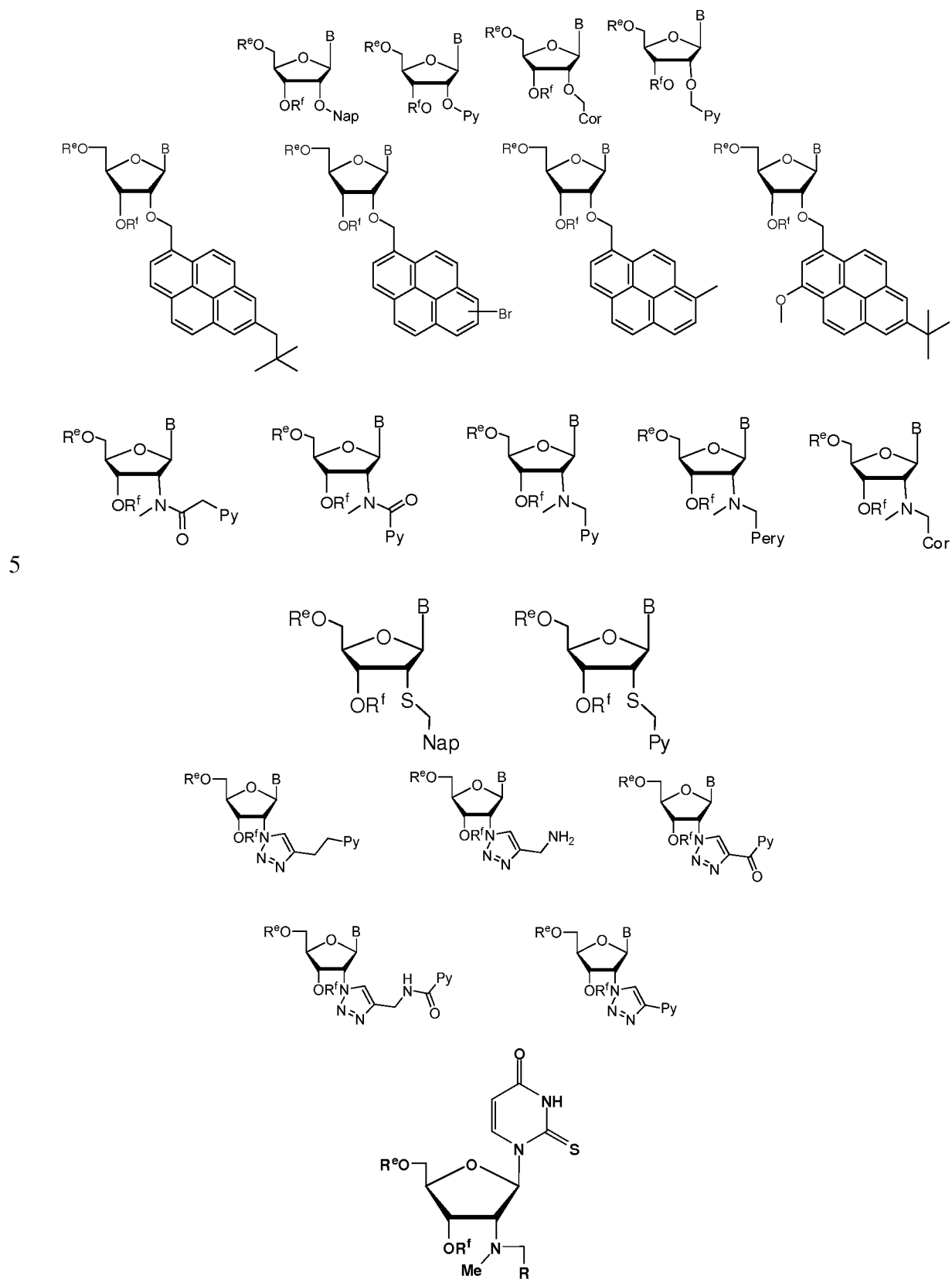
, or



wherein B is selected from uracil, guanine, cytosine, adenine, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one or derivatives thereof.

Particular disclosed embodiments of the probe comprise monomers having any one of the following structures:

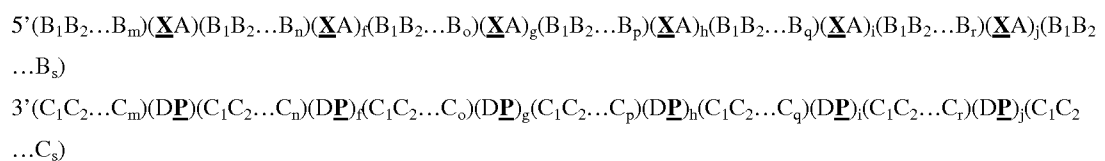




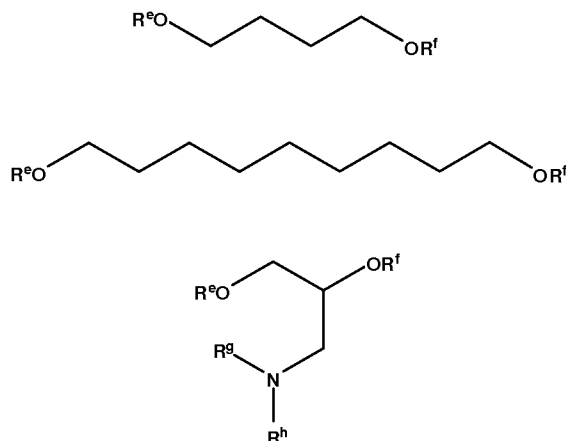
wherein B is selected from uracil, guanine, cytosine, adenine, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one or derivatives thereof, R^e is H, DMTr, or phosphate, such as provided by a phosphodiester bond in an oligonucleotide and R^f is H, (N(i-Pr)₂)P(OCH₂CH₂CN), or phosphate, such as provided by a phosphodiester bond in an oligonucleotide; Nap refers to naphthyl, such as 2-naphthyl, Cor to coronenyl, such as coronen-1-yl, Py to pyrenyl, such as pyren-1-yl, pyren-2-yl and pyren-4-yl and Pery to perylenyl, such as to perylene-3-yl.

In particular disclosed embodiments, the at least one natural nucleotide may be selected from adenine, guanine, cytosine, uracil, thymine and derivatives thereof, and the at least one non-natural nucleobase may be selected from C5-functionalized pyrimidines, C6-functionalized pyrimidines, C7-functionalized 7-deazapurines, C8-functionalized purines, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one or derivatives thereof. In certain disclosed embodiments, the probe comprises at least one natural nucleotide, unnatural nucleotide, and combinations thereof, which are selected to substantially match at least one nucleotide of a corresponding nucleic acid sequence.

Particular disclosed embodiments concern a probe having the following formula:

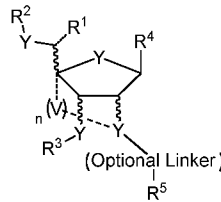


wherein B₁, B₂ and B_{m-s} may be any natural or non-natural nucleotide, wherein m-s ranges from zero to about 28; f, g, h, i and j range from 0 to 10, more likely 0 to 5, and typically 0 to 3; X is the first monomer; A is the complement Watson-Crick base pairing nucleotide of P; C is any natural or non-natural nucleotide capable of Watson-Crick base pairing with any one of B₁, B₂ and B_{m-s}; P is the second monomer, and D is the complement Watson-Crick base pairing nucleotide of X. In certain disclosed embodiments, the probe is selected to recognize a predetermined sequence of a nucleic acid target, which may be single-stranded or double-stranded, more commonly double-stranded. In certain disclosed embodiments, the probe may additionally comprise one or more monomers at any given position that do not participate in base pairing, such as the following structures, wherein R^e is H, DMTr, or phosphate, such as provided by a phosphodiester bond in an oligonucleotide and R^f is H, (N(i-Pr)₂)P(OCH₂CH₂CN), or phosphate, such as provided by a phosphodiester bond in an oligonucleotide, and R^g and R^h are, by way of example and without limitation, independently selected from hydrogen, aliphatic, particularly alkyl, such as C1-C10 alkyl, cyclic, heterocyclic, aromatic, heteroaromatic, amides, and carbamates.



Particular disclosed embodiments concern a probe wherein the first monomer and the second monomer are arranged in a manner that substantially weakens the duplex's thermal stability. In particular disclosed embodiments, the probe may have a thermal melting temperature which is comparable (slightly lower, similar or slightly higher) to that of a corresponding (i.e., isosequential) unmodified nucleic acid duplex, which typically does not comprise a monomer having the formulas disclosed herein. The probe may comprise a pair of monomers wherein the first monomer and the second monomer are arranged in a +n or -n interstrand zipper orientation, wherein n ranges from 0 to about 10, more typically from 0 to about 2, more typically the first monomer and the second monomer are arranged in a +n orientation, wherein n is 1. The probe may also comprise one or more additional pairs of monomers; a signal generating moiety capable of being detected, selected from a fluorophore, a member of a specific binding pair (e.g. biotin), a nanoparticle, a signal quenching moiety, such as a quencher of fluorescence (e.g., Black Hole Quencher); permanent or inducible crosslinking reagents (such as psoralen) capable of forming bonds between the probe and nucleic acids, proteins, sugars, lipids and other biomolecules; a nucleic acid cargo (e.g., single-stranded DNA, single-stranded RNA, double-stranded DNA, double-stranded RNA, plasmid, gene); and combinations thereof. In particular disclosed embodiments, the secondary entity facilitates cell-uptake and/or cellular compartmentalization and includes peptides [NLS, CPP, KDEL], and small molecules with nuclear affinity (e.g. Hoechst-type dyes, ethidium, acridine and thiazole orange). The probe may be used in solution, on a solid surface (e.g. multi-well plates; noble metal surfaces, such as electrodes), or in combination with a colloidal material and/or nanomaterials (e.g. gold nanoparticles, quantum dots).

Certain disclosed embodiments concern a method for detecting a target, comprising: selecting a probe comprising a monomer having a formula



where each Y independently is selected from oxygen, sulfur, and NR^b, wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl; V is selected from carbon, oxygen, sulfur, and NR^b; n ranges from 0 to 4; R¹ and R² are selected from hydrogen, aliphatic, aryl, and a heteroatom-containing moiety; R³ is a heteroatom-containing functional group; R⁴ is selected from any natural or non-natural nucleobase; R⁵ is selected from any functional group suitable for coupling to or associating with a nucleic acid and at least one natural nucleotide, unnatural nucleotide, and combinations thereof; exposing a nucleic acid target to the probe; and detecting the probe. In particular disclosed embodiments of the method, the probe is selected to substantially match a region of the target nucleic acid, particularly double stranded nucleic acid target regions, which may or may not comprise one or more polypurine stretches; more typically the nucleic acid is a mixed sequence of nucleotides, structured nucleic acid, particularly double-stranded nucleic acid sequences (dsDNA), even more particularly double stranded DNA, such as by way of example a mixed-sequence, hairpin DNA targets, PCR amplicons, genomic DNA, etc. which are isosequential with the probe. In particular disclosed embodiments, exposing the target to the probe comprises incubating the probe with the nucleic acid target. In certain disclosed embodiments, the nucleic acid target is incubated with an excess, such as about a 5-fold excess of the probe up to at least about a 5,000-fold excess of the probe, more typically up to about a 500-fold excess of the probe, and even more typically about a 5-fold excess of the probe to about a 200-fold excess of the probe. In particular disclosed embodiments, the probe and probe-target (recognition) complex, is detected by fluorescence spectroscopy, electrophoresis, absorption spectroscopy, fluorescence microscopy, flow cytometer, and combinations thereof.

A person of ordinary skill in the art will appreciate that the target sequences for disclosed probe embodiments can vary. For certain disclosed embodiments, the method is particularly useful for gender determination in mammals. For example, the method can be used for gender determination of ungulates and ruminates, particularly bovines, equines or porcines. For other embodiments, the target is isosequential (relative to a probe) to double stranded DNA target regions, including stems of molecular beacons, target regions embedded within PCR amplicons, target regions embedded within circular or linearized plasmids, target regions embedded within genomic DNA, and target regions embedded within microorganisms. The target also can be selected from a nucleic acid sequence associated with a proliferative disorder, such as B cell and T cell leukemias, lymphomas, breast cancer, colon cancer, and neurological cancers. In yet other embodiments, the target is

selected from a virus or other microorganism and the probe is used to detect and/or identify the microorganism.

Kits comprising a nucleic acid probe also are disclosed. Such kits typically comprise a probe comprising at least one disclosed monomer, and may comprise at least one bulge monomer.

5 Such kits also may include a sequence selected from SEQ ID NOs. 1-254. Certain kit embodiments are particularly useful for gender determination in mammals.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram that illustrates a particular embodiment of a method for detecting a target using the disclosed probe.

FIG. 2 is an image of an embodiment of the disclosed probe.

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FIG. 3A is a schematic drawing illustrating the concept of using bulge monomers.

FIG. 3B is a schematic drawing illustrating probes with two +1 interstrand arrangements of disclosed monomers (green) and one to four non-pairing bulged monomers (red loop), such as **402-4**, **402-N** and **402-9**.

20 **FIG. 4** is an image of a thermal denaturation curve between an exemplary embodiment of disclosed probes and a nucleic acid target illustrating characteristics of duplexes between one of the (two) probe strands and one of the (two) target strands.

FIG. 5 is an image of a thermal denaturation curve between an exemplary embodiments of disclosed probes and a nucleic acid target illustrating characteristics of duplexes between one of the (two) probe strands and one of the (two) target strands.

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FIG. 6 is an image of a thermal denaturation curve between an exemplary embodiment of disclosed probes and a nucleic acid target illustrating characteristics of duplexes between one of the (two) probe strands and one of the (two) target strands.

FIG. 7 are absorption spectra obtained from an exemplary embodiment and duplexes formed with a nucleic acid target.

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FIG. 8 are absorption spectra obtained from an exemplary probe embodiment and the duplexes formed with a nucleic acid targets.

FIG. 9 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

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FIG. 10 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 11 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 12 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 13 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

5 **FIG. 14** are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 15 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

10 **FIG. 16** are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 17 are fluorescence emission spectra of an exemplary probe embodiment in the presence or absence of a nucleic acid target.

FIG. 18 is a schematic drawing of a target used in an exemplary method of using a disclosed probe.

15 **FIG. 19** is a schematic drawing of a target and exemplary embodiments of the disclosed probe used in an exemplary method of using the probe.

FIG. 20 is an image of a gel obtained using gel electrophoresis analysis of different exemplary embodiments of the probe and their ability to bind to a particular target.

20 **FIG. 21** is schematic drawing of a target used to analyze the ability of the disclosed probe to suppress gene expression.

FIG. 22 is schematic drawing of exemplary embodiments of disclosed probes and the results obtained using such probes to suppress gene expression in a particular target.

FIG. 23 is schematic drawing of the results obtained from a dose-dependent study using exemplary embodiments of the disclosed probe and a particular target.

25 **FIG. 24** is schematic drawing of an exemplary embodiment wherein the probe is used to target a mixed-sequence, structured nucleic acid target.

FIG. 25 is an image of a gel obtained from gel electrophoresis analysis of an exemplary embodiment of the disclosed probe illustrating its ability to complex with a mixed-sequence, structured nucleic acid target.

30 **FIG. 26** is an image of the results obtained using a variety of exemplary embodiments of the disclosed probes as well as embodiments used as controls (e.g. single-stranded probe precursor and a control with no probe) to target and form a complex with a mixed-sequence, structured nucleic acid target.

35 **FIG. 27** are absorption spectra obtained using exemplary embodiments of a disclosed probe comprising a triazole moiety.

FIG. 28 are steady-state fluorescence emission spectra obtained using exemplary embodiments of a disclosed probe comprising a triazole moiety.

FIG. 29 is schematic drawing illustrating a putative mechanism of the disclosed probe wherein universal hybridization occurs.

FIG. 30 illustrates recognition of structured dsDNA targets by probes using electrophoretic mobility shift assays: (a) schematic illustration of recognition process; (b) structures of dsDNA-targets with isosequential (**SL1**) or non-isosequential (**SL2** and **SL3**) stem regions (arrows denote deviation points); (c), (d) and (e) recognition of **SL1** using escalating excess of **126W2:126W5**, **120Q2:120Q5** or **D1:D2**, respectively; (f) incubation of **SL1** with 100-fold excess of single-stranded **126W2**, **126W5**, **120Q2** or **120Q5**; (g) and (h) incubation of **SL1-SL3** with 100-fold excess of **126W2:126W5** or **120Q2:120Q5**, respectively. Probe-target incubation: 3 hours at 20 °C; 15% non-denaturing PAGE; DIG: digoxigenin.

FIG. 31 are dose-response curves for recognition of structured dsDNA **SL1** by the following probes (top to bottom): **126W2:126W5**, **126X2:126X5**, **126Y2:126Y5**, **120Q2:120Q5**, **120Y2:120Y5** (**P2:P5**), **124X6:124X8** (**K6:K8**) and **120'W6:120'W8** (**M6:M8**). For experimental conditions see FIG. 30. Dose response curves are fitted to a logarithmic equation.

FIG. 32 illustrates the results of a control experiment. Incubation of **SL1** with 100-fold excess of single-stranded **120'W6** (**M6**), **120'W8** (**M8**), **120Y2** (**P2**) or **120Y5** (**P5**). For experimental conditions and sequence of **SL1**, see FIG. 30.

FIG. 33 illustrates the results of a control experiment. Incubation of **SL1-SL3** with 100-fold excess of a) **126X2:126X5**, b) **126Y2:126Y5** or c) **124X6:124X8**. For experimental conditions and sequence of **SL1-SL3**, see FIG. 30.

FIG. 34 illustrates targeting of structured dsDNA **HP1** using different probes modified with monomer **120Y** (200-fold molar excess) as monitored by the gel mobility shift assay (electrophoretic mobility shift assay; for concept see FIG 30). **HP1**: (5'-GGTATATATAGGC-(T10)-GCCTATATATACC (34.4 nM) incubated at room temperature. **HP1** alone (lane 1); **HP1** incubated with 200-excess of **120Y-P1** (lane 2), **120Y-P2** (lane 3), **120Y-P3** (lane 4), **120Y-P4** (lane 5), **120Y-P5** (lane 6), **120Y-P6** (lane 7) or **120Y-P7** (lane 8).

FIG. 35 illustrates targeting of structured dsDNA **HP1** using various concentrations of a selected probe (**120Y-P4**).

FIG. 36 illustrates the results of a control experiment demonstrating the recognition specificity of the disclosed probes.

FIG. 37 provides proof for a proposed recognition mechanism, demonstrating the disclosed mode of nucleic acid targeting using disclosed probes. Lane 1: DIG-labeled **HP1** only; lane 2: DIG-labeled 'upper' probe strand only (5'-GG(**120Y**)A(**120Y**)A TAT AGG C-DIG); lane 3: DIG-labeled 'lower' probe strand only (3'-DIG-CCA (**120Y**)A(**120Y**) ATA TCC G); lane 4: probe with DIG-labeled upper strand (5'-GG(**120Y**)A(**120Y**)ATATAGGC-DIG + 3'-CCA(**120Y**)A(**120Y**)ATATCCG) incubated with unlabeled structured target **HP1**; lane 5: probe with DIG-labeled lower strand (5'-GG(**120Y**)A(**120Y**)ATATAGGC + 3'-DIG-

CCA(**120Y**)A(**120Y**)ATATCCG) incubated with unlabeled structured target **HP1**; lane 6: unlabeled probe (5'-GG(**120Y**)A(**120Y**)ATATAGGC + 3'-CCA(**120Y**)A(**120Y**)ATATCCG) incubated with labeled structured target **HP1**.

FIG. 38 provides information that both strands of a double-stranded probe facilitate recognition of dsDNA target regions. Lane 1: only **HP1**; lane 2: **HP1** + upper strand of **120Y-P4** (i.e., 5'-GG(**120Y**)A(**120Y**)ATATAGGC); lane 3: **HP1** + lower strand of **120Y-P4** (i.e., 3'-CCA(**120Y**)A(**120Y**)ATATCCG); lane 4: **HP1** + **120Y-P4**. Probes are used in 200-fold molar excess relative to structured target **HP1**. Incubated in Hepes buffer for 15 hours at room temperature.

FIG. 39 is a gel illustrating the results of incubation of **HP1** with increasing concentrations of an isosequential and unmodified dsDNA probe. 5x-500x refers to molar fold excess of dsDNA with respect to **HP1**

FIG. 40 is a graph of linker chemistry versus invasion% for symmetric bulges illustrating the results of targeting structured dsDNA (stem-loop) target using probe with one or more bulges, where Dig-labeled structured dsDNA target was incubated with 200-fold excess of probe in Hepes buffer for 15 hours followed by electrophoresis, imaging, and quantification.

FIG. 41 is a graph of linker chemistry versus invasion% for an up stranded bulge illustrating the results of targeting structured dsDNA (stem-loop) target using probe with one or more bulges where Dig-labeled structured dsDNA target was incubated with 200-fold excess of probe in Hepes buffer for 15 hours followed by electrophoresis, imaging, and quantification.

FIG. 42 is a graph of linker chemistry versus invasion% for a down stranded bulge illustrating the results of targeting structured dsDNA (stem-loop) target using probe with one or more bulges where Dig-labeled structured dsDNA target was incubated with 200-fold excess of probe in Hepes buffer for 15 hours followed by electrophoresis, imaging, and quantification.

FIG. 43 is an illustration on the use of double-stranded probes with +1 interstrand zipper arrangements that are additionally modified with a fluorophore-quencher pair. Binding to the nucleic acid target results in generation of an optical signal, more commonly a fluorescent signal.

SEQUENCE LISTING

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NOs: 1-4, 11 and 240-245 are nucleotide sequences of structured dsDNA targets with isosequential or non-isosequential stem regions.

SEQ ID NOs: 5-10, 12-239, 246-251 and 254 are nucleotide sequences of oligonucleotide probes and target regions.

SEQ ID NOs: 252 and 253 are nucleotide sequences of double-stranded probes containing a non-pairing bulge.

DETAILED DESCRIPTION

5 I. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII*, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Publishers, 1994 (ISBN 0632021829);
10 and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar references.

As used herein, the singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context
15 clearly indicates otherwise. Also, as used herein, the term “comprises” means “includes.” Hence “comprising A or B” means including A, B, or A and B.

A wavy line (“~”) indicates a bond disconnection. A dashed line (“- -”) illustrates that a bond may be formed at a particular position.

All nucleotide sizes or amino acid sizes, and all molecular weight or molecular mass values,
20 given for nucleic acids or polypeptides or other compounds are approximate, and are provided for description.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below.

25 All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

30 In order to facilitate review of the various examples of this disclosure, the following explanations of specific terms are provided:

Aliphatic: Any open or closed chain molecule, excluding aromatic compounds, containing only carbon and hydrogen atoms which are joined by single bonds (alkanes), double bonds (alkenes), or triple bonds (alkynes). This term encompasses substituted aliphatic compounds, saturated aliphatic compounds, and unsaturated aliphatic compounds.

35 **Analog, Derivative or Mimetic:** An analog is a molecule that differs in chemical structure from a parent compound, for example a homolog (differing by an increment in the chemical structure, such as a difference in the length of an alkyl chain), a molecular fragment, a structure that differs by

one or more functional groups, a change in ionization. Structural analogs are often found using quantitative structure activity relationships (QSAR), with techniques such as those disclosed in Remington (*The Science and Practice of Pharmacology*, 19th Edition (1995), chapter 28). A derivative is a biologically active molecule derived from the base structure. A mimetic is a molecule that mimics the activity of another molecule, such as a biologically active molecule. Biologically active molecules can include chemical structures that mimic the biological activities of a compound.

Aromatic: A term describing conjugated rings having unsaturated bonds, lone pairs, or empty orbitals, which exhibit a stabilization stronger than would be expected by the stabilization of conjugation alone. It can also be considered a manifestation of cyclic delocalization and of resonance.

Aryl: A substantially hydrocarbon-based aromatic compound, or a radical thereof (e.g. C₆H₅) as a substituent bonded to another group, particularly other organic groups, having a ring structure as exemplified by benzene, naphthalene, phenanthrene, anthracene, etc. This term also encompasses substituted aryl compounds.

Aryl alkyl: A compound, or a radical thereof (C₇H₇ for toluene) as a substituent bonded to another group, particularly other organic groups, containing both aliphatic and aromatic structures.

Complementary: The natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. Complementarity may exist when only some of the nucleic acids bind, or when total complementarity exists between the nucleic acids.

Conjugating, joining, bonding or linking: Joining one molecule to another molecule to make a larger molecule. For example, making two polypeptides into one contiguous polypeptide molecule, or covalently attaching a hapten or other molecule to a polypeptide, such as an scFv antibody. In the specific context, the terms include reference to joining a ligand, such as an antibody moiety, to an effector molecule. The linkage can be either by chemical or recombinant means. "Chemical means" refers to a reaction between the antibody moiety and the effector molecule such that there is a covalent bond formed between the two molecules to form one molecule.

Coupled: The term "coupled" means joined together, either directly or indirectly. A first atom or molecule can be directly coupled or indirectly coupled to a second atom or molecule. A secondary antibody provides an example of indirect coupling. Coupling can occur via covalent, non-covalent, and ionic bond formation.

Derivative: In chemistry, a derivative is a compound that is derived from a similar compound or a compound that can be imagined to arise from another compound, for example, if one atom is replaced with another atom or group of atoms. The latter definition is common in organic chemistry. In biochemistry, the word is used for compounds that at least theoretically can be formed from the precursor compound.

Deviation from additivity (DA): The DA value for a probe ONX:ONY is defined as: $DA_{\text{ONX:ONY}} \equiv \Delta T_m(\text{ONX:ONY}) - [\Delta T_m(\text{ONX:DNA X}) + \Delta T_m(\text{DNA Y:ONY})]$, where ONX:ONY is a double-stranded probe with certain interstrand zipper arrangements of disclosed monomers and

'DNA X' and 'DNA Y' are the complementary single-stranded nucleic acid targets of ONX and ONY, respectively. The term "Thermal advantage" is significantly related to DA, i.e., TA = - DA.

Displace (ment) (ed): A reaction in which an atom, radical, or molecule (anionic or neutral) replaces another in a compound.

5 **Double Stranded Nucleic Acid:** An oligonucleotide containing a region of two or more nucleotides having a double stranded motif.

Epitope: An antigenic determinant. These are particular chemical groups or contiguous or non-contiguous peptide sequences on a molecule that are antigenic, that is, that elicit a specific immune response.

10 **Fluorescence:** The emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength.

Fluorophore: A functional group of a molecule which causes the molecule to be fluorescent. Typically, the functional group can absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength.

15 **Human epidermal growth factor receptor (Her) family:** A family of structurally related proteins, including at least Her1, Her2, Her3 and Her4 (aka EGFR1, EGFR2, EGFR3 and EGFR4, respectively, or ErbB-1, ErbB-2, ErbB-3 and ErbB-4, respectively). Her1, Her2 and Her4 are receptor tyrosine kinases; although Her3 shares homology with Her1, Her2 and Her4, Her3 is kinase inactive. Included in the Her family is p95, a truncated form of Her2 lacking portions of the Her2
20 extracellular domain (see, e.g., Arribas *et al.*, *Cancer Res.*, 71:1515-1519, 2011; Molina *et al.*, *Cancer Res.*, 61:4744-4749, 2001).

The human epidermal growth factor family of receptors mediate cell growth and are dysregulated in many types of cancer. For example Her1 and Her2 are upregulated in many human cancers, and their excessive signaling may be critical factors in the development and malignancy of
25 these tumors. See, e.g., Herbst, *Int. J. Radiat. Oncol. Biol. Phys.*, 59:21-6, 2004; Zhang *et al.*, *J. Clin. Invest.* 117 (8): 2051-8, 2007. Receptor dimerization is essential for Her pathway activation leading to receptor phosphorylation and downstream signal transduction. Unlike Her1, -3 and -4, Her2 has no known ligand and assumes an open conformation, with its dimerization domain exposed for interaction with other ligand-activated Her receptors.

30 Approximately 30% of breast cancers have an amplification of the Her2 gene or overexpression of its protein product. Her2 overexpression also occurs in other cancer types, such as ovarian cancer, stomach cancer, and biologically aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma. See, e.g., Santin *et al.*, *Int. J. Gynaecol. Obstet.*, 102 (2): 128-31, 2008. Her2-containing homo- and hetero-dimers are transformation competent protein complexes.
35 Trastuzumab, a humanized antibody that prevents Her2 homodimerization is used to treat certain Her2 overexpressing cancers, including breast cancer. Additionally, the level of Her2 expression in cancer tissue is predictive of patient response to Her2 therapeutic antibodies (e.g., Trastuzumab).

Because of its prognostic role as well as its ability to predict response to Trastuzumab, tumors (e.g., tumors associated with breast cancer) are routinely checked for overexpression of Her2.

The Her pathway is also involved in ovarian cancer pathogenesis. Many ovarian tumor samples express all members of the Her family. Co-expression of Her1 and Her2 is seen more frequently in ovarian cancer than in normal ovarian epithelium, and overexpression of both receptors correlates with poor prognosis. Preferred dimerization with Her2 (Her1/Her2, Her2/Her3) and subsequent pathway activation via receptor phosphorylation have also been shown to drive ovarian tumor cell proliferation, even in the absence of Her2 overexpression. Pertuzumab, a humanized antibody that prevents Her2 dimerization (with itself and with Her3) has been shown to provide therapeutic benefit to patients with Her2 and/or Her3 expressing ovarian cancer.

Examples of Her1 amino acid sequence include NCBI/Genbank accession Nos. NP_005219.2, CAA25240.1, AAT52212.1, AAZ66620.1, BAF83041.1, BAH11869.1, ADZ75461.1, ADL28125.1, BAD92679.1, AAH94761.1. Examples of, Her2, amino acid sequences include NCBI/Genbank accession BAI17684.1, P04626.1, AAI67147.1, NP_001005862.1, NP_004439.2, AAA75493.1, AAO18082.1. Examples of Her3 amino acid sequences include NCBI/Genbank accession Nos. NP_001973.3, P21860.1, AAH82992.1, AAH02706.1, AAA35979.1. Examples of Her4 amino acid sequences include NCBI/Genbank accession Nos., AAI43750, Q15303.1, NP_005226.1, NP_001036064.1, AAI43748.1.

Heteroaliphatic: An aliphatic group, which contains one or more atoms other than carbon and hydrogen, such as, but not limited to, oxygen, sulfur, nitrogen, phosphorus, chlorine, fluorine, bromine, iodine, and selenium.

Homology: As used herein, “homology” refers to a degree of complementarity. Partial homology or complete homology can exist. Partial homology involves a nucleic acid sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid.

Homopolymer: This term refers to a polymer formed by the bonding together of multiple units of a single type of molecular species, such as a single monomer (for example, an amino acid).

Interstrand Zipper Nomenclature (+1/-1, etc...): The “interstrand zipper arrangement” nomenclature is used to describe 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.

Isolated: An “isolated” microorganism (such as a virus, bacterium, fungus, or protozoan) has been substantially separated or purified away from microorganisms of different types, strains, or species. Microorganisms can be isolated by a variety of techniques, including serial dilution and culturing.

An "isolated" biological component (such as a nucleic acid molecule, protein or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins, and organelles. Nucleic acids and proteins that have been
5 "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically synthesized nucleic acids or proteins, or fragments thereof.

Leaving Group: A molecular fragment that departs with a pair of electrons after heterolytic bond cleavage. Leaving groups can be anions or neutral molecules. Common anionic leaving groups
10 can include halides, such as Cl^- , Br^- , and I^- , and sulfonate esters, such as *para*-toluenesulfonate (TsO^-), trifluoromethanesulfonate (TfO^-). Common neutral molecule leaving groups can include H_2O , NH_3 , alcohols, and gases (N_2 , O_2 , CO_2 , CO , and SO_2).

Lewis acid: A chemical substance that can accept a pair of electrons from a **Lewis base**, B, which acts as an electron-pair donor, forming an adduct, AB as given by the following: $\text{A}^+:\text{B} \rightarrow$
15 $\text{A}-\text{B}$.

Linker: As used herein, a linker is a molecule or group of atoms positioned between two moieties.

Lower alkyl: Any aliphatic chain that contains 1-10 carbon atoms.

Modified: As used herein, "modified" refers to an oligonucleotide that has a non-natural
20 composition, in that it comprises one or more synthetic nucleobases which can pair with a natural base.

Molecule of interest or Target: A molecule for which the presence, location and/or concentration is to be determined.

Nucleobase: As used herein, "nucleobase" includes naturally occurring nucleobases as well
25 as non-natural nucleobases. A person of ordinary skill in the art will recognize that "nucleobase" encompasses purine and pyrimidine derivatives, as well as heterocyclic derivatives and tautomers thereof.

Nucleophile: A reagent that forms a chemical bond to its reaction partner (the electrophile) by donating both bonding electrons. A molecule or ion with a free pair of electrons can act as
30 nucleophile.

Nucleotide: Phosphorylated nucleosides are "nucleotides," which are the molecular building-blocks of DNA and RNA.

Nucleoside: A glycoside of a heterocyclic base. The term "nucleoside" is used broadly as to include non-naturally occurring nucleosides, naturally occurring nucleosides as well as other
35 nucleoside analogues. Illustrative examples of nucleosides are ribonucleosides comprising a ribose moiety as well as deoxyribo-nucleosides comprising a deoxyribose moiety. With respect to the bases of such nucleosides, it should be understood that this may be any of the naturally occurring bases, e.g.

adenine, guanine, cytosine, thymine, and uracil, as well as any modified variants thereof or any possible unnatural bases.

Oligonucleotide: A plurality of joined nucleotides joined by native phosphodiester bonds. An oligonucleotide is a polynucleotide of between at least 2 and about 300 nucleotides in length.

5 Typically, an oligonucleotide is a polynucleotide of between about 5 and about 50 nucleotides. An oligonucleotide analog refers to moieties that function similarly to oligonucleotides but have non-naturally occurring portions. For example, oligonucleotide analogs can contain non-naturally occurring portions, such as altered sugar moieties or inter-sugar linkages, such as a phosphorothioate oligodeoxynucleotide. Functional analogs of naturally occurring polynucleotides can bind to RNA or
10 DNA, and include locked nucleic acid (LNA) and peptide nucleic acid (PNA) molecules.

Pharmaceutically acceptable salt: Pharmaceutically acceptable salts are more soluble in aqueous solutions than the corresponding free acids and bases from which the salts are produced; however, salts having lower solubility than the corresponding free acids and bases from which the salts are produced may also be formed. Pharmaceutically acceptable salts are typically
15 counterbalanced with an inorganic base, organic base, or basic amino acid if the salts are positively charged; or the salt is counterbalanced with an inorganic acid, organic acid, or acidic amino acid if they are negatively charged. Pharmaceutically acceptable salts can also be zwitterionic in form. Salts can be formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc, and from bases such as ammonia, ethylenediamine, N-methyl-glutamine, lysine,
20 arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chlorprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethylammonium hydroxide. Other elements capable of forming salts are well-known to those of ordinary skill in the art, e.g. all elements from the main groups I to V of the Periodic Table of the Elements, as well as the elements from the subgroups I to VIII. Any chemical compound
25 recited in this specification may alternatively be administered as a pharmaceutically acceptable salt thereof. "Pharmaceutically acceptable salts" are also inclusive of the free acid, base, and. Descriptions of suitable pharmaceutically acceptable salts can be found in *Handbook of Pharmaceutical Salts, Properties, Selection and Use*, Wiley VCH (2002), which we herein incorporate by reference.

30 **Polypeptide:** A polymer in which the monomers are amino acid residues that are joined together through amide bonds. When the amino acids are α -amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins,
35 as well as those which are recombinantly or synthetically produced.

The term "residue" or "amino acid residue" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Protecting Group: A moiety that can be introduced into a molecule by chemical modification of a functional group. Protecting groups often are used to protect one functional group in order to obtain chemoselectivity in a chemical reaction with a different functional group. Suitable protecting groups are well known to those of ordinary skill in the art and can include aryl groups, aliphatic groups, heteroaliphatic groups, heteroaryl groups.

Protein: A molecule, particularly a polypeptide, comprised of amino acids.

Purified: The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified compound is one that is isolated in whole or in part from other contaminants. Generally, substantially purified peptides, proteins, conjugates, oligonucleotides, or other active compounds for use within the disclosure comprise more than 80% of all macromolecular species present in a preparation prior to admixture or formulation of the peptide, protein, conjugate or other active compound with a pharmaceutical carrier, excipient, buffer, absorption enhancing agent, stabilizer, preservative, adjuvant or other co-ingredient in a complete pharmaceutical formulation for therapeutic administration. More typically, the peptide, protein, conjugate or other active compound is purified to represent greater than 90%, often greater than 95% of all macromolecular species present in a purified preparation prior to admixture with other formulation ingredients. In other cases, the purified preparation may be essentially homogeneous, wherein other macromolecular species are not detectable by conventional techniques.

Quantum Yield: A measure of the efficiency of the fluorescence process. The "quantum yield" of a radiation-induced process indicates the number of times that a defined event occurs per photon absorbed by the system.

Reactive Groups: Formulas throughout this application refer to "reactive groups," which can be any of a variety of groups suitable for undergoing a chemical transformation as described herein. For example, the reactive group might be an amine-reactive group, such as an isothiocyanate, an isocyanate, an acyl azide, an NHS ester, an acid chloride, such as sulfonyl chloride, aldehydes and glyoxals, epoxides and oxiranes, carbonates, arylating agents, imidoesters, carbodiimides, anhydrides, and combinations thereof. Suitable thiol-reactive functional groups include haloacetyl and alkyl halides, maleimides, aziridines, acryloyl derivatives, arylating agents, thiol-disulfide exchange reagents, such as pyridyl disulfides, TNB-thiol, and disulfide reductants, and combinations thereof. Suitable carboxylate-reactive functional groups include diazoalkanes, diazoacetyl compounds, carbonyldiimidazole compounds, and carbodiimides. Suitable hydroxyl-reactive functional groups include epoxides and oxiranes, carbonyldiimidazole, *N,N'*-disuccinimidyl carbonates or *N*-hydroxysuccinimidyl chloroformates, periodate oxidizing compounds, enzymatic oxidation, alkyl halogens, and isocyanates. Aldehyde and ketone-reactive functional groups include hydrazines, Schiff bases, reductive amination products, Mannich condensation products, and combinations thereof. Active hydrogen-reactive compounds include diazonium derivatives, Mannich condensation products, iodination reaction products, and combinations thereof. Photoreactive

chemical functional groups include aryl azides, halogenated aryl azides, benzophenones, diazo compounds, diazine derivatives, and combinations thereof.

Sample: A biological specimen from a subject, such as might contain genomic DNA, RNA (including mRNA), protein, or combinations thereof. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy, surgical specimen, amniocentesis samples and autopsy material.

Single nucleotide polymorphism: A nucleic acid sequence variation occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual. For example, two sequenced nucleic acid fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide.

Substantially complementary: As used herein, “substantially complementary refers to the oligonucleotides of the disclosed methods that are at least about 50% homologous to target nucleic acid sequence they are designed to detect, more preferably at least about 60%, more preferably at least about 70%, more preferably at least about 80%, more preferably at least about 90%, more preferably at least about 90%, more preferably at least about 95%, most preferably at least about 99%.

Thermal advantage: Thermal Advantage (TA) is defined as $TA (ONX:ONY) \equiv T_m (ONX:DNA X) + T_m (DNA Y:ONY) - T_m (ONX:ONY) - T_m (DNA X:DNA Y)$, where ONX:ONY is a double-stranded probe with certain interstrand zipper arrangements of disclosed monomers, ONX:DNA X and DNA Y:ONY are the duplexes between individual probe strands and nucleic acid targets, and DNA X:DNA Y is the double-stranded nucleic acid target. A large, positive TA-value signifies significant potential for probe ONX:ONY to target DNA X:DNA Y. The term “Deviation from Additivity” is significantly related to ‘TA’ by the equation: $TA = -DA$ (highly positive TA or highly negative DA values demonstrate significant targeting potential).

Transition metal: Any of the metallic elements within Groups 3 to 12 in the Periodic Table that have an incomplete inner electron shell and that serve as transitional links between the most and the least electropositive in a series of elements.

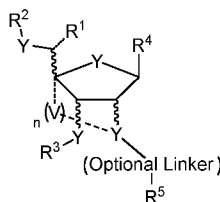
II. INTRODUCTION

Disclosed herein is a probe for targeting nucleic acids and particular sequences thereof. In particular disclosed embodiments, the probe comprises one or more pairs of monomers capable of intercalating with one or more nucleic acid targets. In particular disclosed embodiments, the probe comprises one or more pairs of monomers comprising a first monomer and a second monomer arranged on opposite nucleic acid strands. Certain disclosed embodiments concern a probe wherein one or more monomers are arranged in a manner that promotes thermal instability of the probe and increases the probe’s ability to detect a target.

III. EMBODIMENTS OF DISCLOSED PROBES

A. Monomers

In particular disclosed embodiments, the disclosed probe may comprise one or more monomers capable of coupling with a nucleic acid. In particular disclosed embodiments, each monomer independently may have a Formula 1, illustrated below.



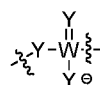
Formula 1

With reference to Formula 1, each Y may independently be selected from oxygen, sulfur, a triazole, oxazole, tetrazole, isoxazole, and NR^b, wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl, and V may be selected from carbon, oxygen, sulfur, and NR^b, wherein R^b is as previously recited. The variable "n" may range from 0 to 4; more typically, n is 1 or zero. A person of ordinary skill in the art will recognize that when n is 1 or greater, V may or may not be bound to Y, as indicated by the dashed line connecting these two variables in Formula 1. R¹ may be selected from hydrogen, aliphatic, such as alkyl, more typically lower alkyl, such as methyl, ethyl, propyl, butyl, etc., alkenyl, alkynyl, aryl, aryl aliphatic, such as aryl alkyl, and a heteroatom-containing moiety. The heteroatom-containing moiety may be selected from, but not limited to, ether (R^aOR^b), hydroxyl (R^aOH), silyl ether (R^aR^bR^cSiOR^d), phosphine (PR^aR^bR^c), thiol (R^aSH), thioether/sulfide (R^aSR^b), disulfide (R^aSSR^b), isothiocyanate (R^aNCS), isocyanate (R^aNCO), amine (NH₂, NHR^a, NR^aR^b), amide (R^aNR^bC(O)R^c), ester (R^aOC(O)R^b), halogen (I, Br, Cl, F), carbonate (R^aOC(O)OR^b), carboxyl (R^aC(O)OH), carboxylate (R^aCOO⁻), ester (R^aC(O)OR^b), ketone (R^aC(O)R^b), phosphate (R^aOP(O)(OH)₂), phosphoryl (R^aP(O)(OH)₂), sulfinyl (R^aS(O)R^b), sulfonyl (R^aSO₂R^b), carbonothioyl (R^aC(S)R^b or R^aC(S)H), sulfinio (R^aS(O)OH), sulfo (R^aSO₃H), amide (R^aC(O)NR^bR^c), azide (N₃), nitrile (R^aCN), isonitrile (R^aN⁺C⁻), and nitro (R^aNO₂). With reference to all the heteroatom-containing moieties disclosed herein, R^a represents the remaining monomer structure, which is attached to the abovementioned functional groups at the position indicated for R¹; and R^b, R^c, and R^d independently are hydrogen, aliphatic, aryl, heteroaliphatic, heteroaryl, and any combination thereof. The optional linker may be selected from alkyl, amide, carbamate, carbonate, urea, and combinations thereof.

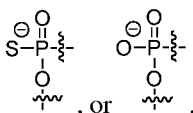
R² may be selected from hydrogen, aliphatic, aryl, and any one of the heteroatom-containing moieties described herein. In particular embodiments, R² may be selected from a protecting group known to those of ordinary skill in the art, such as, but not limited to, 4,4'-dimethoxytrityl, trityl, 9-arylthioxanthenyl, mesyl (Ms), tosyl (Ts), besoyl (Bs), trifluoromethane (CF₃), and

trifluoromethanesulfonyl. In certain disclosed embodiments, R^2 may be one or more nucleotides or monomers.

R^3 typically may be a heteroatom-containing functional group. In particular disclosed embodiments, the heteroatom may be selected from phosphorous, sulfur, nitrogen, oxygen, selenium, and/or a metal. Certain disclosed embodiments utilize R^3 substituents having a formula



where Y is selected from oxygen, sulfur, NR^b where R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, heteroaryl, and W is selected from phosphorus, SH, or SeH. Certain species of R^3 substituents include, without limitation, the following:

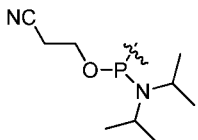


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In other disclosed embodiments, R^3 has a formula



where W is phosphorus, and each Z independently is selected from ether, thioether, hydroxyl, and NR^b . In particular disclosed embodiments, R^3 may be



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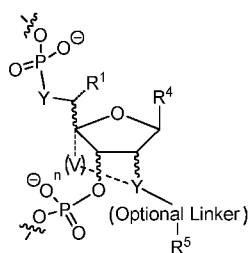
R^4 may be selected from any natural or non-natural nucleobase. In particular disclosed embodiments, R^4 is a natural nucleobase selected from uracil, adenine, thymine, cytosine, guanine. A person of ordinary skill in the art will recognize that R^4 may also be any non-natural, or synthetically developed nucleobase including those presently known or developed in the future. In particular disclosed embodiments, R^4 may be selected from C-5 functionalized pyrimidines, C6-functionalized pyrimidines, C7-functionalized 7-deazapurines, C8-functionalized purines, 2,6-diaminopurine, 2-thiouracil, 4-thiouracil, deoxyinosine and 3-(2'-deoxy- β -D-ribofuranosyl)pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one.

R^5 may be an intercalator capable of intercalating within a nucleic acid. In particular disclosed embodiments, R^5 may be any moiety capable of intercalating with single stranded nucleic acids, double stranded nucleic acids, and/or triple stranded nucleic acids. In particular disclosed embodiments, R^5 may be a planar moiety capable of maintaining a flat orientation when inserted into a nucleic acid. R^5 may be a hydrocarbon selected from pyrene, coronene, perylene, anthracene, naphthalene, and functionalized derivatives thereof; or an aromatic heterocycle, such as a porphyrin, a nucleobase (such as pyrimidines, purines, size-expanded nucleobases), a metal chelator (such as phenanthroline, DPPZ), an azapyrene, thiazole orange, ethidium, a diazobenzene, an indole, a

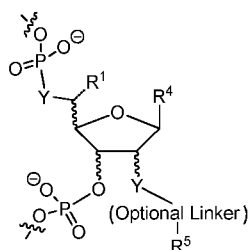
pyrrole, benzimidizoles, and modified analogs thereof. R^5 may be modified to include various aliphatic, aryl, or heteroatom-containing functional groups.

Particular disclosed embodiments concern a probe comprising one or more monomers selected from any one of Formulas 3-5.

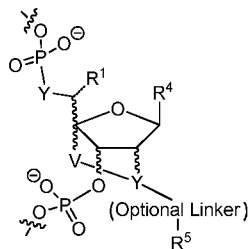
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Formula 3



Formula 4

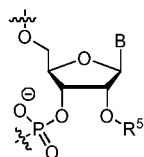


Formula 5

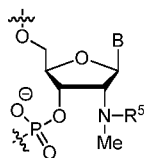
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In certain disclosed embodiments, the probe may comprise one or more monomers having any one of Formulas 6 and 7. With reference to Formulas 6 and 7, B may be selected from uracil, adenine, thymine, guanine, cytosine and 2-thiouracil with or without common protecting groups, and R^5 may be selected from naphth-2-yl, pyren-1-yl, coronen-1-yl, CH_2 -pyren-1-yl, CH_2 -coronen-1-yl, CO-pyren-1-yl, COCH_2 -pyren-1-yl, CH_2 -perylene-3-yl; CH_2 -1-(7-neopentylpyrenyl), CH_2 -1-(6-bromopyrenyl), CH_2 -1-(8-bromopyrenyl), CH_2 -1-(8-methylpyrenyl), CH_2 -1-(7-tert-butyl-3-methoxypyrenyl), CH_2 -pyren-2-yl and CH_2 -pyren-4-yl.

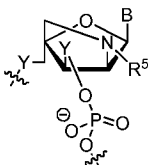
15



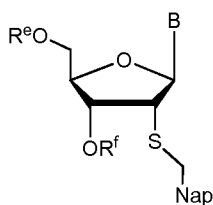
Formula 6



Formula 7

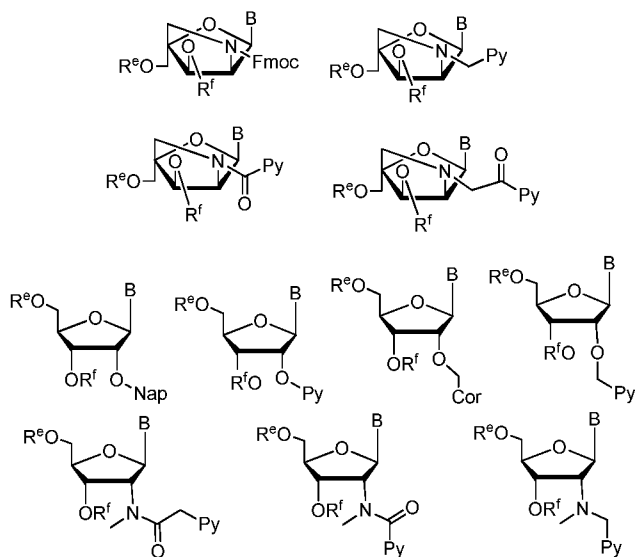


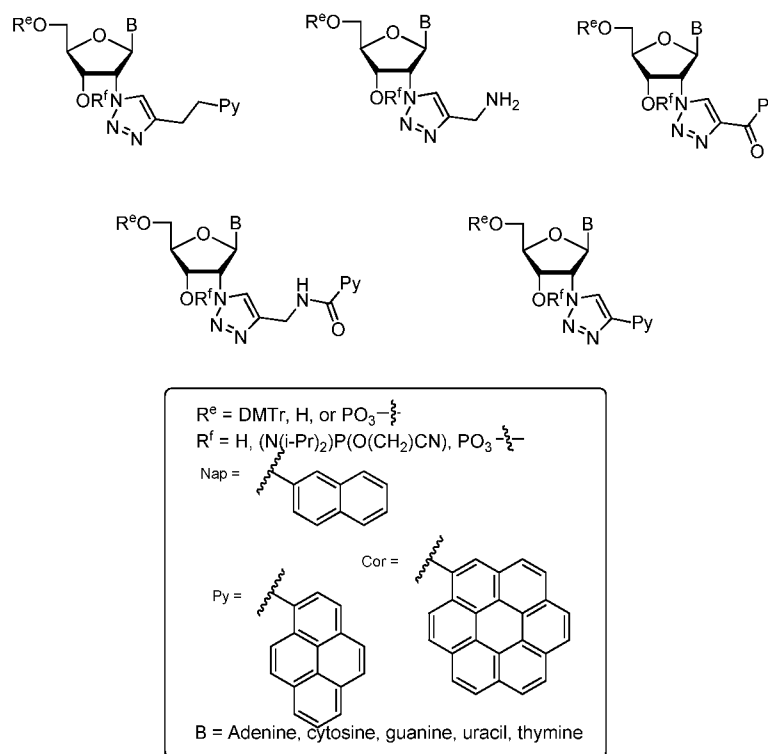
Formula 8



Formula 9

Exemplary working embodiments include the following compounds:





5 Particular disclosed embodiments concern using one or more monomers that do not participate in base pairing when either forming the probe or when the probe is reacted with a target. The monomers that do not participate in base pairing are herein referred to as “bulge monomers.” In particular disclosed embodiments, the bulge monomers may comprise one or more aliphatic, abasic sites, heteroalkyl groups, natural/modified nucleotides, and combinations thereof. One or more of the

10 bulge monomers may be included in the probe.

B. Single-Stranded Probe Precursor

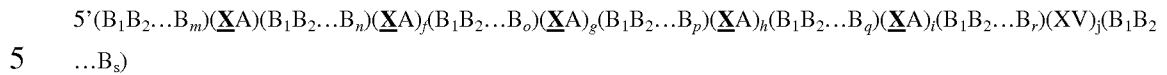
Particular disclosed embodiments concern a single-stranded probe precursor wherein one or more of the disclosed monomers are contained within a single-stranded oligonucleotide. In certain

15 disclosed embodiments, the single-stranded probe precursor may comprise one or more monomers arranged sequentially or in a manner wherein one or more natural or non-natural nucleotides are located between two or more monomers.

In particular disclosed embodiments, a single-stranded probe may serve as a precursor to a duplex comprising one or more of the disclosed monomers, or it may be used in the disclosed method

20 discussed herein. Certain disclosed embodiments concern a single-stranded probe having a general

Formula 9, illustrated below. The disclosed single-stranded probe precursor may comprise locked monomers, unlocked monomers, or combinations thereof.

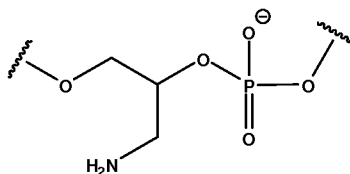
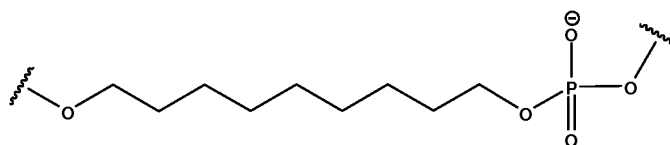
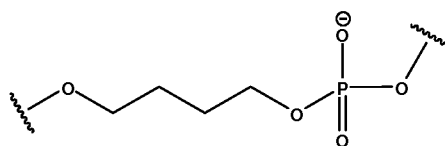


Formula 9

With reference to Formula 9, B₁B₂...B_{m-s} may be any natural or non-natural nucleotide presently known or discovered in the future, wherein *m-s* may range from zero to about 28. In particular disclosed embodiments, *f*, *g*, *h*, *i* and *j* may range from 0 to 10, more typically 0 to 5, and even more typically 0 to 1. Each X independently may be selected from any of the disclosed monomers and A may be a Watson-Crick base pairing nucleotide, or derivative thereof, which is capable of coupling with a complementary Watson-Crick base pairing nucleotide, or derivative thereof, in a target. In particular disclosed embodiments, each X independently may be a monomer having any one of formulas 1-7 and A may be selected from uracil, adenine, guanine, thymine, cytosine, and derivatives thereof. In particular disclosed embodiments, the single-stranded probe precursor may have a Formula 9 wherein the variables are defined according to any one of the following: *m* = 2, *n* = 5, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 4, *n* = 3, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 9, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 4, *n* = 7, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 6, *n* = 5, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 8, *n* = 3, *o* = *p* = *q* = *r* = *s* = 0, *f* = *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 0, *o* = 7, *p* = *q* = *r* = *s* = 0, *f* = 1, *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 2, *o* = 5, *p* = *q* = *r* = *s* = 0, *f* = 1, *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 4, *o* = 3, *p* = *q* = *r* = *s* = 0, *f* = 1, *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 0, *o* = 11, *p* = *q* = *r* = *s* = 0, *f* = 1, *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers comprising a uracil nucleobase, and A is adenin-9-yl DNA nucleotide; *m* = 2, *n* = 7, *o* = 4, *p* = *q* = *r* = *s* = 0, *f* = 1, *g* = *h* = *i* = *j* = 0, X is any one of the disclosed monomers

comprising a uracil nucleobase, and *A* is adenin-9-yl DNA nucleotide; $m = 4$, $n = 3$, $o = p = q = r = s = 0$, $f = g = h = i = j = 0$, **X** is any one of the disclosed monomers comprising an adenine nucleobase, and *A* is thymine-1-yl DNA nucleotide; $m = 4$, $n = 3$, $o = p = q = r = s = 0$, $f = g = h = i = j = 0$, **X** is any one of the disclosed monomers comprising a cytosine nucleobase, and *A* is guanine-9-yl DNA nucleotide; $m = 4$, $n = 3$, $o = p = q = r = s = 0$, $f = g = h = i = j = 0$, **X** is any one of the disclosed monomers comprising a guanine nucleobase, and *A* is cytosine-1-yl DNA nucleotide; $m = 4$, $n = 3$, $o = p = q = r = s = 0$, $f = g = h = i = j = 0$, **X** is any one of the disclosed monomers comprising an adenine nucleobase, and *A* is a guanine-9-yl DNA nucleotide; and $m = 4$, $n = 3$, $o = p = q = r = s = 0$, $f = g = h = i = j = 0$, **X** is any one of the disclosed monomers comprising an cytosine nucleobase, and *A* is an adenin-9-yl DNA nucleotide.

One or more bulge monomers also may be inserted at any position within a single stranded or double stranded probe. Probes may be additionally modified with one or more non-pairing modifications (a non-pairing modification is anything that can be incorporated internally within an oligonucleotide), which serves to decrease the thermostability of the probe, which facilitates the dsDNA-recognition reaction. The following provides examples of structures of non-pairing bulges that have been incorporated into disclosed probe embodiments.



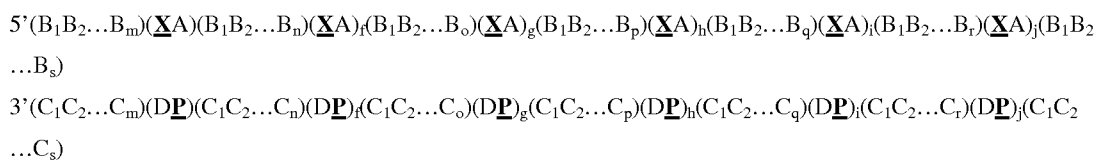
C. Probe Duplex

Particular disclosed embodiments concern a probe that can be used to identify, locate, bind, and/or modify a target. The probe may be a duplex comprising two strands of oligonucleotides comprising one or more pairs of the disclosed monomers. In particular disclosed embodiments, the one or more pairs of the disclosed monomers are arranged in a manner that substantially decrease the duplex's thermal stability, thereby providing the probe with the ability to bind to the target. Without being limited to a single theory of operation, it is currently believed that arranging the monomers in the particular manner disclosed herein provides the probe with sufficient energy to dissociate (or denature) into two strands that then couple with the target (FIG. 1).

The probe may comprise more than one pairs of monomers; for example, the probe may comprise anywhere from 1 pair of monomers to about 5 pairs of monomers. In particular disclosed embodiments, a pair of monomers can comprise two monomers, having any of the formulas disclosed herein, that are located on opposite strands of the probe (e.g. opposite strands of the duplex). In particular disclosed embodiments, a first monomer may be positioned at any location on one of the probe strands, with the second monomer of the pair being positioned at a particular location relative to the first monomer on the other probe strand. Certain disclosed embodiments concern a probe having one or more pairs of monomers arranged in a (+/-)n zipper arrangement, wherein n can range from 0 to about 10; more typically from 0 to about 3; even more typically from at least 1 to about 2. Particular disclosed embodiments concern a probe having at least one pair of monomers arranged in a +n zipper arrangement. Without being limited to a particular theory of operation, it currently is believed that certain arrangements of the interstrand monomers result in destabilization of the probe, such as illustrated in FIGS. 1-2. In particular disclosed embodiments, the pair of monomers may be arranged in a -n zipper arrangement. Exemplary embodiments of the disclosed probe typically comprise a pair of monomers comprising a first monomer and a second monomer arranged in a (+1) interstrand zipper arrangement. Other exemplary embodiments concern a probe comprising two pairs of monomers, with the first monomer and the second monomer of each pair being arranged in a (+1) interstrand zipper arrangement and each pair of monomers being separated by at least 0 to about 10 natural or non-natural nucleotides or bulge monomers.

Particular disclosed embodiments of the probe may have a Formula 10, illustrated below.

The disclosed probe may comprise locked monomers, unlocked monomers, and combinations thereof.



Formula 10

With reference to Formula 10, $B_1, B_2 \dots B_{m-s}$ may be any natural or non-natural nucleotide, presently known or discovered in the future, wherein $m-s$ may range from zero to about 28. In particular disclosed embodiments, f, g, h, i and j may range from 0 to 1,000, such as 0 to 900, such as 0 to 800, such as 0 to 700, such as 0 to 600, such as 0 to 500, such as 0 to 400, such as 0 to 300, such as 0 to 200, such as 0 to 100, such as 0 to 50, typically 0 to 10, and even more typically 0-5. $\underline{X}$ may be selected from any of the disclosed monomers and A may be a Watson-Crick base pairing nucleotide, or derivative thereof, or another disclosed monomer, which is capable of coupling with a complementary Watson-Crick base pairing nucleotide, or derivative thereof, in a target. In particular disclosed embodiments, $\underline{X}$ may be a monomer having any one of Formulas 1-7, and A may be selected from nucleotides with uracil, adenine, guanine, thymine or cytosine nucleobases, or nucleotides with pseudocomplementary nucleobases (e.g., 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one). C may be any natural or non-natural nucleotide, presently known or discovered in the future, that is capable of Watson-Crick base pairing with any one of $B_1, B_2 \dots B_{m-s}$. “B” and “C” also can be pseudocomplementary base pairs that do not form strong (or any base pairs); $\underline{P}$ may be selected from any of the disclosed monomers, and D may be a Watson-Crick base pairing nucleotide, or derivative thereof, or another disclosed monomer, which is capable of coupling with a complementary Watson-Crick base pairing nucleotide, or derivative thereof, in a target. In particular disclosed embodiments, $\underline{P}$ may be a monomer having any one of Formulas 1-7, and D may be selected from uracil, adenine, guanine, thymine, and cytosine.

Particular disclosed embodiments concern a probe having a Formula 10, wherein the variables are defined according to any one of the following: $m = 2, n = 5, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 9, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 4, n = 7, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 6, n = 5, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 8, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 0, o = 7, p = q = r = s = 0, f = 1, g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 2, o = 5, p = q = r = s = 0, f = 1, g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 4, o = 3, p = q = r = s = 0, f = 1, g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = o = p = q = r = s = 0, f = g = h = 1, i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 13, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{X} = \underline{P}$ are monomers with a uracil nucleobase, and $V = Y$ are adenine-9-yl DNA nucleotide; $m = 2, n = 0, o = 11,$

$p = q = r = s = 0, f = 1, g = h = i = j = 0, \underline{\mathbf{X}} = \underline{\mathbf{P}}$ are monomers with a uracil nucleobase, and $V = Y$ are adenin-9-yl DNA nucleotide; $m = 2, n = 7, o = 4, p = q = r = s = 0, f = 1, g = h = i = j = 0, \underline{\mathbf{X}} = \underline{\mathbf{P}}$ are monomers with a uracil nucleobase, and $V = Y$ are adenin-9-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{\mathbf{X}} = \underline{\mathbf{P}}$ are monomers with an adenine nucleobase, and $V = Y$ are thymine-1-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{\mathbf{X}} = \underline{\mathbf{W}}$ are monomers with a cytosine nucleobase, and $V = Y$ are guanin-9-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{\mathbf{X}} = \underline{\mathbf{W}}$ are monomers with a guanine nucleobase, and $V = Y$ are cytosin-1-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{\mathbf{X}}$ is a monomer with an adenine nucleobase, $\underline{\mathbf{W}}$ is a monomer with a cytosine nucleobase, Y is a thymine-1-yl DNA nucleotide and V is a guanin-9-yl DNA nucleotide; $m = 4, n = 3, o = p = q = r = s = 0, f = g = h = i = j = 0, \underline{\mathbf{X}}$ is a monomer with a cytosine nucleobase, $\underline{\mathbf{W}}$ is a monomer with a thymine nucleobase, Y is a guanin-9-yl DNA nucleotide and V is an adenin-9-yl DNA nucleotide.

FIGS. 3A and B illustrates the concept of using bulge monomers. With reference to FIGS. 3A and B green denotes disclosed the intercalator-functionalized monomers; red represents a non-nucleosidic linker. One or more bulge monomers can be added to any of the embodiments defined by the above formula.

Exemplary embodiments of the disclosed probe are provided in Table 1. A person of ordinary skill in the art will recognize that any of the disclosed embodiments of the probe can be made from two single-stranded precursors that are combined (hybridized) to form the probe. The embodiments disclosed in Table 1 concern a probe wherein $\underline{\mathbf{X}}$ is a monomer disclosed herein and R^4 is defined as a uracil or thymine nucleobase; however a person of ordinary skill in the art would recognize that any of the disclosed monomers may be used.

Table 1
Exemplary Embodiments of Certain Disclosed Probes and Corresponding Target

Probe	Target region
5'-d(GTG A $\underline{\mathbf{X}}$ A TGC)	5'-GTG ATA TGC
3'-d(CAC TA $\underline{\mathbf{X}}$ ACG	3'-CAC TAT ACG

5'-d(GG <u>X</u> ATA TAT AGG C) 3'-d(CCA <u>X</u> AT ATA TCC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GGT A <u>X</u> A TAT AGG C) 3'-d(CCA TA <u>X</u> ATA TCC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GGT ATA <u>X</u> AT AGG C) 3'-d(CCA TAT A <u>X</u> A TCC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GGT ATA TA <u>X</u> AGG C) 3'-d(CCA TAT ATA <u>X</u> CC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GG <u>X</u> A <u>X</u> A TAT AGG C) 3'-d(CCA <u>X</u> A <u>X</u> ATA TCC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GG <u>X</u> ATA <u>X</u> AT AGG C) 3'-d(CCA <u>X</u> AT A <u>X</u> A TCC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(GG <u>X</u> ATA TA <u>X</u> AGG C) 3'-d(CCA <u>X</u> AT ATA <u>X</u> CC G)	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-d(G <u>G</u> <u>X</u> A <u>X</u> AT AAG CAG C) 3'-d(C CA <u>X</u> A <u>X</u> A TTC GTC G)	5'-G GTA TAT AAG CAG C 3'-C CAT ATA TTC GTC G
5'-GGT A <u>X</u> A <u>X</u> AT AGG C 3'-CCA TA <u>X</u> A <u>X</u> A TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT ATA <u>X</u> A <u>X</u> AGG C 3'-CCA TAT A <u>X</u> A <u>X</u> CC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GG <u>X</u> A <u>X</u> A <u>X</u> A <u>X</u> AGG C 3'-CCA <u>X</u> A <u>X</u> A <u>X</u> A <u>X</u> CC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G

Additional exemplary embodiments are disclosed in Table 2. The embodiments disclosed in Table 2 concern a probe wherein the monomer in each strand (e.g. the strands designated as 5' and 3') may have any one of formulas 1-7, wherein the R⁴ moiety is selected from adenine (A), cytosine (C), guanine (G), uracil (U), or thymine (T).

Table 2
Exemplary Embodiments of Certain Disclosed Probes and Corresponding Target Regions

Probe	Target region
5'-d(GTG A <u>A</u> T TGC) 3'-d(CAC TT <u>A</u> ACG)	5'-GTG AAT TGC 3'-CAC TTA ACG
5'-d(GTG A <u>A</u> G TGC) 3'-d(CAC TT <u>C</u> ACG)	5'-GTG AAG TGC 3'-CAC TTC ACG
5'-d(GTG A <u>A</u> C TGC) 3'-d(CAC TT <u>G</u> ACG)	5'-GTG AAC TGC 3'-CAC TTG ACG
5'-d(GTG A <u>C</u> G TGC) 3'-d(CAC TG <u>C</u> ACG)	5'-GTG ACG TGC 3'-CAC TGC ACG
5'-d(GTG A <u>C</u> C TGC) 3'-d(CAC TG <u>G</u> ACG)	5'-GTG ACC TGC 3'-CAC TGG ACG
5'-d(GTG A <u>C</u> A TGC) 3'-d(CAC TG <u>T</u> ACG)	5'-GTG ACA TGC 3'-CAC TGT ACG
5'-d(GTG A <u>G</u> C TGC) 3'-d(CAC TC <u>G</u> ACG)	5'-GTG AGC TGC 3'-CAC TCG ACG
5'-d(GTG A <u>G</u> A TGC) 3'-d(CAC TC <u>T</u> ACG)	5'-GTG AGA TGC 3'-CAC TCT ACG
5'-d(GTG A <u>T</u> T TGC) 3'-d(CAC TA <u>A</u> ACG)	5'-GTG ATT TGC 3'-CAC TAA ACG

5'-d(GTG A <u>T</u> A TGC)	5'-GTG ATA TGC
3'-d(CAC TAT <u>T</u> ACG)	3'-CAC TAT ACG

Additional working embodiments of probes are provided below in Table 3 with 0-arrangements.

5

Table 3**Working Embodiments of Probes with 0-Arrangement**

Probe	Target Region
5'-GGT <u>T</u> ATA TAT AGG C 3'-CCA <u>A</u> TAT ATA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT A <u>T</u> A TAT AGG C 3'-CCA T <u>A</u> T ATA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT ATA <u>T</u> AT AGG C 3'-CCA TAT <u>A</u> TA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT ATA TAT <u>T</u> AGG C 3'-CCA TAT ATA <u>A</u> TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT <u>A</u> <u>T</u> A TAT AGG C 3'-CCA <u>A</u> <u>T</u> <u>A</u> T ATA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT <u>T</u> ATA <u>T</u> AT AGG C 3'-CCA <u>A</u> TAT <u>A</u> TA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT <u>T</u> ATA TAT <u>T</u> AGG C 3'-CCA <u>A</u> TAT ATA <u>A</u> TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT A <u>T</u> A <u>T</u> AT AGG C 3'-CCA T <u>A</u> T <u>A</u> TA TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT ATA <u>T</u> <u>A</u> T AGG C 3'-CCA TAT <u>A</u> <u>T</u> <u>A</u> TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G
5'-GGT <u>A</u> <u>T</u> A <u>T</u> <u>A</u> T AGG C 3'-CCA <u>A</u> <u>T</u> <u>A</u> T <u>A</u> <u>T</u> A TCC G	5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G

Tables 4-6 outline additional working embodiments of certain exemplary probes targeting different DNA regions, i.e., second insulin [INSB], PPAR gamma and CEBP promoters.

10

Table 4
Probes Targeting Second Insulin Promoter [INSB]

Probe	Target region
5'-G <u>GTA</u> TAT AAG CAG CAC A 3'-C CAT <u>ATA</u> TTC GTC GTG T	5'-G GTA TAT AAG CAG CAC A 3'-C CAT ATA TTC GTC GTG T
5'-G <u>GTA</u> <u>TAT</u> AAG CAG CAC A 3'-C CAT <u>ATA</u> <u>TTC</u> GTC GTG T	5'-G GTA TAT AAG CAG CAC A 3'-C CAT ATA TTC GTC GTG T
5'-AGG AAG <u>GTA</u> <u>TAT</u> AAG CA 3'-TCC TTC CAT <u>ATA</u> TTC GT	5'-AGG AAG GTA TAT AAG CA 3'-TCC TTC CAT ATA TTC GT
5'-ACT <u>ATA</u> GAA TAC TCA AG 3'-TGA <u>TAT</u> CTT ATG AGT TC	5'-ACT ATA GAA TAC TCA AG 3'-TGA TAT CTT ATG AGT TC

5

Table 5
Additional Examples of Probes for PPAR Gamma

Probe	Target region
5'-CCC <u>ACG</u> <u>TTA</u> GCA GTT 3'-GGG <u>TGC</u> <u>AAT</u> CGT CAA	5'-CCC ACG TTA GCA GTT 3'-GGG TGC AAT CGT CAA
5'-CCC ACG <u>TTA</u> <u>GCA</u> GTT 3'-GGG TGC <u>AAT</u> <u>CGT</u> CAA	5'-CCC ACG TTA GCA GTT 3'-GGG TGC AAT CGT CAA
5'-AGA CAA <u>AAC</u> <u>ACC</u> AGT 3'-TCT GTT TTG <u>TGG</u> <u>TCA</u>	5'-AGA CAA AAC ACC AGT 3'-TCT GTT TTG TGG TCA
5'-AGA <u>CAA</u> <u>AAC</u> ACC AGT 3'-TCT <u>GTT</u> TTG <u>TGG</u> TCA	5'-AGA CAA AAC ACC AGT 3'-TCT GTT TTG TGG TCA
5'-CTA <u>CAT</u> <u>TGT</u> CTC GCC 3'-GAT <u>GTA</u> <u>ACA</u> GAG CGG	5'-CTA CAT TGT CTC GCC 3'-GAT GTA ACA GAG CGG
5'-CTA <u>CAT</u> <u>TGT</u> CTC GCC 3'-GAT <u>GTA</u> <u>ACA</u> GAG CGG	5'-CTA CAT TGT CTC GCC 3'-GAT GTA ACA GAG CGG
5'-CGT <u>CAT</u> <u>CGT</u> GCT CGC 3'-GCA <u>GTA</u> GCA <u>CGA</u> GCG	5'-CGT CAT CGT GCT CGC 3'-GCA GTA GCA CGA GCG

Table 6
Additional Examples of Probes for CEBP

Probe	Target region
5'-CGG ACC ACG <u>TGT</u> GTG 3'-GCC TGG TGC <u>ACA</u> <u>CAC</u>	5'-CGG ACC ACG TGT GTG 3'-GCC TGG TGC ACA CAC
5'-CGG ACC <u>C</u> ACG TGT GTG 3'-GCC TGG <u>TGC</u> ACA <u>CAC</u>	5'-CGG ACC ACG TGT GTG 3'-GCC TGG TGC ACA CAC
5'-GTC <u>AGT</u> GGG CGT TGC 3'-CAG <u>TCA</u> <u>CCC</u> GCA ACG	5'-GTC AGT GGG CGT TGC 3'-CAG TCA CCC GCA ACG
5'-GTC <u>AGT</u> GGG CGT TGC 3'-CAG <u>TCA</u> <u>CCC</u> GCA <u>ACG</u>	5'-GTC AGT GGG CGT TGC 3'-CAG TCA CCC GCA ACG
5'-CCT CTA <u>TAA</u> AAG CGG 3'-GGA GAT <u>ATT</u> TTC GCC	5'-CCT CTA TAA AAG CGG 3'-GGA GAT ATT TTC GCC
5'-CCT CTA <u>TAA</u> AAG CGG 3'-GGA <u>GAT</u> <u>ATT</u> TTC GCC	5'-CCT CTA TAA AAG CGG 3'-GGA GAT ATT TTC GCC

5

Additional working embodiments of probes that may be used for gender determination in animals, more commonly, in bovine, are shown in Table 7, where Cy3 is a Cy3 fluorophore; underlined A/C/G/T are monomers; and underlined B is a bulged (non-pairing) monomer .

10

Table 7
Bovine Series

Probe	Target Region
5'-AGC CCT <u>GTG</u> CCC TG 3'-TCG GGA <u>CAC</u> GGG AC	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC
5'-CCT <u>GTG</u> CCC TG 3'-GGA <u>CAC</u> GGG AC	5'-CCT GTG CCC TG 3'-GGA CAC GGG AC
5'-CCT <u>GTG</u> CCC TG 3'-GGA <u>CAC</u> GGG <u>AC</u>	5'-CCT GTG CCC TG 3'-GGA CAC GGG AC

5'- <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'-TCG GGA <u>CAC</u> GGG <u>AC</u>	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC
5'-CTG <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'-GAG TCG GGA <u>CAC</u> GGG <u>AC</u>	5'-CTG AGC CCT GTG CCC TG 3'-GAG TCG GGA CAC GGG AC
5'- <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'-TCG GGA <u>CAC</u> GGG <u>AC</u>	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>B</u> CCC <u>TG</u> 3'- TCG GGA <u>CAC</u> <u>B</u> GGG <u>AC</u> <u>Cy3</u>	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>B</u> CCC <u>TG</u> 3'- TCG GGA <u>CAC</u> GGG <u>AC</u> <u>Cy3</u>	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'- TCG GGA <u>CAC</u> <u>B</u> GGG <u>AC</u> <u>Cy3</u>	5'-AGC CCT GTG CCC TG 3'-TCG GGA CAC GGG AC

IV. TARGETS

Particular disclosed embodiments concern targeting and binding to a particular target using the disclosed probe. In particular disclosed embodiments, the target may be a nucleic acid, such as, but not limited to, single-stranded DNA (and derivatives thereof), double-stranded DNA (and derivatives thereof), and any combinations thereof. Particular disclosed embodiments concern targeting isosequential (relative to the probe) double stranded DNA target regions, including: stems of molecular beacons, target regions embedded within PCR amplicons, target regions embedded within circular or linearized plasmids, target regions embedded within genomic DNA (crude, purified, cell culture, in vivo, embryos, etc.), target regions embedded within microorganisms, and the like. This list of targets is meant to be exemplary and is not intended to be limiting. In particular disclosed embodiments, the target may be selected by identifying an RNA target, such as those pursued in antisense/siRNA/anti-miRNA clinical trials and pre-clinical trials (e.g. those used in modulation of gene expression and/or identification of biomarkers) and design a target comprising the corresponding DNA to this particular RNA target. Solely by way of example, specific targets include linearized plasmids (e.g. against T7 promotor, as illustrated by FIGS. 18-20); circular plasmids (e.g. against insulin B promotor in circular plasmids, as illustrated by FIGS. 21-23); genomic DNA; structured dsDNA targets (FIGS 24-26 and FIGS 30-42).

A target nucleic acid sequence can vary substantially in size. Without limitation, the nucleic acid sequence can have a variable number of nucleic acid residues. For example a target nucleic acid

sequence can have at least about 2 nucleic acid residues, typically at least about 10 nucleic acid residues, or at least about 20, 30, 50, 100, 150, 500, 1000 residues.

In specific, non-limiting examples, a protein is produced by a target nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) associated with a neoplasm (for example, a cancer).

5 Numerous chromosome abnormalities (including translocations and other rearrangements, amplification or deletion) have been identified in neoplastic cells, especially in cancer cells, such as B cell and T cell leukemias, lymphomas, breast cancer, colon cancer, neurological cancers and the like. Therefore, in some examples, at least a portion of a protein is produced by a nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) that is amplified or deleted in at least a subset of cells in
10 a sample.

Oncogenes are known to be responsible for several human malignancies. For example, chromosomal rearrangements involving the SYT gene located in the breakpoint region of chromosome 18q11.2 are common among synovial sarcoma soft tissue tumors. The t(18q11.2) translocation can be identified, for example, using the disclosed probe. In other examples, a protein
15 produced from a nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) is selected that is a tumor suppressor gene that is deleted (lost) in malignant cells. In particular disclosed embodiments, this type of target may also be detected, identified, the expression of the gene reduced and/or the gene modified using disclosed probe embodiments. For example, the p16 region (including D9S1749, D9S1747, p16(INK4A), p14(ARF), D9S1748, p15(INK4B), and D9S1752) located on chromosome
20 9p21 is deleted in certain bladder cancers. Chromosomal deletions involving the distal region of the short arm of chromosome 1 (that encompasses, for example, SHGC57243, TP73, EGFL3, ABL2, ANGPTL1, and SHGC-1322), and the pericentromeric region (*e.g.*, 19p13-19q13) of chromosome 19 (that encompasses, for example, MAN2B1, ZNF443, ZNF44, CRX, GLTSCR2, and GLTSCR1) are characteristic molecular features of certain types of solid tumors of the central nervous system.

25 The aforementioned examples are provided solely for purpose of illustration and are not intended to be limiting. Numerous other cytogenetic abnormalities that correlate with neoplastic transformation and/or growth are known to those of ordinary skill in the art. Target proteins that are produced by nucleic acid sequences (*e.g.*, genomic target nucleic acid sequences), which have been correlated with neoplastic transformation and which are useful in the disclosed methods, also include
30 the EGFR gene (7p12; *e.g.*, GENBANK™ Accession No. NC_000007, nucleotides 55054219-55242525), the C-MYC gene (8q24.21; *e.g.*, GENBANK™ Accession No. NC_000008, nucleotides 128817498-128822856), D5S271 (5p15.2), lipoprotein lipase (LPL) gene (8p22; *e.g.*, GENBANK™ Accession No. NC_000008, nucleotides 19841058-19869049), RB1 (13q14; *e.g.*, GENBANK™ Accession No. NC_000013, nucleotides 47775912-47954023), p53 (17p13.1; *e.g.*,
35 GENBANK™ Accession No. NC_000017, complement, nucleotides 7512464-7531642)), N-MYC (2p24; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 151835231-151854620), CHOP (12q13; *e.g.*, GENBANK™ Accession No. NC_000012,

complement, nucleotides 56196638-56200567), FUS (16p11.2; *e.g.*, GENBANK™ Accession No. NC_000016, nucleotides 31098954-31110601), FKHR (13p14; *e.g.*, GENBANK™ Accession No. NC_000013, complement, nucleotides 40027817-40138734), as well as, for example: ALK (2p23; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 29269144-29997936), Ig heavy chain, CCND1 (11q13; *e.g.*, GENBANK™ Accession No. NC_000011, nucleotides 69165054..69178423), BCL2 (18q21.3; *e.g.*, GENBANK™ Accession No. NC_000018, complement, nucleotides 58941559-59137593), BCL6 (3q27; *e.g.*, GENBANK™ Accession No. NC_000003, complement, nucleotides 188921859-188946169), MALF1, AP1 (1p32-p31; *e.g.*, GENBANK™ Accession No. NC_000001, complement, nucleotides 59019051-59022373), TOP2A (17q21-q22; *e.g.*, GENBANK™ Accession No. NC_000017, complement, nucleotides 35798321-35827695), TMPRSS (21q22.3; *e.g.*, GENBANK™ Accession No. NC_000021, complement, nucleotides 41758351-41801948), ERG (21q22.3; *e.g.*, GENBANK™ Accession No. NC_000021, complement, nucleotides 38675671-38955488); ETV1 (7p21.3; *e.g.*, GENBANK™ Accession No. NC_000007, complement, nucleotides 13897379-13995289), EWS (22q12.2; *e.g.*, GENBANK™ Accession No. NC_000022, nucleotides 27994271-28026505); FLI1 (11q24.1-q24.3; *e.g.*, GENBANK™ Accession No. NC_000011, nucleotides 128069199-128187521), PAX3 (2q35-q37; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 222772851-222871944), PAX7 (1p36.2-p36.12; *e.g.*, GENBANK™ Accession No. NC_000001, nucleotides 18830087-18935219), PTEN (10q23.3; *e.g.*, GENBANK™ Accession No. NC_000010, nucleotides 89613175-89716382), AKT2 (19q13.1-q13.2; *e.g.*, GENBANK™ Accession No. NC_000019, complement, nucleotides 45431556-45483036), MYCL1 (1p34.2; *e.g.*, GENBANK™ Accession No. NC_000001, complement, nucleotides 40133685-40140274), REL (2p13-p12; *e.g.*, GENBANK™ Accession No. NC_000002, nucleotides 60962256-61003682) and CSF1R (5q33-q35; *e.g.*, GENBANK™ Accession No. NC_000005, complement, nucleotides 149413051-149473128).

In other examples, a target protein is selected from a virus or other microorganism associated with a disease or condition. Detection of the virus- or microorganism-derived target nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) in a cell or tissue sample is indicative of the presence of the organism. The disclosed probe may be used to detect and/or identify these types of targets. Also, a gene encoding a critical enzyme for the survival of a microorganism can be targeted by disclosed probe embodiments, which can cause the death of the microorganism. For example, the target protein can be selected from the genome of an oncogenic or pathogenic virus, a bacterium or an intracellular parasite (such as *Plasmodium falciparum* and other *Plasmodium* species, *Leishmania* (sp.), *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia lamblia*, as well as *Toxoplasma*, *Eimeria*, *Theileria*, and *Babesia* species).

In some examples, the target protein is produced from a nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) from a viral genome. Exemplary viruses and corresponding

genomic sequences may selected from the following (GENBANK™ RefSeq Accession No. in parentheses): human adenovirus A (NC_001460), human adenovirus B (NC_004001), human adenovirus C (NC_001405), human adenovirus D (NC_002067), human adenovirus E (NC_003266), human adenovirus F (NC_001454), human astrovirus (NC_001943), human BK polyomavirus (V01109; GI:60851) human bocavirus (NC_007455), human coronavirus 229E (NC_002645), human coronavirus HKU1 (NC_006577), human coronavirus NL63 (NC_005831), human coronavirus OC43 (NC_005147), human enterovirus A (NC_001612), human enterovirus B (NC_001472), human enterovirus C (NC_001428), human enterovirus D (NC_001430), human erythrovirus V9 (NC_004295), human foamy virus (NC_001736), human herpesvirus 1 (Herpes simplex virus type 1) (NC_001806), human herpesvirus 2 (Herpes simplex virus type 2) (NC_001798), human herpesvirus 3 (Varicella zoster virus) (NC_001348), human herpesvirus 4 type 1 (Epstein-Barr virus type 1) (NC_007605), human herpesvirus 4 type 2 (Epstein-Barr virus type 2) (NC_009334), human herpesvirus 5 strain AD169 (NC_001347), human herpesvirus 5 strain Merlin Strain (NC_006273), human herpesvirus 6A (NC_001664), human herpesvirus 6B (NC_000898), human herpesvirus 7 (NC_001716), human herpesvirus 8 type M (NC_003409), human herpesvirus 8 type P (NC_009333), human immunodeficiency virus 1 (NC_001802), human immunodeficiency virus 2 (NC_001722), human metapneumovirus (NC_004148), human papillomavirus-1 (NC_001356), human papillomavirus-18 (NC_001357), human papillomavirus-2 (NC_001352), human papillomavirus-54 (NC_001676), human papillomavirus-61 (NC_001694), human papillomavirus-cand90 (NC_004104), human papillomavirus RTRX7 (NC_004761), human papillomavirus type 10 (NC_001576), human papillomavirus type 101 (NC_008189), human papillomavirus type 103 (NC_008188), human papillomavirus type 107 (NC_009239), human papillomavirus type 16 (NC_001526), human papillomavirus type 24 (NC_001683), human papillomavirus type 26 (NC_001583), human papillomavirus type 32 (NC_001586), human papillomavirus type 34 (NC_001587), human papillomavirus type 4 (NC_001457), human papillomavirus type 41 (NC_001354), human papillomavirus type 48 (NC_001690), human papillomavirus type 49 (NC_001591), human papillomavirus type 5 (NC_001531), human papillomavirus type 50 (NC_001691), human papillomavirus type 53 (NC_001593), human papillomavirus type 60 (NC_001693), human papillomavirus type 63 (NC_001458), human papillomavirus type 6b (NC_001355), human papillomavirus type 7 (NC_001595), human papillomavirus type 71 (NC_002644), human papillomavirus type 9 (NC_001596), human papillomavirus type 92 (NC_004500), human papillomavirus type 96 (NC_005134), human parainfluenza virus 1 (NC_003461), human parainfluenza virus 2 (NC_003443), human parainfluenza virus 3 (NC_001796), human parechovirus (NC_001897), human parvovirus 4 (NC_007018), human parvovirus B19 (NC_000883), human respiratory syncytial virus (NC_001781), human rhinovirus A (NC_001617), human rhinovirus B (NC_001490), human spumaretrovirus

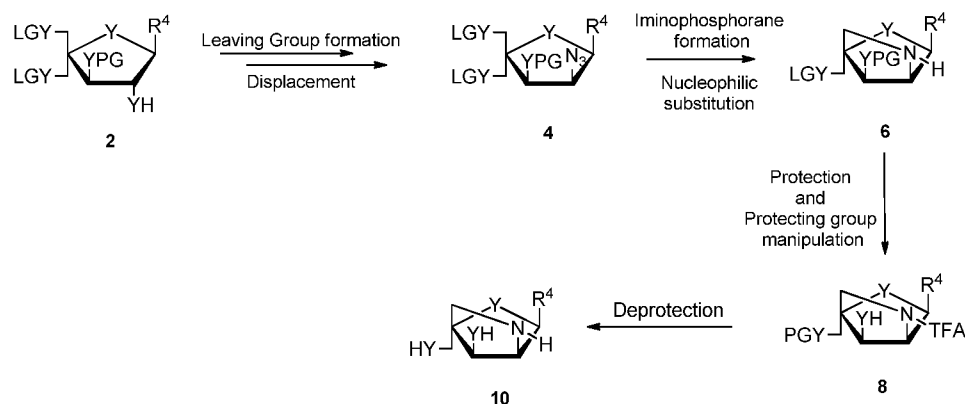
(NC_001795), human T-lymphotropic virus 1 (NC_001436), human T-lymphotropic virus 2 (NC_001488).

In certain examples, the target protein is produced from a nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) from an oncogenic virus, such as Epstein-Barr Virus (EBV) or a Human Papilloma Virus (HPV, *e.g.*, HPV16, HPV18). In other examples, the target protein produced from a nucleic acid sequence (*e.g.*, genomic target nucleic acid sequence) is from a pathogenic virus, such as a Respiratory Syncytial Virus, a Hepatitis Virus (*e.g.*, Hepatitis C Virus), a Coronavirus (*e.g.*, SARS virus), an Adenovirus, a Polyomavirus, a Cytomegalovirus (CMV), or a Herpes Simplex Virus (HSV). Other targets contemplated by the present disclosure include Her1, Her2, Her3 and Her4 (*e.g.* EGFR1, EGFR2, EGFR3 and EGFR4, respectively, or ErbB-1, ErbB-2, ErbB-3 and ErbB-4, respectively).

V. METHOD OF MAKING MONOMERS

Particular disclosed embodiments concern a method of making the disclosed monomers. In certain disclosed embodiments, the method may concern synthesizing locked monomers (*e.g.* monomers having Formulas 1, 2, or 3); and in other disclosed embodiments, the method may concern synthesizing unlocked monomers (*e.g.* monomers having Formulas 1, 2, or 4).

In particular disclosed embodiments, the monomer may be a locked monomer, which may be synthesized from a precursor **2** using the synthetic sequence illustrated in Scheme 1.

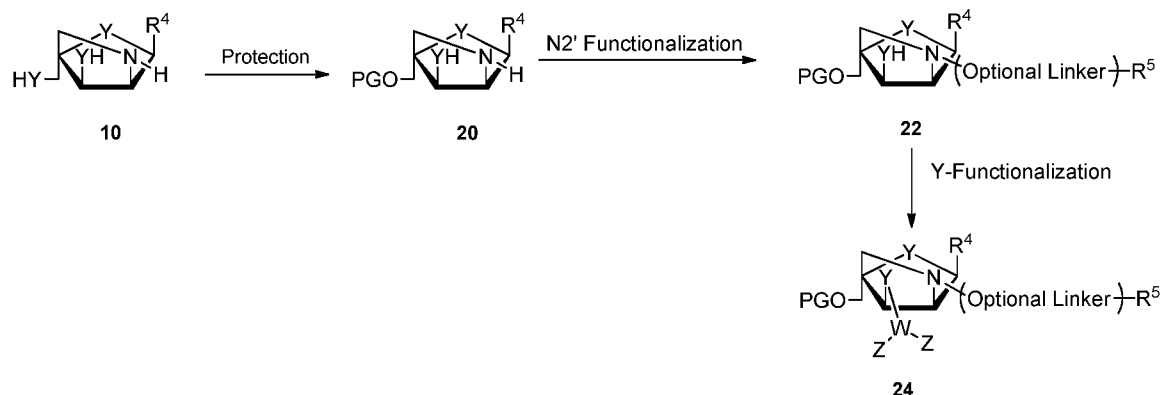


Scheme 1

According to Scheme 1, precursor nucleoside **2** may be converted to an N2'-functionalized locked nucleic acid using a number of chemical transformations. Treating precursor nucleoside **2** may be converted to azide **4** using methods known to those of ordinary skill in the art, such as by converting the YH group of precursor nucleoside **2** to a leaving group, such as a mesylate, triflate, and/or tosylate, and displacing the leaving group using a nucleophilic azide compound, such as sodium azide. Azide **4** can then be converted to locked nucleoside **6** using a tandem Staudinger reaction (iminophosphorane formation)/intramolecular nucleophilic substitution sequence. Protection

and protecting group manipulation of N2' nucleoside **6** followed by deprotection ultimately provides locked nucleoside **10** in a number of steps.

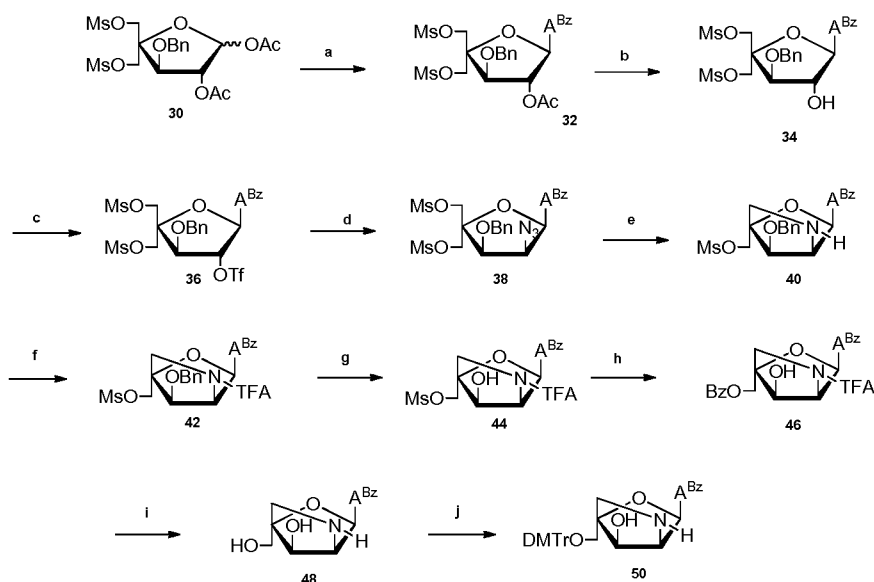
In particular disclosed embodiments, the locked nucleoside **10** may be converted to a monomer suitable for implementation into the disclosed probe. Scheme 2 illustrates the conversion of locked nucleoside **10** to such a monomer.



Scheme 2

With reference to Scheme 2, the locked nucleoside **10** can be converted to protected nucleoside **20**. N2' functionalization of protected nucleoside **20** using a variety of substituents can be carried out using conditions known to a person of ordinary skill in the art as being suitable for coupling an amine with various functional groups. These conditions include, for example, reductive amination with aromatic aldehydes (ArCHO) using triacetoxyborohydride or acylation using aromatic carboxylic acids (ArCOOH) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or 2-(1H-7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) as a coupling reagent. N2' functionalized nucleoside **22** can comprise an optional linker and an R⁵ moiety selected from any of the R⁵ moieties disclosed herein. After functionalization of the YH group of N2' functionalized nucleoside **22** using methods known to those having ordinary skill in the art, such as by base-mediated substitution, a monomer **24** can be made. Monomer **24** is suited for further incorporation into a nucleic acid sequence.

An exemplary method of making the disclosed monomers is illustrated below in Schemes 3 and 4. See, N. K. Andersen, J. Wengel and P. J. Hrdlicka, "N2'-Functionalized 2'-Amino- α -L-LNA Adenine Derivatives – Efficient Targeting of Single Stranded DNA," *Nucleosides Nucleotides Nucleic Acids*, **2007**, 26, 1415-1417, which is incorporated herein by reference.

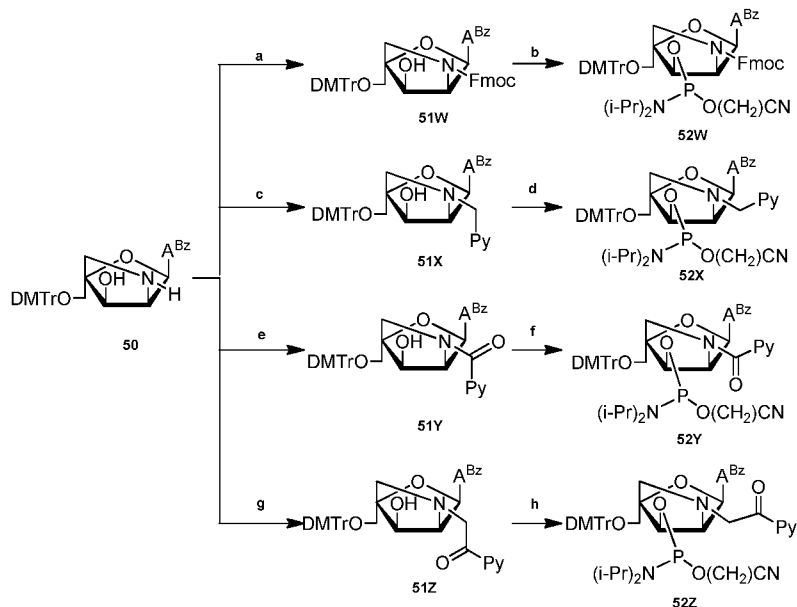


Scheme 3 Reagents and conditions: a) BSA, TMSOTf, A^{Bz}, an. 1,2-DCE, reflux 70h; b) 1) Guanidinium nitrate, NaOMe, MeOH, DCM, 30min, rt. or 2) ½ sat. NH₃/MeOH, 0 °C, 1.5h; c) (CF₃SO₂)₂O pyridine, an. DCM -78 °C, 3h; d) NaN₃, 15-C-5, an. DMF, 40 °C, 16h; e) 2M aq. NaOH, PMe₃, THF, rt. 16h; f) (CF₃CO)₂O, an. pyridine, an. CH₂Cl₂, 0 °C, 2h; g) BCl₃, an. CH₂Cl₂, -78 °C to rt., 17h; h) NaOBz, 15-crown-5, an. DMF, 90 °C, 5h; i) 2M aq. NaOH, 1,4-dioxane/water, 0 °C, 2h; j) DMTrCl, an. pyridine, 0 °C to rt., 23h. Key: A^{Bz} = 6-*N*-benzoyl-adenine. 1,2-DCE = 1,2-dichloroethane

According to Scheme 3, conversion of diacetate **30** to the desired β -nucleoside **32** was carried out using a one-pot reaction, under modified Vorbrüggen conditions, with *in-situ* persilylation of the benzoyl protected adenine nucleobase using N,O-bis(trimethylsilyl)acetamide (BSA) and TMSOTf at reflux in 1,2-dichloroethane. Nucleoside **32** was then subjected to chemoselective O2'-deacetylation using half saturated methanolic ammonia to afford nucleoside **34**. Nucleoside **34** was reacted with trifluoromethanesulfonic anhydride to facilitate formation of the O2'-triflate **36** which was subsequently without intermediate purification treated with sodium azide and 15-crown-5 in anhydrous DMF to afford azide **38**. IR spectroscopy verified the presence of the azide functionality (sharp band at 2115 cm⁻¹) and provided along with NMR and HRMS-MALDI, evidence for the proposed structure of azide **38**. Azide **38** was converted into the desired bicyclic nucleoside **40** in 80% yield using a one-pot tandem Staudinger/intramolecular nucleophilic substitution reaction. Protecting nucleoside **40** with a trifluoroacetyl group using trifluoroacetic anhydride in anhydrous dichloromethane and anhydrous pyridine facilitated formation of nucleoside **42** in 70% yield. Nucleoside **42** was subsequently subjected to benzylic ether cleavage conditions using BCl₃ in anhydrous dichloromethane affording debenzylated nucleoside **44** in yields of 65-87%. Debenzylation was followed by exchanging the methanesulfonyl protecting group at C-5' with a benzoyl protecting group. The reaction was carried out under anhydrous conditions using sodium benzoate and 15-crown-5 in DMF affording nucleoside **46** in isolated yields of 70 – 83%. Subjecting

nucleoside **46** to sodium hydroxide in water and 1,4-dioxane cleaved both the 5'-benzoyl and trifluoroacetic acid protecting groups. Purification of the amino diol afforded target nucleoside **48** in 60-80% isolated yield. The hydroxyl group of nucleoside **48** was subsequently protected at the 5'-position by 4,4'-dimethoxytrityl (DMTr) to afford the DMTr-protected nucleoside **50**.

5 Scheme 4 illustrates an exemplary method of converting a DMTr-protected nucleoside **50** to different exemplary embodiments of the disclosed monomers.



Scheme 4 Reagents and conditions: a) Fmoc-Cl, an. pyridine, rt, 6h, 51%; b) 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite, *N*-methylimidazole, DIPEA, CH₂Cl₂, rt, 4h, 47%; c) 1-pyrenecarboxaldehyde, NaBH(OAc)₃, 1,2-DCE, rt, 17h, 68%; d) 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite, 20% DIPEA in CH₂Cl₂, rt, 21h, 51%; e) 1-pyrenecarboxylic acid, EDC·HCl, CH₂Cl₂, rt, 45h, 64%, or 1-pyrenecarboxylic acid, HATU, DIPEA, DMF, 0°C to rt, 5h, 74%; f) 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite, 20% DIPEA in CH₂Cl₂, rt, 22h, 67%; g) 1-pyreneacetic acid, EDC·HCl, CH₂Cl₂, rt, 3.5h, 79%; h) 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite, DIPEA, CH₂Cl₂, rt, 71%.

According to Scheme 4, monomer **51W** was synthesized from nucleoside **50** using 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) in anhydrous pyridine at 0°C for 6h, and isolated in 51% yield. Nucleoside **51W** was converted into the corresponding amidite **52W** for use during oligonucleotide synthesis using 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite and diisopropylethylamine in anhydrous dichloromethane.

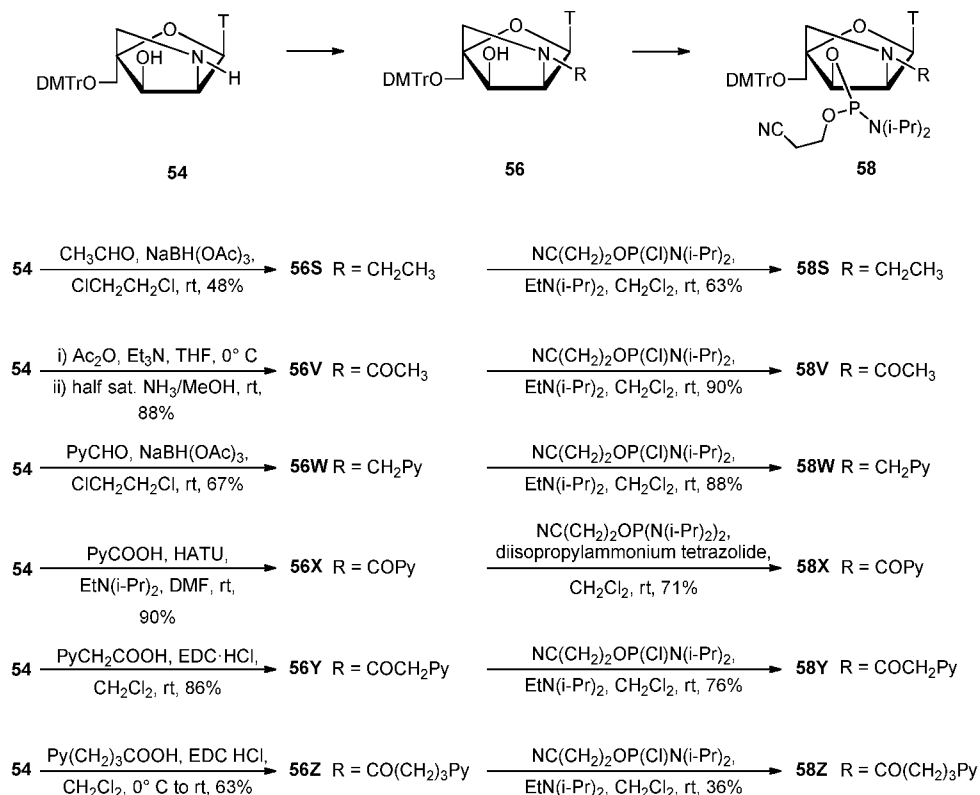
Pyrenylmethyl derivative **51X** was synthesized from nucleoside **50** via reductive amination, using pyrene carboxaldehyde and sodium triacetoxy borohydride in 1,2-dichloroethane at room temperature for 24h, and was isolated in 60% yield. The functionalized nucleoside **51X** was converted into the corresponding amidite **52X** for use during oligonucleotide synthesis, using 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite and 20% diisopropylethylamine in anhydrous dichloromethane.

5 The amide bond of **51Y** was formed via a 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide hydrochloride (EDC·HCl) mediated coupling. The reaction was performed by dissolving bicyclic nucleoside **50** in anhydrous dichloromethane and adding pyrenecarboxylic acid and EDC·HCl. The reaction mixture was stirred at room temperature for 45h. The reaction mixture was subsequently subjected to standard aqueous workup and purification, isolating nucleoside **51Y** in 64% yield. Nucleoside **51Y** was converted into the corresponding phosphoramidite **52Y** using 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite and 20% diisopropylethylamine in anhydrous dichloromethane. Similar

10 In addition, bicyclic nucleoside **50** was converted to monomer **51Z** by adding pyrenecarboxylic acid and EDC·HCl. The reaction mixture was stirred at room temperature for 2.5h and was subjected to standard aqueous workup and purification, isolating nucleoside **51Z** in 79% yield. Nucleoside **51Z** was converted into the corresponding phosphoramidite **52Z** using 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite and diisopropylethylamine in anhydrous dichloromethane.

15 Also disclosed are embodiments wherein the R^4 moiety is selected to be thymine. An exemplary method for synthesizing monomers having R^4 = thymine is illustrated below in Scheme 5. See, T. S. Kumar, A. S. Madsen, M. E. Østergaard, S. P. Sau, J. Wengel and P. J. Hrdlicka*, "Functionalized 2'-Amino- α -L-LNA - Directed Positioning of Intercalators for DNA Targeting", *J. Org. Chem.* **2009**, 74, 1070-1081, which is incorporated herein by reference.

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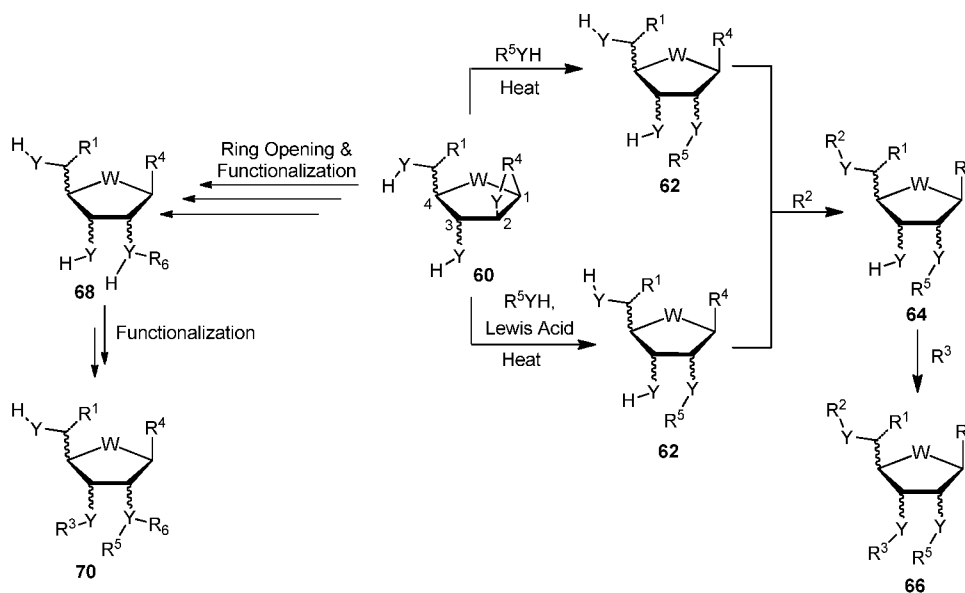


Scheme 5

With reference to Scheme 5, O5'-tritylated bicyclic nucleoside **54**, which may be obtained from commercially available diacetone- α -D-glucose in 5% overall yield over seventeen steps involving eight chromatographic purification steps, was used as a suitable starting material for the synthesis of N2'-functionalized 2'-amino- α -L-LNA phosphoramidites **58Q-58Z**. The disclosed monomers are selected to probe the available structural space in nucleic acids and fall into two groups based on the nature of the N2'-moiety (e.g. monomers with small non-aromatic units [monomers **58Q**, **58S**, and **58V**] or with aromatic units [monomers **58W-58Z**]). Sodium triacetoxymethylborohydride mediated reductive amination of secondary amine **54** with acetaldehyde or 1-pyrenecarbaldehyde provided tertiary amines **56S** and **56W** in 48% and 67% yield, respectively. Chemoselective *N*-acylation of amino alcohol **54** was achieved using two different strategies. Treatment of amino alcohol **54** with slight excess of acetic anhydride followed by selective O3'-deacylation using dilute methanolic ammonia provided nucleoside **56V** in excellent 88% yield over two steps. EDC-mediated coupling of amino alcohol **54** with 1-pyrenylcarboxylic acid, 1-pyrenylacetic acid or 4-(1-pyrenyl)butyric acid afforded nucleosides **56X**, **56Y** and **56Z** in 62%, 86% and 63% yield, respectively. A HATU-mediated coupling procedure were used in order to improve the yield of **56X** to 90%. Disappearance of ^1H NMR signals of the exchangeable 3'-OH protons upon D_2O addition confirmed the N2'-functionalized constitution of nucleosides **56S-56Z**, which

subsequently were converted to the corresponding phosphoramidites **58S-58Z** using 2-cyanoethyl *N,N'*-(diisopropyl)-phosphoramidochloridite and diisopropylethyl amine (Hünig's base). While amidites **58S-58Y** were obtained in good to excellent yields (60-90 %), **58Z** is obtained in 36% yield. The yield of **58X** was improved using bis-(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine in dichloromethane with diisopropylammonium tetrazolide as an activator.

Other disclosed embodiments concern unlocked monomers and a method of making these monomers. In particular disclosed embodiments, the unlocked monomers may be made in approximately three steps from a particular compound. A particular disclosed embodiment of a method of making the unlocked monomers is illustrated in Scheme 6.

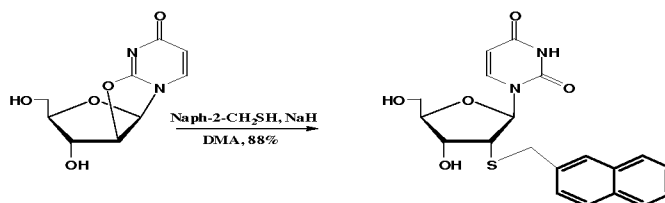


Scheme 6

According to Scheme 6, bicyclic nucleoside **60** may be converted to nucleoside **62** via methods known to those of ordinary skill in the art, such as Lewis acid-mediated ring-opening and/or heat-catalyzed nucleophilic addition. In particular disclosed embodiments, an alcohol, thiol, or amine may be used to open the ring illustrated in Scheme 6. These reagents may be combined with a Lewis acid, such as a borane, and heat to facilitate functionalization and conversion of the bicyclic nucleoside **60** into nucleoside **62**. Subsequently, nucleoside **62** can be converted to protected nucleoside **64**, which may then be further protected to produce monomer **66**. Also according to Scheme 6, nucleoside **62** may be converted to a compound having a di-functionalized $Y2'$ moiety at the C2' position, such as compound **68**, which can be made by methods known in the art, such as via ring-opening and functional group manipulation. In order to make monomer **70**, compound **68** can be exposed to coupling conditions known to those of ordinary skill in the art, such as, but not limited to activated couplings and base mediated couplings. In particular disclosed embodiments, activating agents, such as *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC), 1-ethyl-3-(3-

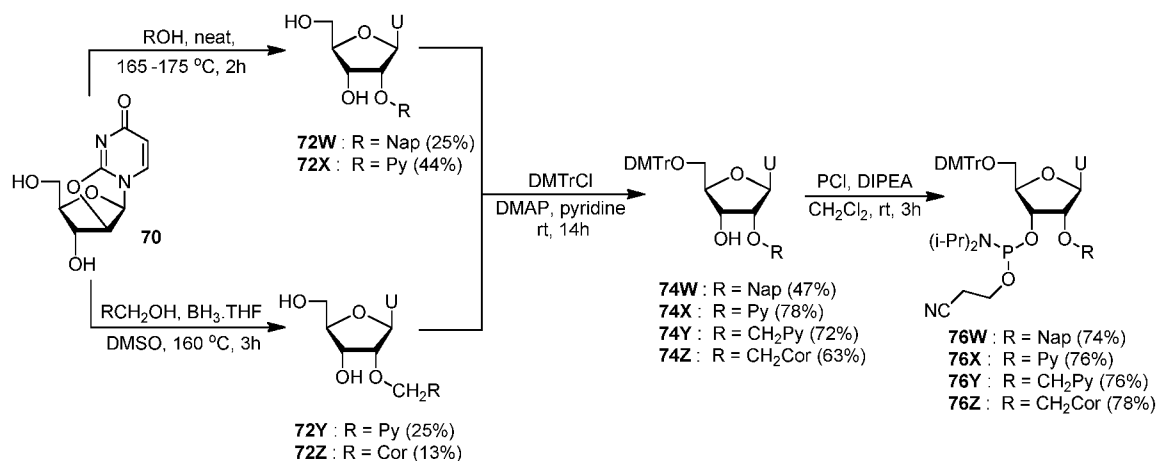
dimethylaminopropyl)carbodiimide hydrochloride (EDCI), dicyclohexylcarbodiimide (DCC), carbonyl diimidazole (CDI), 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-aza-benzotriazole (HOAt), and o-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) may be used in the activated coupling. In certain disclosed embodiments, coupled nucleosides may also be obtained by treating nucleoside **68** with a base, such as an aliphatic amine base (e.g. triethylamine, 1,8-diazabicycloundec-7-ene, 1,4-diazabicyclo[2.2.2]octane, and diisopropylethylamine), and an electrophile, such as a compound comprising an R⁴ moiety and a leaving group, wherein the leaving group may be selected from a halide, a mesylate, a triflate, and a tosylate. Monomer **70** is made via base-mediated functionalization. In certain embodiments, an optional linker is inserted between Y and R⁵ and/or R⁶.

Sulfur analogs can be used in the general approach illustrated in Scheme 6. An example of this approach is illustrated below in Scheme 6A where a sulfur nucleophile is introduced using a nucleophile R⁵YH and a base (e.g., NaH).



Scheme 6A

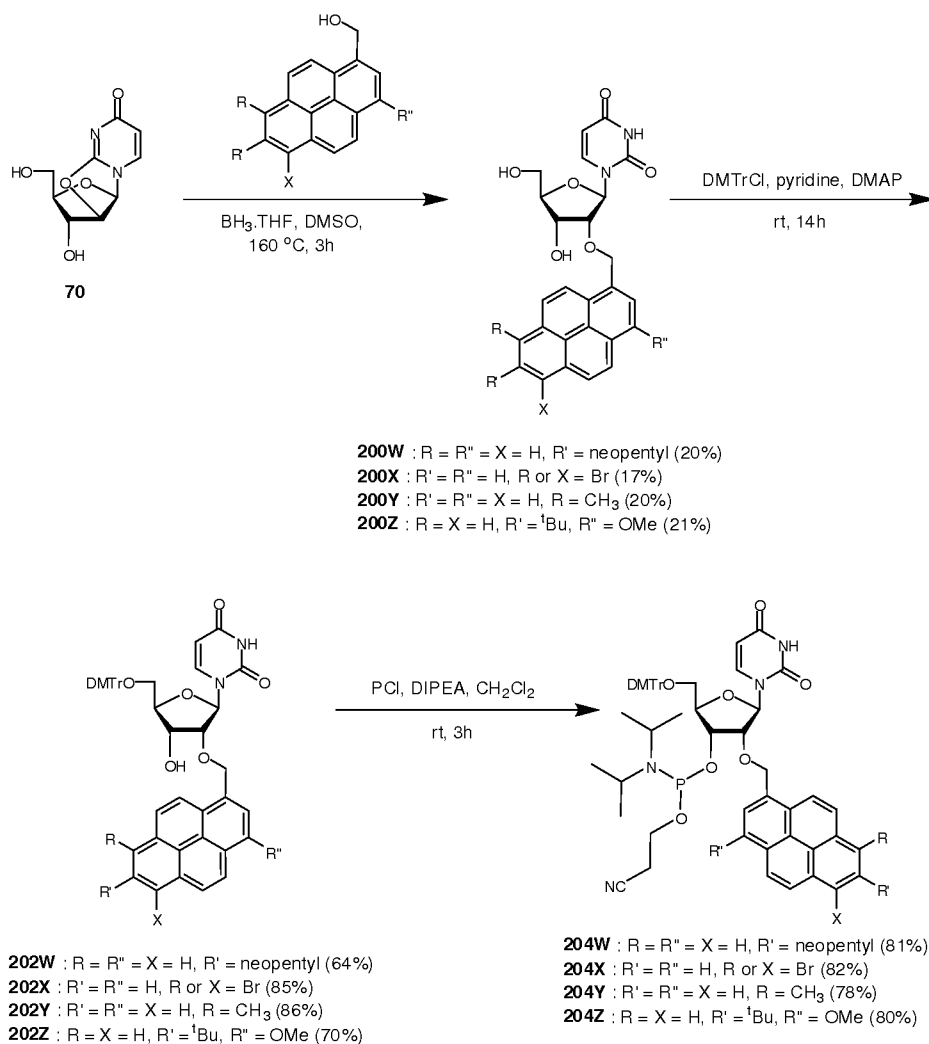
In particular disclosed embodiments, the monomer may be synthesized using the methods illustrated in Scheme 7, below. A person of ordinary skill in the art will recognize that the methods of Scheme 7 are exemplary only and are not intended to be limiting.



Scheme 7A

According to Scheme 7A, 2,2'-anhydrouridine **70** is treated with neat phenols, such as 2-naphthol and 1-pyrenol, to afford O2'-arylated uredines **72W** and **72X** in 25% and 44% yield, respectively. This method was adapted in order to obtain nucleosides **72Y** and **72Z** by treating bicyclic nucleoside **70** with tris(pyrene-1-ylmethyl) or tris(coronene-1-ylmethyl) borate - generated *in situ* upon addition of pyren-1-ylmethanol or coronen-1-ylmethanol to borane. This modification afforded nucleosides **72Y** and **72Z** in reproducible yields. Subsequent O5'-dimethoxytritylation afforded nucleosides **74W-74Z** in 47-78% yield, which upon treatment with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI-reagent) and diisopropylethylamine (Hünig's base) provided target phosphoramidites **76W-76Z** in 74-78% yield (Scheme 7).

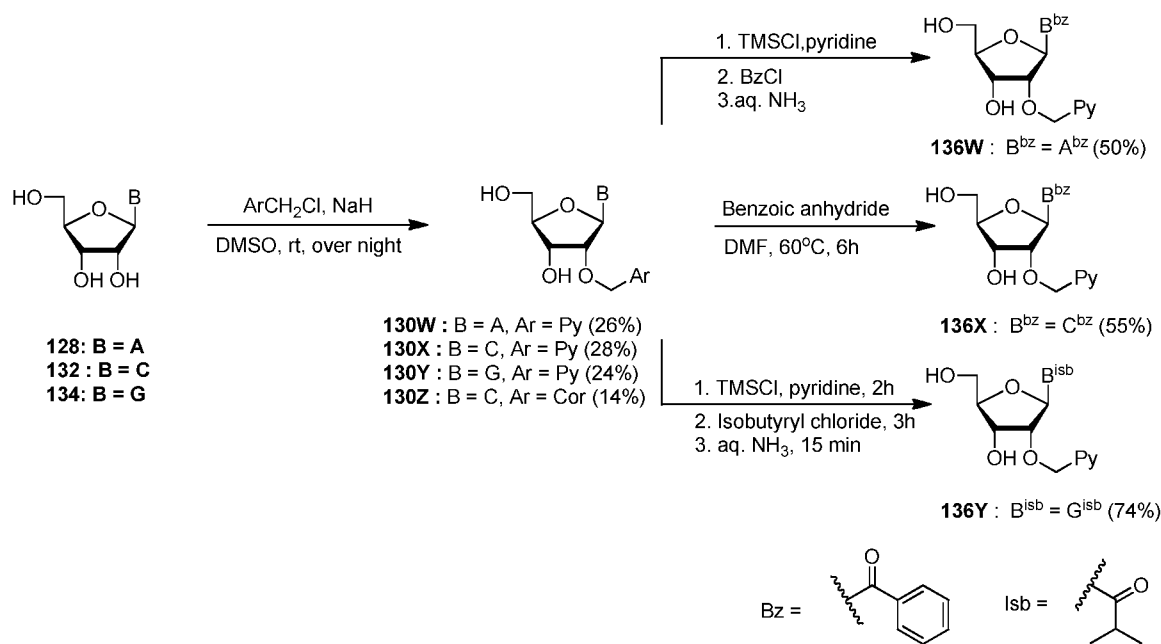
Scheme 7B provides additional examples of monomers obtained via this method.



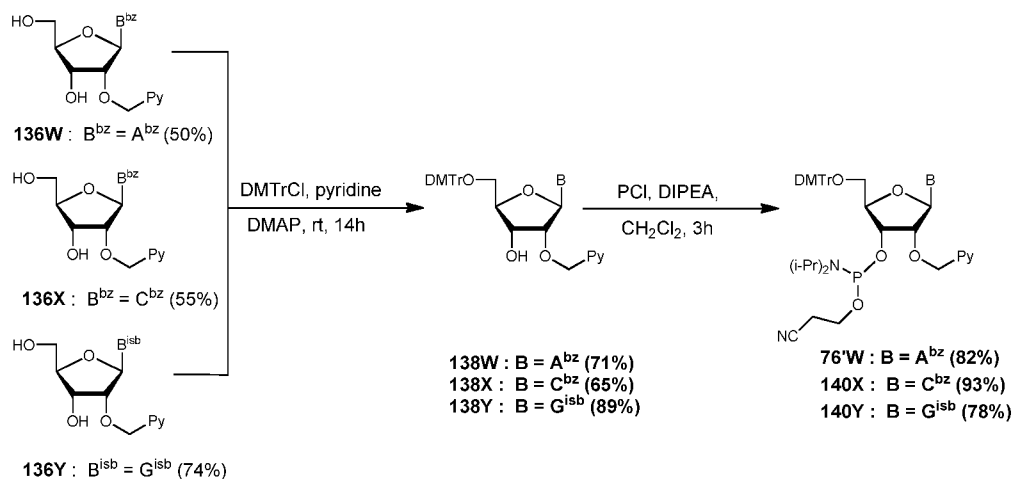
Scheme 7B

Other particular disclosed embodiments of making the disclosed monomers are illustrated in Schemes 8 and 9. With reference to Scheme 8, and concerning the exemplary conversion of **128** to **130W**, certain embodiments used O2' alkylation, typically using a haloalkane and base, such as arylmethylhalide and sodium hydride.

5



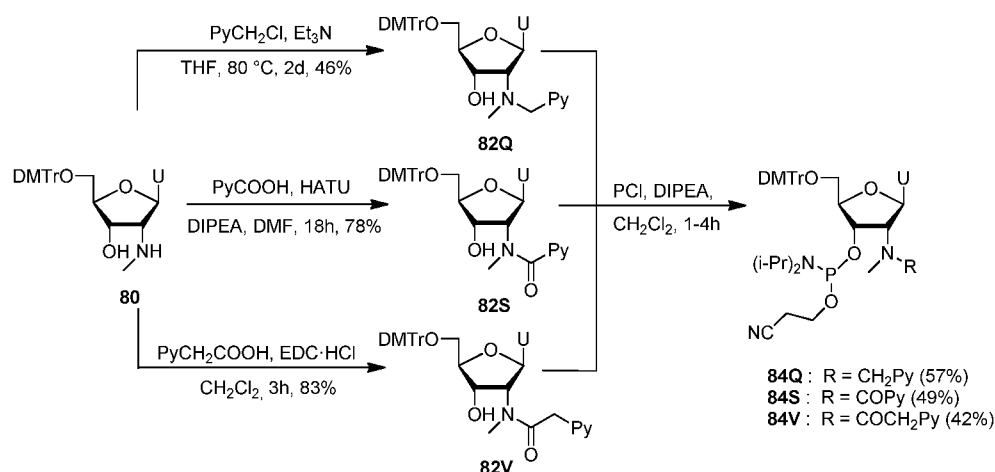
Scheme 8



Scheme 9

Exemplary embodiments of the disclosed monomer may also be obtained using the methods illustrated in Scheme 10. According to Scheme 10, methylamine nucleoside **80** (made from 5'-O-dimethoxytrityl-2,2'-anhydrouridine in ~73% yield over three steps) may undergo direct N2'-

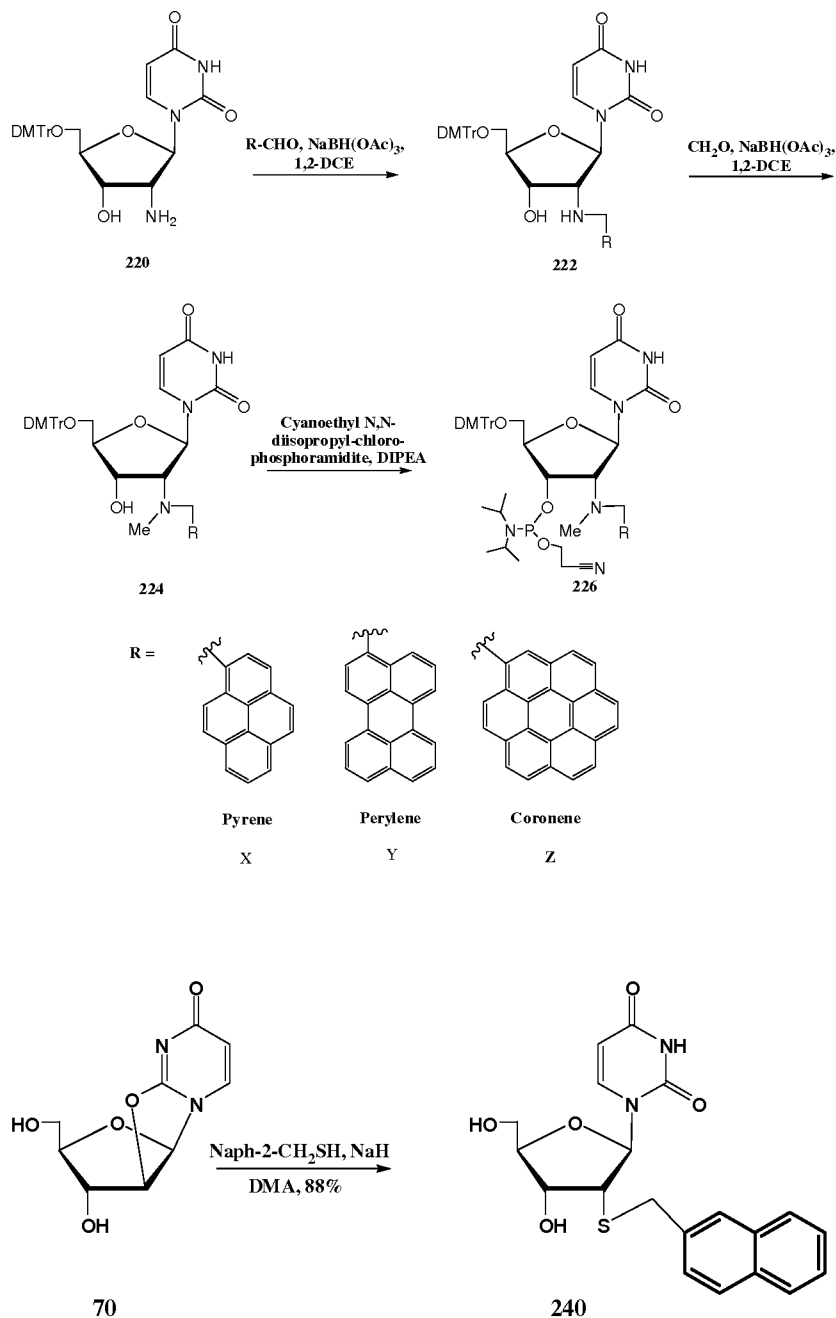
alkylation using pyren-1-ylmethyl chloride afforded the desired product **82Q** in 46% yield. Interestingly, reductive amination of **80** using 1-pyrenecarbaldehyde and sodium triacetoxymethylborohydride or sodium cyanoborohydride failed to afford **82Q** in acceptable yields due to prominent formation of the corresponding cyclic N2',O3'-hemiaminal ether. While formation of this byproduct was avoided by prior protection of the O3'-position of **80** as a TBDMS-ether, the increased steric bulk resulted in low yields during the subsequent reductive amination.



Scheme 10

Still with reference to Scheme 10 HATU-mediated coupling between nucleoside **80** and 1-pyrenecarboxylic acid afforded N2'-acylated nucleoside **82S** in 78% yield, while EDC-mediated coupling between nucleoside **80** and 1-pyreneacetic acid furnished **82V** in 83% yield. Subsequent O3'-phosphitylation of **82Q/82S/82V** using similar conditions as for the synthesis of **76W-76Z** afforded phosphoramidites **84Q/84S/84V** only in moderate yields (42-57%), presumably due to the increased steric bulk at the N2'-position.

With reference to Scheme 11 reductive amination of **220** using an aromatic aldehyde (e.g., 1-pyrenecarbaldehyde, 3-perylenecarbaldehyde or 1-coronencarbaldehyde) affords **222** in 43-95% yield. Subsequent N-methylation of **222** via reductive amination affords **224** in 89-99% yield (note **224X** = **82Q** & **226X** = **84Q**). Subsequent O3'-phosphitylation (e.g., using cyanoethyl N,N-diisopropyl-chloro-phosphoramidite, i.e., PCI-reagent) provides **226** in 62-90% yield.

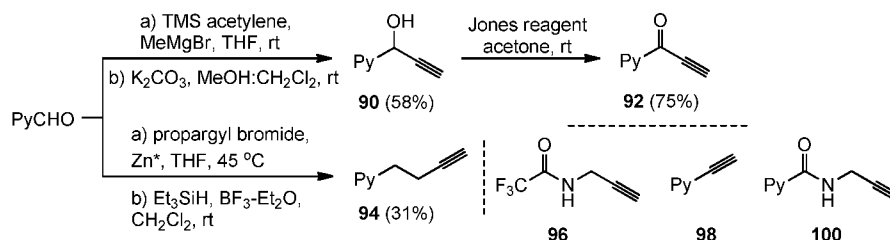


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Scheme 11

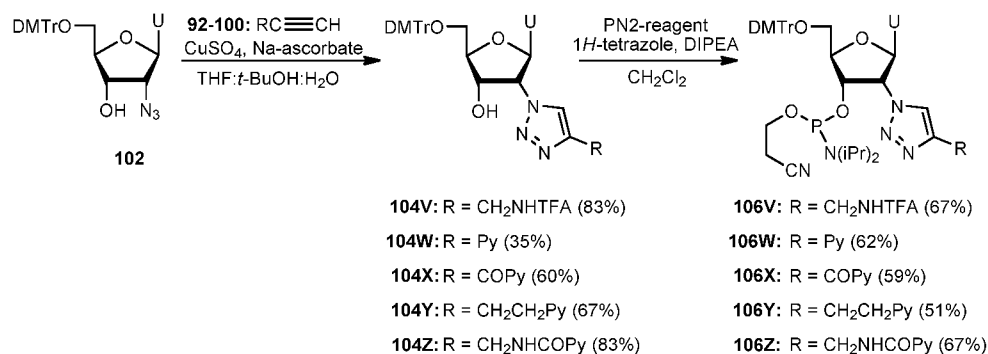
Other disclosed embodiments concern monomers comprising a triazole moiety, wherein the triazole moiety allows the nucleoside to be coupled with a variety of R^5 moieties. Schemes 12 and 13 illustrate an exemplary embodiment of making a monomer comprising a triazole moiety. Scheme 12 illustrates an exemplary method for making the necessary coupling reagents used in producing the triazole moieties, and Scheme 13 illustrates how these coupling reagents are ultimately used in triazole synthesis.

10



Scheme 12

With reference to Scheme 12, 2,2,2-trifluoro-*N*-(prop-2-ynyl)acetamide **96**, 1-ethynylpyrene **98** and *N*-(prop-2-ynyl)pyrene-1-carboxamide **100** were prepared according to methods readily known in the art. In contrast, 1-(pyren-1-yl)-prop-2-yn-1-one **92** and 4-(pyren-1-yl)-but-1-yne **94** were obtained via substantially different methods. According to Scheme 12, nucleophilic addition of MgC≡CTMS (generated *in situ* from trimethylsilylacetylene and MeMgBr in THF) to pyrene-1-carboxaldehyde followed by desilylation using potassium carbonate provided **90** in 58% yield. Subsequent Jones oxidation afforded **92** in 75% yield. Similarly, nucleophilic addition of HC≡CCH₂ZnBr (generated *in situ* from propargyl bromide and activated zinc in THF) to pyrene-1-carboxaldehyde, followed by deoxygenation of the resultant homopropargyl alcohol using trifluoroboron etherate and triethylsilane, afforded **94** in 31% yield.

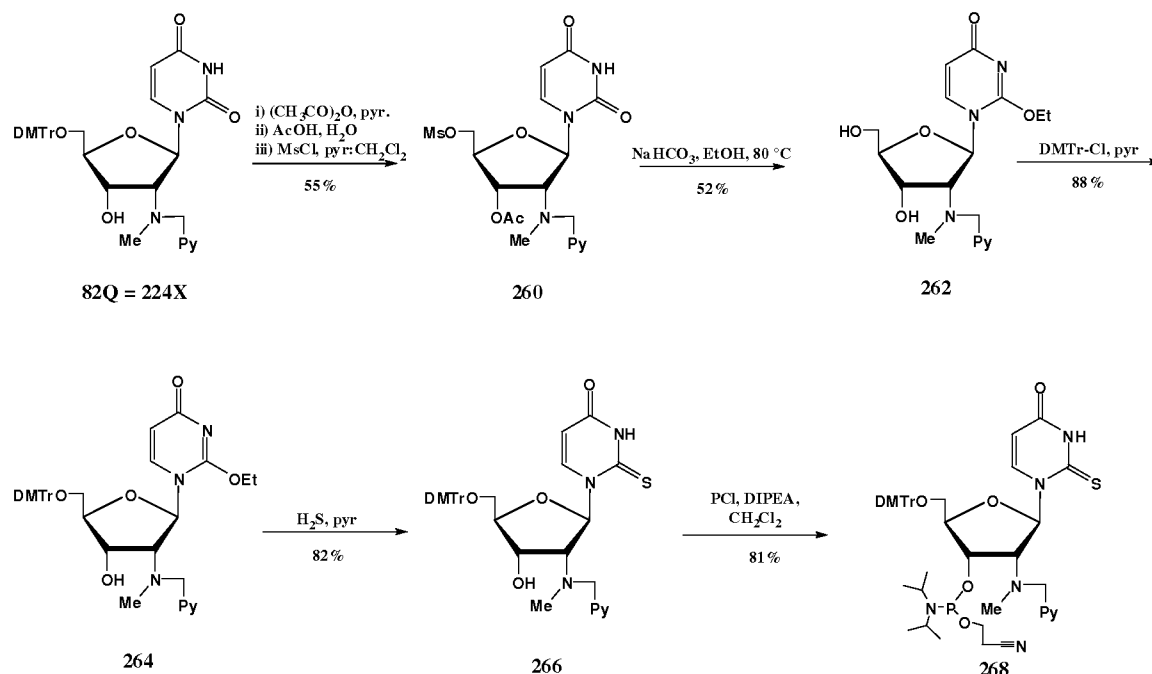


Scheme 13

With reference to Scheme 13, room temperature reactions between **102** and terminal alkynes **92-100** provided the corresponding triazoles **104V-104Z** in robust yields (60-83%), except for the reaction involving 1-ethynylpyrene **98**, which required heating (75 °C) to afford nucleoside **104W** in 35% yield (Scheme 2). Nucleosides **104V-104Z** were subsequently converted into phosphoramidites **106V-106Z** (51-67% yield) using 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (PN2-reagent) and 1*H*-tetrazole as an activator.

Other disclosed embodiments concern monomers comprising pseudocomplementary nucleobases (in particular those involving C2-thiouracil as the nucleobase moiety). With reference to Scheme 14, protecting group manipulations on nucleoside **82Q** (= **224X**) converted it to nucleoside **260** in 55% yield. Base-mediated anhydronucleoside formation and nucleophilic cleavage hereof, provided **262** in 52% yield. O5'-protection hereof provided **264**, which upon treatment with a sulphur

source produced 2-thiouracil derivative **266**. Subsequent O3'-phosphitylation using standard conditions, afforded amidite **268** as a suitable building block for oligonucleotide synthesis.



Scheme 14

VI. METHOD OF MAKING DISCLOSED PROBE EMBODIMENTS

Disclosed embodiments concern a method of making a probe capable of recognizing a target, particularly a double stranded DNA target. In particular disclosed embodiments, the probe is a nucleic acid duplex that comprises at least one pair of monomers that are capable of reducing the thermal stability of the duplex and thus promote dissociation of two strands of the duplex.

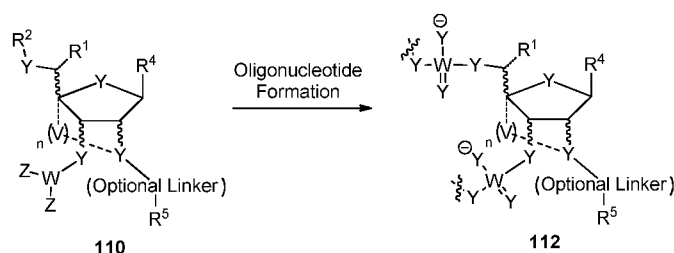
In certain disclosed embodiments, the probe may be able to identify an isosequential nucleic acid sequence. For example, the probe may be made by first identifying a desired target, such as a particular nucleic acid sequence, and then constructing the probe to be a complement to such sequence by developing a probe having nucleotides capable of Watson-Crick base pairing with the target. The probe may be modified with at least one pair of monomers, whereas the isosequential target lacks such a modification.

In particular disclosed embodiments, the probe is constructed by converting one or more of the monomers disclosed herein to an oligonucleotide, wherein the monomer is coupled with one or more natural or non-natural nucleobases to form a modified oligonucleotide. The probe may be designed as a double stranded DNA probe (e.g. monomers and natural and/or non-natural nucleobases bound together via phosphate moieties), a double stranded phosphorothioate-DNA probe (e.g. monomers and natural and/or non-natural nucleobases bound together via one or more

phosphorothioate moieties), a triazole-linked DNA or RNA probe, an unmodified RNA probe, modified RNA probe and/or other non-natural DNA/RNA strands now known or hereafter discovered or made.

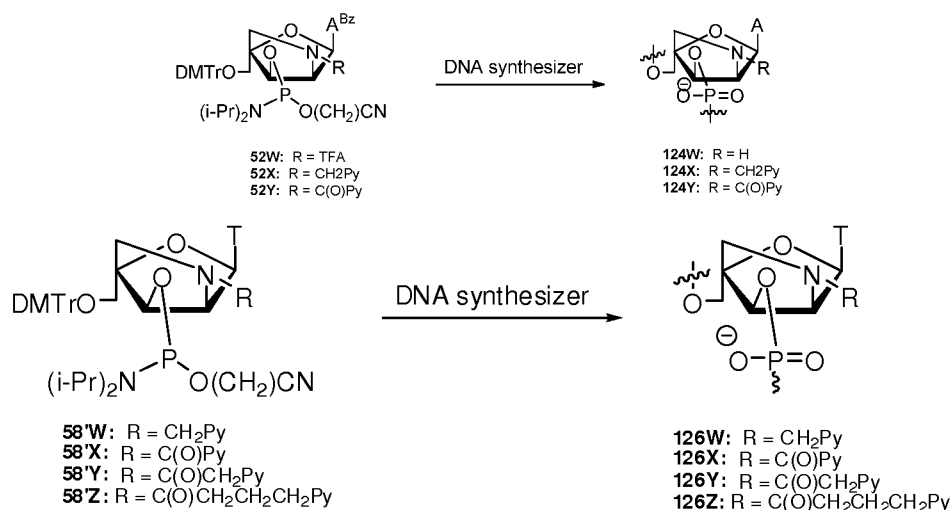
Scheme 14 illustrates a particular disclosed embodiment of a method for making the probe.

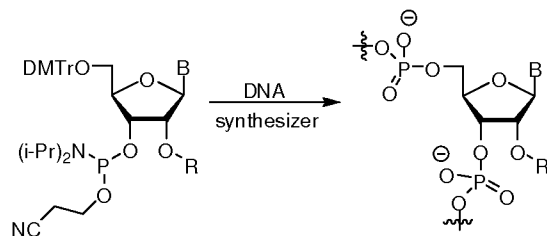
- 5 According to Scheme 14, a monomer may be incorporated into an oligonucleotide using methods known to a person of ordinary skill in the art, such as by using a nucleic acid synthesizer. With reference to Scheme 15, monomer **110** may be converted into oligonucleotide **112**, wherein the wavy lines indicate the position at which one or more natural or non-natural nucleotides and/or additional identical or different monomers may be coupled. The transformation illustrated in Scheme 14 can be
- 10 obtained by any methods known to those of ordinary skill in the art, such as by using an activator, such as an imidazole, triazole, or tetrazole compound, and an oxidant, such as iodine or a peroxide compound. Particular embodiments utilize dicyanoimidazole as the activator. Examples of peroxide compounds include, but are not limited to, hydrogen peroxide or tert-butyl hydrogen peroxide.



Scheme 15

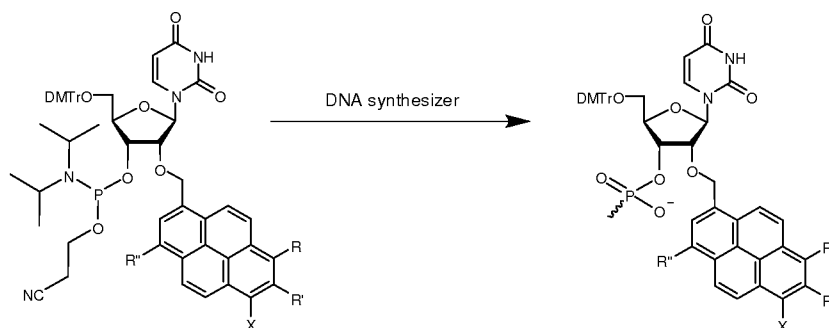
Particular disclosed embodiments may concern making the disclosed probe according to the method illustrated in Scheme 16.





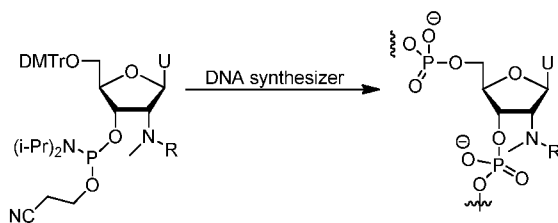
76W : R = Nap, B = uracil
76X : R = Py, B = uracil
76Y : R = CH₂Py, B = uracil
76Z : R = CH₂Cor, B = uracil
76'W : R = CH₂Py, B = adenine-Bz
76'X : R = CH₂Py, B = thymine
140X : R = CH₂Py, B = cytosine-Bz
140Y : R = CH₂Py, B = guanine-iBu

120W : R = Nap, B = uracil
120X : R = Py, B = uracil
120Y : R = CH₂Py, B = uracil
120Z : R = CH₂Cor, B = uracil
120'W : R = CH₂Py, B = adenine
120'X : R = CH₂Py, B = thymine
140'X : R = CH₂Py, B = cytosine
140'Y : R = CH₂Py, B = guanine



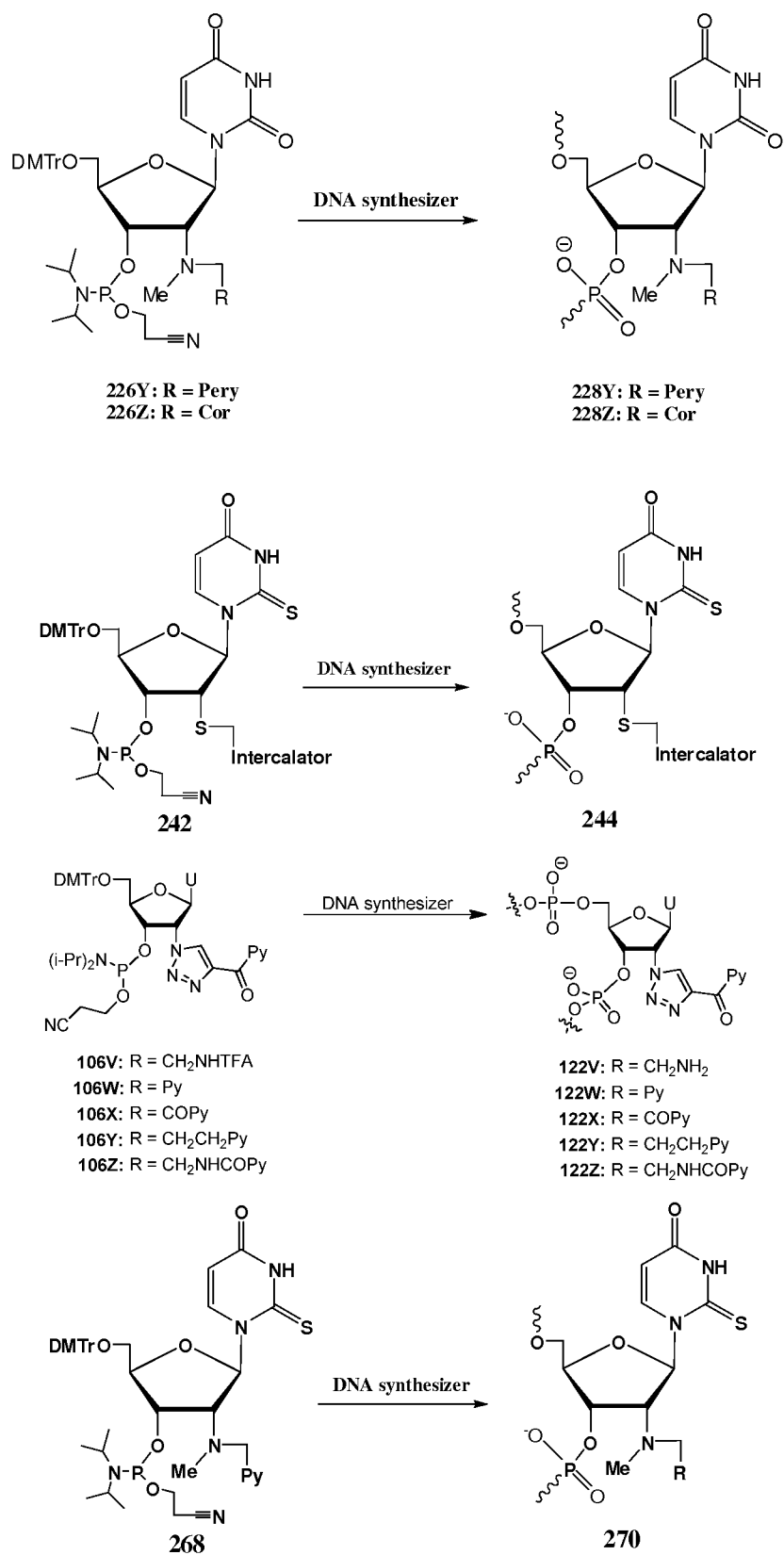
204W : R = R' = X = H, R' = neopentyl
204X : R' = R'' = H, R or X = Br
204Y : R' = R'' = X = H, R = CH₃
204Z : R = X = H, R' = ^tBu, R'' = OMe

Monomer **208W** : R = R' = X = H, R' = neopentyl
 Monomer **208X** : R' = R'' = H, R or X = Br
 Monomer **208Y** : R' = R'' = X = H, R = CH₃
 Monomer **208Z** : R = X = H, R' = ^tBu, R'' = OMe



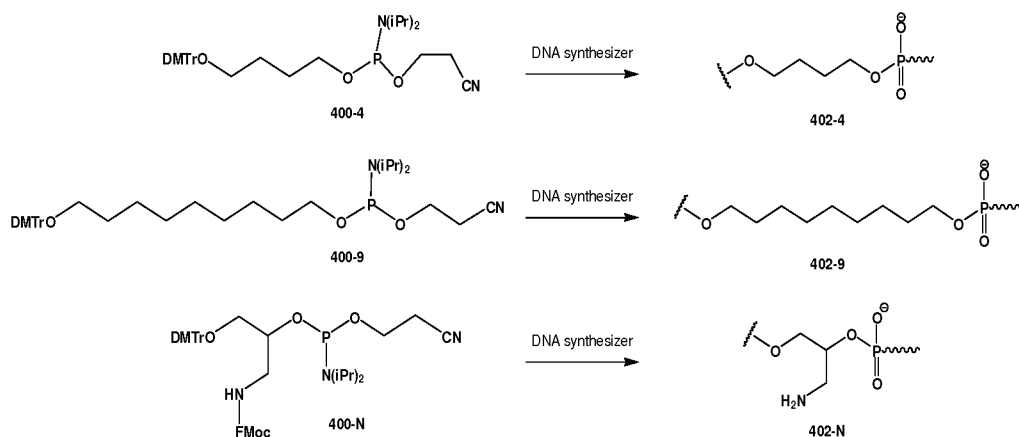
84Q : R = CH₂Py
84S : R = COPy
84V : R = COCH₂Py

120Q : R = CH₂Py
120S : R = COPy
120V : R = COCH₂Py



According to Scheme 16, intercalator-functionalized phosphoramidites **120W-120Z**, **120Q-120V**, **122W-122Z**, and **124W-124Y** (a person of ordinary skill in the art will realize that this can apply to all disclosed monomer embodiments) were incorporated into oligonucleotides via machine-assisted solid-phase DNA synthesis using an activator, such as an activator selected from 4,5-dicyanoimidazole and 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole, for time periods ranging from about 1 minute to about 40 minutes; more typically from about 10 minutes to about 35 minutes.

Particular embodiments concern probes simultaneously comprising one or more of the disclosed monomers and one or more non-pairing or bulge monomers. According to Scheme 17 below, non-pairing or bulge monomers **402-4**, **402-9** and **402-N** were incorporated into oligonucleotides via machine-assisted solid-phase DNA synthesis as recommended by commercial vendors and/or in an equivalent manner as described for the disclosed intercalator-functionalized monomers.



Scheme 17

In order to determine the efficiency of probes disclosed herein, the thermal affinity of the probe toward complementary DNA or RNA targets can be evaluated via UV thermal denaturation experiments using medium salt buffers that mimic physiological ionic strengths ($[Na^+] = 110$ mM, Tables 8 and 9). In particular disclosed embodiments, denaturation curves may display sigmoidal monophasic transitions, such as those exemplary embodiments illustrated in FIGS. 4-5. Changes in thermal denaturation temperatures (T_m -values) of modified duplexes are discussed relative to T_m -values of unmodified reference duplexes, unless otherwise mentioned.

Certain embodiments entail single-stranded probes comprising the disclosed monomers. It currently is believed that certain disclosed monomers result in significantly increased thermal affinity toward single-stranded nucleic acid targets, more commonly single-stranded DNA. For example, 9-mer oligonucleotides (ONs), which are modified with locked monomers **126W-Z**, display

extraordinary thermal affinity toward complementary DNA relative to unmodified ONs (ΔT_m up to +19.5 °C, Table 8).

Without being limited to one theory of operation, incorporation of monomers with short linkers appears to result in greater duplex stabilization than monomers with long linkers

- 5 (126X>126Y>>126Z); still without being limited to one operation of theory, monomers with acyl linkers seem preferred over those with alkyl linkers (126X>126W). Significantly similar trends seem to be observed for ONs modified with N2'-pyrene-functionalized 2'-amino- α -L-LNA adenine monomers 124X and 124Y (Table 9).

- 10 Control ONs that are singly modified with 2'-oxy- α -L-LNA thymine monomer O (i.e., without an intercalator) only display moderately increased thermal affinity toward DNA complements (see Table 8). ONs modified with unfunctionalized 2'-amino- α -L-LNA thymine monomer N (i.e., without an intercalator) also display low thermal affinity toward DNA (see Table 8).. This suggests that the intercalators of the disclosed monomers have stabilizing roles.

- 15 Without being limited to a single theory, it is currently believed that the observed trends in DNA duplex thermostabilization of probes comprising monomers 120Q-120V indicate that monomers where the intercalator moiety is attached via N2'-alkyl linkers are preferred over those with N2'-alkanoyl linkers (compare ΔT_m /mod for 120S1-120S5 = -6.0 to +4.0 °C with data for 120Q1-120Q5, Table 8) and that extending the N2'-alkanoyl linker between the furanose and intercalator moiety as in oligonucleotides modified with 2'-N-(pyren-1-ylmethycarbonyl)-2'-aminouridine monomer 120V, partially reverses the detrimental effects of N2'-acylation on DNA duplex thermostability (monomer 120S $\rightarrow$ monomer 120V, ΔT_m /mod = -0.5 to +6.5 °C, Table 8). Also, without being limited to a single theory of operation, it is currently believed that the rigid 2'-N-alkanoyl linker positions the intercalator in an unsuitable position for affinity-enhancing intercalation and/or that increased solvation of the linker stabilizes the single-stranded state rendering
- 25 hybridization less energetically favorable.

- Without being limited to a single theory of operation, it is currently believed that the observed trends in DNA affinity of ONs modified with unlocked monomers, such as monomer 120Q or 120Y are substantially similar to those obtained with locked monomers, such as 126W-126Z (Table 8). Significantly similar trend is observed for probes modified with monomers 120'W and
- 30 124X bearing adenines as nucleobases (Table 9).

Table 8

ΔT_m values of duplexes between probes comprised of certain disclosed monomers and complementary DNA.^a

		$\Delta T_m/^{\circ}\text{C}$										
ON	Duplex	<u>B</u> =	O	N	126W	126X	126Y	126Z	120Q	120S	120V	120Y
B1	5'-G <u>B</u> G ATA TGC		+2.5	-2.0	+7.0	+10.0	+10.5	+0.5	+5.0	-6.0	-0.5	+5.0

D2 3'-CAC TAT ACG										
B2 5'-GTG A TA TGC	+6.0	+0.5	+14.0	+19.0	+15.5	+6.0	+14.0	+3.0	+6.0	+12.5
D2 3'-CAC TAT ACG										
B3 5'-GTG ATA B GC	+3.0	-1.0	+10.5	+14.0	+11.5	-	-	-	-	+8.0
D2 3'-CAC TAT ACG										
D1 5'-GTG ATA TGC	+3.5	-0.5	+6.5	+10.5	+10.0	+0.5	+1.5	-6.0	+1.0	+3.5
B4 3'-CAC B AT ACG										
D1 5'-GTG ATA TGC	+8.0	+2.5	+15.5	+19.5	+16.5	+6.5	+13.0	+4.0	+6.5	+11.5
B5 3'-CAC TAB A CG										

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); T_m values determined as the first derivative maximum of denaturation curves (A_{260} vs T) recorded in medium salt buffer ([Na⁺] = 110 mM, [Cl⁻] = 100 mM, pH 7.0 (NaH₂PO₄/Na₂HPO₄)), using 1.0 μ M of each strand. T_m values are averages of at least two measurements within 1.0 °C; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer. “-” = not determined.

Table 9
 T_m Values of Duplexes Between Probes Comprised of Certain Disclosed Monomers and Complementary DNA^a

ON	Duplex	$\Delta T_m/^\circ\text{C}$			
		<u>B</u> =	124X	124Y	120'W
B6	5'-GTG <u>B</u> TA TGC				
D2	3'-CAC TAT ACG	+5.0	+11.0	+4.5	
B7	5'-GTG AT <u>B</u> TGC				
D2	3'-CAC TAT ACG	+7.0	+14.0	+8.5	
D1	5'-GTG ATA TGC				
B8	3'-CAC T <u>B</u> T ACG	+6.5	+11.5	+8.5	
D1	5'-GTG ATA TGC				
B9	3'-CAC TAT <u>B</u> CG	+5.5	+12.0	+6.5	

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 for experimental conditions.

In particular disclosed embodiments, the results of thermal DNA affinity of other monomers may vary from $\Delta T_m/\text{mod} = -13.0$ °C to $+20.0$ °C (Table 10). Also suggested by these results is the theory that increasing the intercalator surface area leads to additional increases in DNA duplex thermostability (compare data for probes comprising monomers **120Y** and **120Z**, Tables 8 and 10) and that shortening the linker between the furanose and intercalator moiety results in markedly lower DNA-affinity (compare data for probes comprising monomers **120Y** and **120X**, Tables 8 and 10). Furthermore, it is currently believed that concomitant reduction in aromatic surface area results in

additionally decreased DNA duplex thermostability (compare data for probes comprising monomers **120X** and **120W**, Table 10).

Table 10
 T_m Values of Duplexes Between Probes Comprised of Certain Disclosed Monomers and Complementary DNA^a

		T_m [ΔT_m /mod] (°C)				
ON	Duplex	<u>B</u> =	T	120W	120X	120Z
B1	5'-G <u>B</u> B ATA TGC	29.5	21.5	26.5	34.0	
D2	3'-CAC TAT ACG		[-8.0]	[-3.0]	[+4.5]	
B2	5'-GTG A <u>B</u> A TGC	29.5	24.5	33.5	49.5	
D2	3'-CAC TAT ACG		[-5.0]	[+4.0]	[+20.0]	
B3	5'-GTG ATA <u>B</u> GC	29.5	24.5	26.0	40.5	
D2	3'-CAC TAT ACG		[-5.0]	[-3.5]	[+11.0]	
D1	5'-GTG ATA TGC	29.5	16.5	26.0	36.0	
B4	3'-CAC <u>B</u> AT ACG		[-13.0]	[-3.5]	[+6.5]	
D1	5'-GTG ATA TGC	29.5	24.5	30.5	45.5	
B5	3'-CAC TA <u>B</u> ACG		[-5.0]	[+1.0]	[+16.0]	
D1	5'-GTG ATA TGC	29.5	ND	25.5	47.0	
B6	3'-CAC <u>B</u> A <u>B</u> ACG			[-2.0]	[+8.8]	
B7	5'-G <u>B</u> B A <u>B</u> A <u>B</u> GC	29.5	ND	25.5	ND	
D2	3'-CAC TAT ACG			[-1.3]		

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 above for experimental conditions.

- 10 Certain embodiments entail double-stranded probes with interstrand zipper arrangements of disclosed monomers such as those shown in Table 11, which result in destabilization of the probe. The impact on duplex thermostability upon hybridization of two single-stranded probes that are each modified with one or more of the disclosed monomers can be additive, more-than-additive or less-than-additive relative to the correspondingly singly modified duplexes. This can be readily assessed by the term
- 15 'deviation from additivity' (*DA*) for a probe ONX:ONY, defined as:

$$DA_{\text{ONX:ONY}} \equiv \Delta T_m (\text{ONX:ONY}) - [\Delta T_m (\text{ONX:DNA X}) + \Delta T_m (\text{DNA Y:ONY})]$$

- where **ONX:ONY** is a duplex with an interstrand monomer arrangement and 'DNA X' and 'DNA Y' are the complementary DNA of **ONX** and **ONY**, respectively. It follows that $DA \sim 0$ °C for additive impacts, $DA \gg 0$ °C for more-than-additive impacts, and $DA \ll 0$ °C for less-than-additive impacts
- 20 (see also definitions). The term thermal advantage, *TA*, is strongly related to *DA*, i.e., $TA = -DA$ (see also definitions). Double-stranded probes may display a largely positive *TA* (or largely negative *DA*). This energy difference between probe-target duplexes on one side and double-stranded nucleic acid targets (more commonly dsDNA) and probes on the other side, may provide the driving force for recognition of double-stranded target regions (more commonly dsDNA target regions), via the
- 25 method shown in FIG. 1.

Particular embodiments entail double-stranded probes with +1 interstrand zipper arrangements (see also definitions) of disclosed monomers as shown in Tables 11 and 12, which display significant dsDNA-targeting potential as evidenced by the highly negative DA-values (DA between -40 °C and -12 °C). Other embodiments entail double-stranded probes with +2 zipper arrangements of intercalator-modified monomers, which also display negative DA-values, although the values are generally less prominent than probes with +1 zipper monomer arrangements. Probes with other interstrand zipper arrangements (e.g., +4, -1 and -3) display less regular and/or prominent dsDNA-targeting potential as indicated by DA-values ranging from moderately negative to moderately positive (DA between -8.5 °C and +4 °C). Without being limited to a single theory of operation, probes with +1 interstrand arrangements of disclosed monomers display significant potential for targeting of isosequential dsDNA regions, and enable targeting of isosequential double-stranded nucleic acid regions, more commonly double-stranded DNA, via the method outlined in FIG. 1.

Control duplexes with two conventional 2'-oxy- α -L-LNA thymine monomer **O** or 2'-amino- α -L-LNA thymine monomer **N** in +1 arrangements, display DA values ~0 °C. This implies that the dsDNA-targeting potential is generated by the active involvement of the intercalators moieties.

Table 11
 ΔT_m And DA Values for Double-Stranded Probes with Certain Interstrand Zipper Arrangements of Disclosed Monomers^a

			$\Delta T_m [DA]/^{\circ}\text{C}$										
ON	Zipper	Duplex	<u>B</u> =	O	N	126W	126X	126Y	126Z	120Q	120S	120V	120Y
B1	+4	5'-G <u>B</u> G ATA TGC	+11.0	+1.0	+25.0	+28.5	+26.0	+8.5	+19.5	-3.5	+9.0	+16.5	
B5		3'-CAC TAB <u>B</u> ACG	[+0.5]	[+0.5]	[+2.5]	[-1.0]	[-1.0]	[+1.5]	[+1.5]	[-5.5]	[+3.0]	[0.0]	
B1	+2	5'-G <u>B</u> G ATA TGC	+8.0	-1.5	0.0	+6.5	+12.5	-1.0	-1.5	-17.5	-2.0	-6.0	
B4		3'-CAC <u>B</u> AT ACG	[+2.0]	[+1.0]	[-13.5]	[-14.0]	[-8.0]	[-2.0]	[-8.0]	[-5.5]	[-2.5]	[-14.5]	
B2	+1	5'-GTG A <u>B</u> A TGC	+16.0	-5.5	+2.5	-1.5	+1.0	-5.5	-2.0	-10.0	+0.5	-2.0	
B5		3'-CAC TAB <u>B</u> ACG	[+2.0]	[-8.5]	[-27.0]	[-40.0]	[-31.0]	[-18.0]	[-29.0]	[-17.0]	[-12.0]	[-26.0]	
B2	-1	5'-GTG A <u>B</u> A TGC	+7.0	-5.5	+15.5	+26.0	+26.5	+9.5	+13.0	-8.5	+5.5	+10.5	
B4		3'-CAC <u>B</u> AT ACG	[+2.5]	[-5.5]	[-5.0]	[-3.5]	[+1.0]	[+3.0]	[-2.5]	[-5.5]	[-2.5]	[-5.5]	
B3	-1	5'-GTG ATA <u>B</u> GC	+8.5	-2.5	+18.0	+25.0	+29.5						
B5		3'-CAC TAB <u>B</u> ACG	[-2.5]	[-4.0]	[-8.0]	[-8.5]	[+1.5]						
B3	-3	5'-GTG ATA <u>B</u> GC	+8.0	-2.5	+18.0	+28.5	+22.0						
B4		3'-CAC <u>B</u> AT ACG	[+1.5]	[-1.0]	[+1.0]	[+4.0]	[+0.5]						

^a ΔT_m = change in T_m values relative to unmodified reference DNA duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 for experimental conditions.

Table 12
 ΔT_m And DA Values for Double-Stranded Probes with Certain
 Interstrand Zipper Arrangements of Disclosed Monomers.^a

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ON	Zipper	Duplex	$\Delta T_m [DA]/^{\circ}\text{C}$		
			<u>B</u> =	124X	124Y 120'W
B6	+3	5'-GTG <u>B</u> TA TGC	+12.0	+13.0	+10.0
B9		3'-CAC TAT <u>B</u> CG	[+1.5]	[-10.0]	[-1.0]
B6	+1	5'-GTG <u>B</u> TA TGC	-7.0	-8.0	-7.5
B8		3'-CAC T <u>B</u> T ACG	[-18.5]	[-30.5]	[-20.5]
B7	+1	5'-GTG AT <u>B</u> TGC	-5.0	-7.0	-7.0
B9		3'-CAC TAT <u>B</u> CG	[-17.5]	[-33.0]	[-22.0]
B7	-1	5'-GTG AT <u>B</u> TGC	+14.0	+23.5	+15.0
B8		3'-CAC T <u>B</u> T ACG	[+0.5]	[-2.0]	[-2.0]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5^{\circ}\text{C}$); see Table 8 for experimental conditions.

Certain embodiments entail double-stranded probes with 'mixed' interstrand arrangements of disclosed monomers. Double-stranded probes with 'mixed' interstrand arrangements of **124X** and **124Y** are one representative example of this embodiment (e.g., one strand modified with 2'-amino- α -L-LNA monomer **124X**, the other strand modified with 2'-amino- α -L-LNA monomer **124Y**, Table 13). Significantly negative DA values are observed for these double-stranded probes. For example, probes with 'mixed' +1 zippers display DA values between -19.5 $^{\circ}\text{C}$ and -23.0 $^{\circ}\text{C}$ (Table 13). Without being limited to a single operation of theory, probes with +1 zippers composed of different monomers, display significant dsDNA-targeting potential and enable targeting of dsDNA-regions as described in FIG 1.

Table13
 ΔT_m And DA Values for Double-Stranded Probes with 'Mixed'
 Interstrand Arrangements of Monomer 124X and Monomer 124Y^a

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ON	Zipper	Duplex	$\Delta T_m [DA]/^{\circ}\text{C}$
124X6	+3	5'-GTG <u>K</u> TA TGC	+13.0
124Y9		3'-CAC TAT <u>L</u> CG	[-4.0]
124Y6	+3	5'-GTG <u>L</u> TA TGC	+11.0
124X9		3'-CAC TAT <u>K</u> CG	[-5.5]

124X6	+1	5'-GTG K TA TGC	-3.0
124Y8		3'-CAC T L T ACG	[-19.5]
124Y6	+1	5'-GTG L TA TGC	-4.0
124X8		3'-CAC T K T ACG	[-21.5]
124X7	+1	5'-GTG AT K TGC	-4.0
124Y9		3'-CAC TAT L CG	[-23.0]
124Y7	+1	5'-GTG AT L TGC	0.0
124X9		3'-CAC TAT K CG	[-19.5]
124X7	-1	5'-GTG AT K TGC	+22.0
124Y8		3'-CAC T L T ACG	[+3.5]
124Y7	-1	5'-GTG AT L TGC	+18.0
124X8		3'-CAC T K T ACG	[-2.5]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 for experimental conditions.

Certain embodiments entail double-stranded probes where one strand is modified with one or more monomers comprising a thymine nucleobase, and the other strand is modified is with one or more monomers comprising an adenine nucleobase. Particular embodiments of a double-stranded probe where one strand is modified with a thymine monomer (**126W** or **126X**) and the other strand is modified is with an adenine monomer (**124X** or **124Y**) are given in (Tables 14-17). Probes with 0 or +2 interstrand monomer arrangements generally display significantly negative DA-values (DA-values between -22 °C to -10 °C), indicating significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA, via the method shown in FIGS 1-2. Probes with -2 interstrand arrangements display DA-values ~0 °C. Without being limited to a single theory of operation, probes with 0, +1 or +2 interstrand zipper arrangements of disclosed monomers may display significant potential for targeting of double-stranded nucleic acids, more commonly dsDNA, and may enable targeting of dsDNA as shown FIG 1.

Table 14
 ΔT_m And DA Values for Selected Double-Stranded Probes with ‘Mixed’ Interstrand Zippers Comprised of Monomer 126W And Monomer 124X^a

ON	Zipper	Duplex	ΔT_m [DA]/°C
126W2	+2	5'-GTG A(126W)A TGC	+9.5
124X9		3'-CAC TAT (126X)CG	[-10.0]
124X6	0	5'-GTG (126X)TA TGC	-4.0
126W4		3'-CAC (126W)AT ACG	[-15.5]

126W2	0	5'-GTG A(126W)A TGC	0.0
124X8		3'-CAC T(126X)T ACG	[-20.5]
126W3	0	5'-GTG ATA (126W)GC	-4.5
124X9		3'-CAC TAT (126X)CG	[-20.5]
126W3	-2	5'-GTG ATA (126W)GC	+17.5
124X8		3'-CAC T(126X)T ACG	[+0.5]
124X7	-2	5'-GTG AT(126X) TGC	+14.5
126W4		3'-CAC (126W)AT ACG	[+1.0]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5^\circ\text{C}$); see Table 8 for experimental conditions.

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Table 15
 ΔT_m And DA Values for Selected DNA Duplexes with ‘Mixed’ Interstrand Zippers
Comprised of Monomer 126X And Monomer 124Y^a

ON Zipper	Duplex	$\Delta T_m [DA]/^\circ\text{C}$
126X2	+2 5'-GTG A(126X)A TGC	+12.0
124Y9		3'-CAC TAT (124Y)CG [-19.0]
124Y6	0 5'-GTG (124Y)TA TGC	+11.5
126X4		3'-CAC (126X)AT ACG [-10.0]
126X2	0 5'-GTG A(126X)A TGC	+12.5
124Y8		3'-CAC T(124Y)T ACG [-18.0]
126X3	0 5'-GTG ATA (126X)GC	+6.5
124Y9		3'-CAC TAT (124Y)CG [-19.5]
126X3	-2 5'-GTG ATA (126X)GC	+24.5
124Y8		3'-CAC T(124Y)T ACG [-1.0]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5^\circ\text{C}$); see Table 8 for experimental conditions.

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Table 16
 ΔT_m and DA Values for Selected DNA Duplexes with ‘Mixed’ Interstrand Zippers
Comprised of Monomer 126X) and Monomer 124X^a

ON Zipper	Duplex	$\Delta T_m [DA]/^\circ\text{C}$
126X2	+2 5'-GTG A(126X)A TGC	+12.0
124X9		3'-CAC TAT (124X)CG [-12.5]
124X6	0 5'-GTG (124X)TA TGC	-2.0

126X4		3'-CAC (126X)AT ACG	[-17.5]
126X2	0	5'-GTG A(126X)A TGC	+6.0
124X8		3'-CAC T(124X)T ACG	[-19.5]
124X7	0	5'-GTG AT(124X) TGC	+6.0
126X5		3'-CAC TA(126X) ACG	[-20.5]
126X3	-2	5'-GTG ATA (126X)GC	+21.5
124X8		3'-CAC T(124X)T ACG	[+1.0]
124X7	-2	5'-GTG AT(124X) TGC	+19.5
126X4		3'-CAC (126X)AT ACG	[+2.0]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 for experimental conditions.

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Table 17
 ΔT_m And DA Values for Selected DNA Duplexes with ‘Mixed’ Interstrand
Zipper Comprised of Monomer 126W and Monomer 124Y^a

ON	Zipper	Duplex	ΔT_m [DA]/°C
126W2	+2	5'-GTG A(126W)A TGC	+11.0
124Y9		3'-CAC TAT (124Y)CG	[-15.0]
126W3	0	5'-GTG ATA (126W)GC	+0.5
124Y9		3'-CAC TAT (124Y)CG	[-22.0]
126W3	-2	5'-GTG ATA (126W)GC	+24.0
124Y8		3'-CAC T(124Y)T ACG	[+2.0]

^a ΔT_m = change in T_m values relative to unmodified reference duplex **D1:D2** ($T_m \equiv 29.5$ °C); see Table 8 for experimental conditions.

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Additional embodiments include double-stranded probes with +1 interstrand zipper arrangements of monomers **208W-Z** (Table 18).

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Table 18
Thermal Denaturation Properties and dsDNA-Targeting Potential of Certain Disclosed Probes

<u>B</u> =	5'-GTG ATA TGC 3'-CAC TAT ACG	5'-GTG A <u>B</u> A TGC 3'-CAC TA <u>B</u> ACG	5'-GTG A <u>B</u> A TGC 3'-CAC TAT ACG	5'-GTG ATA TGC 3'-CAC TA <u>B</u> ACG	TA
	T_m (°C)	T_m [ΔT_m](°C)	T_m [ΔT_m](°C)	T_m [ΔT_m](°C)	°C
<u>120Y</u>	29.5	27.5[-2.0]	42.0 [+12.5]	41.0 [+11.5]	+26.0

<u>208W</u>	29.5	25.0 [-4.5]	40.0 [+10.5]	38.0 [+8.5]	+23.5
<u>208X</u>	29.5	27.0 [-2.5]	45.0 [+15.5]	45.0 [+15.5]	+33.5
<u>208Y</u>	29.5	29.0 [-0.5]	46.5 [+17.0]	45.5 [+16.0]	+33.5
<u>208Z</u>	29.5	22.0 [-7.5]	36.0 [+6.5]	35.0 [+5.5]	+19.5

see Table 8 for experimental conditions.

With reference to Table 18 above, thermal denaturation temperatures for model DNA duplex target (column 2 of 6), probe (column 3 of 6), probe-target duplex involving upper (column 4 of 6) or lower probe strand (column 5 of 6) are given. Thus, probes with +1 interstrand zipper arrangement of the monomers (column 3) display similar or lower thermostability than model DNA duplex target (column 2; note negative delta T_m values in column 3), while probe-target duplexes (columns 4 and 5) may be greatly stabilized (note highly positive delta T_m values). Without being limited to a single theory of operation, probes with +1 interstrand arrangements of monomers **120Y/208X/208Y/208Z** display significantly positive thermal advantage (TA) values and, thus, significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA, via the method outlined in FIGS 1-2.

Additional particular embodiments entailing double-stranded probes with +1 interstrand zipper arrangements of certain disclosed monomers are given below in Table 19. Without being limited to a single theory of operation, probes with +1 interstrand arrangements of monomers **228X-Z** display significantly positive thermal advantage (TA) values and, thus, significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA, via the method outlined in FIGS 1-2.

Table 19
Exemplary Embodiments of Double-stranded Probes with +1 Interstrand Zipper Arrangements Comprising Certain Disclosed Monomers

Sequence	Probe T _m [°C]	Upper probe strand vs DNA T _m [°C]	Lower probe strand vs DNA T _m [°C]	dsDNA target T _m [°C]	TA [°C]
5' – GTG A(228X)A TGC 3' – CAC TA(228X) ACG	29.5	43.5	42.5	29.5	+27.0
5' – GTG A(228Y)A TGC 3' – CAC TA(228Y) ACG	32.5	48.0	51.5	29.5	+37.5

5' – GTG A(228Z)A TGC	41.0	50.5	49.5	29.5	+29.5
3' – CAC TA(228Z) ACG					

see Table 8 above for experimental conditions.

- Additional examples of working embodiments of probes based on monomer **120Y** are provided below in Table 20. Table 20 provides thermal denaturation temperatures for a model DNA duplex target (column 6 of 7), probe (column 5 of 7), probe-target duplexes [i.e., model products from dsDNA recognition] involving upper (column 3 of 7) or lower probe strand (column 4 of 7). Probes with one +1 interstrand zipper arrangement of the monomers (first four entries) display similar or lower thermostability than unmodified dsDNA (representing the target; note negative delta T_m values in column 5), while probe-target duplexes (columns 3 and 4) display far greater stabilization (note highly positive delta T_m values). As previously indicated, probes with other interstrand arrangements may display less positive (or even negative) TA values and may therefore display less dsDNA-targeting potential (i.e., entries in this Table). Probes with two or more +1 interstrand arrangements of the disclosed monomers (bottom six entries), may display very high TA-values, suggesting significant potential for targeting of double-stranded targets, more commonly, dsDNA target regions, via the method shown in FIGS 1-2.

Table 20
Exemplary Probes Based on Monomer 120Y (= T)

Probe	Zipper	Upper probe strand vs DNA T _m [ΔT _m](°C)	Lower probe strand vs DNA T _m [ΔT _m](°C)	Probe T _m [ΔT _m](°C)	dsDNA target T _m (°C)	TA (°C)
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+1	44.5 [+7.0]	47.5 [+10.0]	36.5 [-1.0]	37.5	+18.0
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+1	47.5 [+10.0]	48.5 [+11.0]	36.5 [-1.0]	37.5	+22.0
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+1	48.5 [+11.0]	47.5 [+10.0]	36.5 [-1.0]	37.5	+22.0
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+1	47.5 [+10.0]	46.5 [+9.0]	35.5 [-2.0]	37.5	+21.0
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+3	44.5 [+7.0]	48.5 [+11.0]	54.0 [+16.5]	37.5	+1.5
5'-GGT ATA TAT AGG C 3'-CCA TAT ATA TCC G	+5	44.5 [+7.0]	47.5 [+10.0]	55.0 [+17.5]	37.5	-0.5
5'-GGT ATA TAT AGG C	+7	44.5 [+7.0]	46.5 [+9.0]	53.0 [+15.5]	37.5	+0.5

3'-CCA TAT ATA T CC G						
5'-GGT A T A TAT AGG C 3'-CCA TAT A T A TCC G	+3	47.5 [+10.0]	47.5 [+10.0]	57.0 [+19.5]	37.5	+0.5
5'-GGT A T A TAT AGG C 3'-CCA T AT ATA TCC G	-1	47.5 [+10.0]	47.5 [+10.0]	53.0 [+15.5]	37.5	+4.5
5'-GGT A T A TAT AGG C 3'-CCA TAT ATA T CC G	+5	47.5 [+10.0]	46.5 [+9.0]	56.0 [+18.5]	37.5	+0.5
5'-GGT ATA T AT AGG C 3'-CCA TAT ATA T CC G	+3	48.5 [+11.0]	46.5 [+9.0]	56.0 [+18.5]	37.5	+1.5
5'-GGT ATA T AT AGG C 3'-CCA TA T ATA TCC G	-1	48.5 [+11.0]	48.5 [+11.0]	55.0 [+17.5]	37.5	+4.5
5'-GGT ATA T AT AGG C 3'-CCA T AT ATA TCC G	-3	48.5 [+11.0]	47.5 [+10.0]	57.0 [+19.5]	37.5	+1.5
5'-GGT ATA TA T AGG C 3'-CCA TAT A T A TCC G	-1	47.5 [+10.0]	47.5 [+10.0]	54.0 [+16.5]	37.5	+3.5
5'-GGT ATA TA T AGG C 3'-CCA TA T ATA TCC G	-3	47.5 [+10.0]	48.5 [+11.0]	57.0 [+19.5]	37.5	+1.5
5'-GGT ATA TA T AGG C 3'-CCA T AT ATA TCC G	-5	47.5 [+10.0]	47.5 [+10.0]	57.0 [+19.5]	37.5	+0.5
5'-GG T A T A TAT AGG C 3'-CCA T A T ATA TCC G	2x +1	51.5 [+14.0]	55.5 [+18.0]	40.0 [+2.5]	37.5	+29.5
5'-GG T ATA T AT AGG C 3'-CCA T AT A T A TCC G	2x +1	53.5 [+16.0]	56.5 [+19.0]	49.0 [+11.5]	37.5	+23.5
5'-GG T ATA TA T AGG C 3'-CCA T AT ATA T CC G	2x +1	52.5 [+15.0]	55.5 [+18.0]	49.0 [+11.5]	37.5	+21.5
5'-GGT A T A T AT AGG C 3'-CCA TAT A T A TCC G	2x +1	55.5 [+18.0]	55.5 [+18.0]	45.0 [+7.5]	37.5	+28.5
5'-GGT ATA T A T AGG C 3'-CCA TAT A T A T CC G	2x +1	54.5 [+17.0]	54.5 [+17.0]	47.5 [+10.0]	37.5	+24.0
5'-GG T A T A T A T AGG C 3'-CCA T A T A T A T CC G	4x +1	65.5 [+28.0]	67.5 [+30.0]	50.0 [+12.5]	37.5	+45.5

”].

Particular embodiments concern probes with 0-arrangements of disclosed monomers, in some particular cases, monomers **120Y** and **120W**. Such probes may display variable TA values ranging from -1 °C to +27 °C, demonstrating that said probes may display significant potential for

targeting of double-stranded nucleic acid targets, more commonly dsDNA targets, via the method outlined in FIGS 1-2.

Table 21
5 Thermal Denaturation Properties of Certain Probes with 0-Zipper Interstrand Arrangements
 Where T is 120Y and A is 120'W

Probe	Upper probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Lower probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Probe $T_m[\Delta T_m](^{\circ}\text{C})$	dsDNA target $T_m(^{\circ}\text{C})$	TA $(^{\circ}\text{C})$
5'-GG <u>T</u> ATA TAT AGG C 3'-CCA <u>A</u> TAT ATA TCC G	44.5 [+7.0]	34.0 [-3.5]	38.0 [+0.5]	37.5	+3.0
5'-GGT A <u>T</u> A TAT AGG C 3'-CCA T <u>A</u> T ATA TCC G	47.5 [+10.0]	31.0 [-6.5]	40.0 [+2.5]	37.5	+1.0
5'-GGT ATA <u>T</u> AT AGG C 3'-CCA TAT <u>A</u> TA TCC G	48.5 [+11.0]	31.0 [-6.5]	40.0 [+2.5]	37.5	+2.0
5'-GGT ATA TAT <u>T</u> AGG C 3'-CCA TAT ATA <u>A</u> TCC G	47.5 [+10.0]	31.0 [-6.5]	40.0 [+2.5]	37.5	+1.0
5'-GG <u>T</u> A <u>T</u> A TAT AGG C 3'-CCA <u>A</u> T <u>A</u> T ATA TCC G	51.5 [+14.0]	27.0 [-10.5]	41.0 [+3.5]	37.5	± 0
5'-GG <u>T</u> ATA <u>T</u> AT AGG C 3'-CCA <u>A</u> TAT <u>A</u> TA TCC G	53.5 [+16.0]	26.0 [-11.5]	41.0 [+3.5]	37.5	+1.0
5'-GG <u>T</u> ATA TAT <u>T</u> AGG C 3'-CCA <u>A</u> TAT ATA <u>A</u> TCC G	52.5 [+15.0]	26.0 [-11.5]	42.0 [+4.5]	37.5	-1.0
5'-GGT A <u>T</u> A <u>T</u> AT AGG C 3'-CCA T <u>A</u> T <u>A</u> TA TCC G	55.5 [+18.5]	26.0 [-11.5]	42.0 [+4.5]	37.5	+2.0
5'-GGT ATA <u>T</u> A <u>T</u> AGG C 3'-CCA TAT <u>A</u> T <u>A</u> TCC G	54.5 [+17.5]	26.0 [-11.5]	42.0 [+4.5]	37.5	+1.0
5'-GG <u>T</u> A <u>T</u> A <u>T</u> A <u>T</u> AGG C 3'-CCA <u>A</u> T <u>A</u> T <u>A</u> T <u>A</u> TCC G	65.5 [+28.0]	43.0 [+5.5]	44.0 [+6.5]	37.5	+27.0

10 Tables 22-24 outline thermal denaturation properties of additional working embodiments of yet further probes comprising unlocked monomers targeting different DNA regions, i.e., second insulin [INSB], PPAR gamma and CEBP promoters). Yet again, the following thermal denaturation temperatures are given: model DNA duplex target (column 5 of 6), probe (column 4 of 6), probe-target duplex [product from dsDNA recognition] involving upper (column 2 of 6) or lower probe strand (column 3 of 6). Probes with one or more +1 interstrand zipper arrangement of the monomers

- display variable thermostability (ranging from strongly destabilized to moderately stabilized relative to unmodified dsDNA (note delta T_m values from -18 to +8 C, column 4). Probe-target duplexes (columns 2 and 3) are greatly stabilized (note highly positive delta T_m values). All probes display positive thermal advantage (TA). Probes with two or more +1 interstrand monomers arrangements display larger TA values (and thus, greater dsDNA-targeting potential) than probes with a single +1 interstrand monomers arrangement. Without being limited to a single theory of operation, probes with more than one +1 zipper arrangement of monomers facilitate dsDNA-targeting.

10 **Table 22**
Probes Targeting Second Insulin Promoter [INSB] Where Y = 120Y and X = 120Q

Probe	Upper probe strand vs DNA T _m [ΔT _m](°C)	Lower probe strand vs DNA T _m [ΔT _m](°C)	Probe T _m [ΔT _m](°C)	DNA target duplex T _m (°C)	TA (°C)
5'-G G <u>Y</u> A TAT AAG CAG CAC A 3'-C C <u>A</u> Y ATA TTC GTC GTG T	58.5 [+6.5]	60.5 [+8.5]	56.0 [+4.0]	52.0	+11.0
5'-G G <u>Y</u> A <u>Y</u> AT AAG CAG CAC A 3'-C C <u>A</u> Y <u>A</u> <u>Y</u> A TTC GTC GTG T	64.5 [+12.5]	65.5 [+13.5]	60.0 [+8.0]	52.0	+18.0
5'-AGG AAG G <u>Y</u> A <u>Y</u> AT AAG CA 3'-TCC TTC C <u>A</u> <u>Y</u> <u>A</u> <u>Y</u> A TTC GT	61.5 [+12.0]	64.0 [+14.5]	53.0 [+3.5]	49.5	+23.0
5'-AC <u>Y</u> <u>A</u> <u>Y</u> A GAA TAC TCA AG 3'-TGA <u>Y</u> <u>A</u> <u>Y</u> CTT ATG AGT TC	57.5 [+12.5]	56.5 [+11.5]	48.0 [+3.0]	45.0	+21.0
5'-G G <u>X</u> A TAT AAG CAG CAC A 3'-C C <u>A</u> <u>X</u> ATA TTC GTC GTG T	60.5 [+8.5]	62.5 [+10.5]	54.0 [+2.0]	52.0	+17.0
5'-G G <u>X</u> A <u>X</u> AT AAG CAG CAC A 3'-C C <u>A</u> <u>X</u> <u>A</u> <u>X</u> A TTC GTC GTG T	65.0 [+13.0]	66.0 [+14.0]	56.0 [+4.0]	52.0	+23.0
5'-AGG AAG G <u>X</u> A <u>X</u> AT AAG CA 3'-TCC TTC C <u>A</u> <u>X</u> <u>A</u> <u>X</u> A TTC GT	62.0 [+12.5]	64.0 [+14.5]	49.0 [-0.5]	49.5	+27.5
5'-AC <u>X</u> <u>A</u> <u>X</u> A GAA TAC TCA AG 3'-TGA <u>X</u> <u>A</u> <u>X</u> CTT ATG AGT TC	58.5 [+13.5]	57.0 [+12.0]	44.0 [-1.0]	45.0	+26.5

Table 23
Additional Examples of probes for PPAR Gamma
 Where T = 120Y; A = 120'W; and C = 140'X

Probe	Upper probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Lower probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Probe $T_m[\Delta T_m](^{\circ}\text{C})$	dsDNA target $T_m(^{\circ}\text{C})$	TA $(^{\circ}\text{C})$
5'-CCC <u>C</u> ACG <u>T</u> <u>T</u> A GCA GTT 3'-GGG <u>T</u> G <u>C</u> AA <u>T</u> CGT CAA	69.0 [+12.0]	68.0 [+11.0]	54.0 [-3.0]	57.0	+26.0
5'-CCC ACG <u>T</u> <u>T</u> A G <u>C</u> A GTT 3'-GGG TGC AA <u>T</u> CG <u>T</u> CAA	71.0 [+14.0]	68.0 [+11.0]	57.0 [± 0]	57.0	+25.0
5'-AGA CAA AA <u>C</u> ACC <u>C</u> AGT 3'-TCT GTT TTG <u>T</u> GG <u>T</u> CA	65.0 [+12.0]	60.0 [+7.0]	54.0 [+1.0]	53.0	+18.0
5'-AGA <u>C</u> AA AA <u>C</u> ACC AGT 3'-TCT G <u>T</u> T TTG <u>T</u> GG TCA	65.0 [+12.0]	58.0 [+5.0]	46.0 [-7.0]	53.0	+24.0
5'-CTA <u>C</u> AT <u>T</u> GT CTC GCC 3'-GAT G <u>T</u> A A <u>C</u> A GAG CGG	66.0 [+10.0]	69.0 [+13.0]	56.0 [± 0]	56.0	+23.0
5'-CTA C <u>A</u> T <u>T</u> GT CTC GCC 3'-GAT GT <u>A</u> A <u>C</u> A GAG CGG	64.0 [+8.0]	68.0 [+12.0]	59.0 [+3.0]	56.0	+17.0
5'-CGT <u>C</u> AT CG <u>T</u> GCT CGC 3'-GCA G <u>T</u> A GCA <u>C</u> GA GCG	73.0 [+9.0]	75.0 [+11.0]	62.0 [-2.0]	64.0	+22.0

Table 24
Additional Examples of Probes for CEBP
 Where T = 120Y; A=120'W; C=140'X; and G=140'Y

Probe	Upper probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Lower probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Probe $T_m[\Delta T_m](^{\circ}\text{C})$	dsDNA target $T_m(^{\circ}\text{C})$	TA $(^{\circ}\text{C})$
5'-CGG ACC ACG <u>TGT</u> GTG 3'-GCC TGG TGC <u>ACA</u> <u>CAC</u>	67.5 [+6.0]	68.5 [+7.0]	46.5 [-15.0]	61.5	+28.0
5'-CGG ACC <u>ACG</u> TGT GTG 3'-GCC TGG <u>TGC</u> ACA <u>CAC</u>	69.5 [+8.0]	70.0 [+8.5]	44.5 [-17.0]	61.5	+33.5
5'-GTC <u>AGT</u> GGG CGT TGC 3'-CAG <u>TCA</u> <u>CCC</u> GCA ACG	70.5 [+9.5]	73.0 [+11.5]	61.5 [± 0]	61.5	+20.5
5'-GTC <u>AGT</u> GGG CGT <u>TGC</u> 3'-CAG <u>TCA</u> <u>CCC</u> GCA <u>ACG</u>	74.5 [+13.0]	70.5 [+9.0]	60.5 [-1.0]	61.5	+23.0
5'-CCT <u>CTA</u> <u>TAA</u> AAG CGG 3'-GGA <u>GAT</u> <u>ATT</u> TTC GCC	64.5 [+14.0]	62.5 [+12.0]	59.5 [+9.0]	50.5	+17.0
5'-CCT <u>CTA</u> <u>TAA</u> AAG CGG 3'-GGA <u>GAT</u> <u>ATT</u> TTC GCC	63.5 [+13.0]	64.5 [+14.0]	47.5 [-3.0]	50.5	+30.0

5

The following Tables 25-27 describe the thermal denaturation properties of probes that may be used for gender determination of individual cells or multicellular assemblies from certain animals and humans; more commonly somatic cells, sperm cells or embryos from certain animals and humans; even more commonly, somatic cells, sperm cells or embryos from bovine.

10

As before, probes display thermostabilities that range from significantly lower to moderately higher than corresponding unmodified double-stranded DNA targets (note delta T_m values from -13 C to +9; column 4), while probe-target duplexes (column 2 and 3) are significantly more thermostable (range from +5 to +24 C). Accordingly, all of the probes (which have between two to five +1 zipper monomer arrangements) display significantly positive TA-values suggesting

15

significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA.

Table 25
Thermal Denaturation Properties of Exemplary Probes
 Where T = 120Y; A = 120'W; C = 140'X and G = 140'Y

Probe	Upper probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Lower probe strand vs DNA $T_m[\Delta T_m](^{\circ}\text{C})$	Probe $T_m[\Delta T_m](^{\circ}\text{C})$	dsDNA target $T_m(^{\circ}\text{C})$	TA $(^{\circ}\text{C})$
5'-AGC CCT <u>T</u> GTG CCC TG 3'-TCG GGA <u>CAC</u> GGG AC	69.5 [+9.0]	74.5 [+14.0]	58.0 [-2.5]	60.5	+25.5
5'-CCT <u>T</u> GTG CCC TG 3'-GGA <u>CAC</u> GGG AC	59.5 [+9.0]	65.5 [+15.0]	48.0 [-2.5]	50.5	+26.5
5'-CCT <u>T</u> GTG CCC TG 3'-GGA <u>CAC</u> GGG <u>AC</u>	59.0 [+8.5]	64.0 [+13.5]	47.0 [-3.5]	50.5	+25.5
5'- <u>AGC</u> CCT <u>T</u> GTG CCC TG 3'-TCG GGA <u>CAC</u> GGG <u>AC</u>	69.5 [+9.0]	75.5 [+15.0]	61.5 [+1.0]	60.5	+23.0
5'-CTG <u>AGC</u> CCT <u>T</u> GTG CCC TG 3'-GAG TCG GGA <u>CAC</u> GGG <u>AC</u>	74.0 [+8.0]	78.0 [+12.0]	57.0 [-9.0]	66.0	+29.0
5'- <u>AGC</u> CCT <u>T</u> GTG CCC TG 3'-TCG GGA <u>CAC</u> GGG <u>AC</u>	70.0 [+9.5]	80.0 [+19.5]	60.0 [-0.5]	60.5	+29.5

5

Yet another working example of a particular embodiment is provided in Table 26 below, which shows thermal denaturation properties and TA-values for probes modified with unlocked monomer **120Q**. Similar patterns as seen for other disclosed monomers are observed, i.e., probes display relatively low thermostability while probe-target duplexes are significantly more

10 thermostable. Probes containing one or more +1 zipper arrangement of unlocked monomer **120Q** therefore display significantly positive TA-values and therefore significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA targets.

Table 26
Thermal Denaturation Properties and TA-Values for Probes
Modified with Unlocked Monomer 120Q

Probe	TA (°C)	Probe T _m (°C)	'upper' probe strand vs DNA T _m (°C)	'lower' probe strand vs DNA T _m (°C)	Target DNA T _m (°C)
5' - GG <u>120Q</u> ATA TAT AGG C 3' - CCA <u>120Q</u> AT ATA TCC G	22.0	33.5	45.5	47.5	37.5
5' - GG <u>X 120Q A120Q</u> A TAT AGG C 3' - CCA <u>120Q A120Q</u> ATA TCC G	25.0	43.5	51.5	54.5	37.5
5' - GG <u>120Q A120Q A 120Q A120Q</u> AGG C 3' - CCA <u>120Q A120Q A120Q A 120Q</u> CC G	56.5	39.5	66.5	67	37.5

5 Particular embodiments entail double-stranded probes with certain zipper arrangements of monomers comprising so-called pseudo-complementary nucleobases (e.g., such as 2-thiouracil, 2,6-diaminopurines, inosine and pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one), more commonly, +1 zipper arrangements of monomers comprising pseudo-complementary nucleobases, even more commonly, +1 zipper arrangements of monomers such as **270**. Examples of working examples of these particular
10 embodiments are given in Table 27 below.

Further particular embodiments entail double-stranded probes with certain zipper arrangements (more commonly +1 zippers) of monomers comprising nucleobases where, in addition, the nucleotide opposite of the disclosed monomer comprising a pseudo-complementary nucleobase, is a nucleotide or disclosed monomer comprising a pseudo-complementary nucleobase (e.g., such as
15 2-thiouracil, 2,6-diaminopurines, inosine and pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one). For a representative working examples, please see entries 2 and 4 in Table 29 below, where D is a DNA monomer with a 2,6-diaminopurine nucleobase (i.e., 2,6-diaminopurine-2'-deoxyriboside). With reference to Table 27 below, it observed that double-stranded probes with -1 or +1 zipper arrangements of monomer **270** display positive TA-values, and therefore significant potential for
20 targeting of double-stranded nucleic acid targets via the method disclosed in FIGS 1-2, more commonly, dsDNA. With further reference to Table 27 below, it is observed that double-stranded probes with -1 or +1 zipper arrangements of monomer **270**, where, in addition, the nucleotide opposite of monomer **270** is D display positive TA-values, and therefore significant potential for
25 targeting of double-stranded nucleic acid targets via the method disclosed in FIGS 1-2, more commonly, dsDNA.

Table 27
Double-Stranded Probes with -1 Or +1 Zipper Arrangements of Monomer 270

Probe	dsDNA target T _m [°C]	Upper probe strand vs DNA T _m [°C]	Lower probe strand vs DNA T _m [°C]	Probe T _m [°C]	TA
5' – GTG A(270)A TGC 3' – CAC T <u>A</u> S ACG	29.5	41.0	40.5	28.5	+23.5
5' – GTG A(270) <u>D</u> TGC 3' – CAC T <u>D</u> (270) ACG	29.5	45.0	45.0	29.5	+31.0
5' – GTG A(270)A TGC 3' – CAC (270)AT ACG	29.5	41.0	32.0	39.5	+4.0
5' – GTG <u>D</u> (270)A TGC 3' – CAC (270) <u>D</u> T ACG	29.5	42.5	33.0	29.0	+17.0

5 Particular embodiments entail double-stranded probes simultaneously comprising one or more arrangements of disclosed monomers and one or more non-pairing monomers (FIG. 3b). Working examples of such embodiments are provided below. Table 28 concerns thermal hybridization properties and dsDNA-targeting potential of 18-mer probes containing non-pairing bulges with a general structure:

10 5' - GG(120Y) GGT CAA **L** CTA TC(120Y) GGA
3' - CCA (140'X)CA GGT **L** GAT AGA (140'X)CT

where “L” denotes the non-pairing monomer; in particular working examples, **L** is selected from monomers 402-4, 402-9 and 402-N. With reference to Table 28 below, “4”, “9” and “N” denotes a single incorporation of 402-4, 402-9 and 402-N, respectively. “444”, “999” and “NNN” denotes three consecutive incorporations of 402-4, 402-9 and 402-N, respectively. Still with reference to Table 28 below, “Up”, “down” and “both” in the third column, denotes whether bulged monomer(s) were included only in the upper probe strand, only in the lower probe strand or in both probe strands, respectively.

With continued reference to the data in Table 28 below, introduction of one or two non-pairing bulges into double-stranded probes, greatly reduces probe thermostability relative to corresponding to double-stranded probes that do not contain non-pairing bulges, denoted control probes (note, T_m values for probes are lower than the 60.5 °C observed for the control probe [i.e., bulge = none] shown in the first entry, column 3). While the stability of the probe-target duplexes also is decreased relative to the control probe (note that T_m values in column 4 and 5 are similar or lower than the T_m values in column 3), probes comprising one or two non-pairing bulges display positive TA-values; in the working examples shown below, several of the probes comprising one or two non-pairing bulges display significantly similar or larger TA-values than the control probe shown in entry 1, suggesting significant potential for targeting of double-stranded nucleic acid targets via the method disclosed in FIGS 1-2, more commonly dsDNA.

Table 28
Thermal Denaturation Properties and TA-Values of Duplexes Involving
Probes Comprising Non-Pairing Monomers

Used bulge	Strand(s) with Bulge(s)	Probe	Upper probe strand vs DNA	Lower probe strand vs DNA	TA
		T _m [°C]	T _m [°C]	T _m [°C]	[°C]
None	-	60.5	68.5	65.5	14.5
4	Up	47.5	60.0	65.5	19.0
	Down	51.5	68.5	58.0	16.0
	Both	40.5	60.0	58.0	18.5
9	Up	45.0	55.5	65.5	17.0
	Down	46.5	55.0	68.5	19.0
	Both	44.5	55.5	53.5	5.5
N	Up	54.0	64.5	65.5	17.0
	Down	58.0	68.5	63.0	14.5
	Both	52.0	64.5	63.0	16.5
444	Up	39.0	53.5	65.5	22.0
	Down	44.0	68.5	53.0	18.5
	Both	37.0	53.0	53.5	10.5
999	Up	43.0	56.0	65.5	19.5
	Down	44.0	68.5	53.0	18.5
	Both	44.0	53.0	56.0	6.0
NNN	Up	47.5	59.0	65.5	18.0
	Down	52.0	68.5	58.0	15.5
	Both	41.0	59.0	58.0	17.0

5

Further working examples of this embodiment are shown in Table 29 below, which illustrates the thermal hybridization properties and dsDNA-targeting potential of 13-mer double-stranded probes comprising monomer **120Y** (= **T**) and non-pairing monomer **402-9** (= **9**).

Introduction of one or two non-pairing monomer **402-9** near the termini of the probes, results in greatly reduced probe thermostability (note that all T_m values are lower than the 45 °C observed for the control probe shown in the first entry, column 2). While the stability of the probe-target duplexes also is decreased relative to the control probe (note that T_m values in columns 3 and 4 are the same or lower than the T_m value in column 3 of entry 1), all probe except for one, positive TA-values. Without being limited to a theory or operation, this suggests that probes with one or more bulges toward probe termini, display significant potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA, via the method shown in FIGS 1-2.

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Table 29
Thermal Hybridization Properties and dsDNA-Targeting Potential
of 13-Mer Double-Stranded Probes Comprising Monomer 120Y

5

Sequence	Probe	Upperprobe strand vs DNA	Lower probe strand vs DNA	TA (° C)
5'- GGT <u>A</u> <u>T</u> <u>A</u> TAT AGG C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TCC G	45.0	55.5	55.5	28.5
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TCC G	<15	44.0	46.5	38.0
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	<15	44.5	43.5	35.5
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	<15	<15	<15	-22.5
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	28.5	44.0	43.5	21.5
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TCC G	32.5	44.5	46.5	21.0
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TCC G	31.5	44.0	55.5	30.5
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TCC G	33.0	44.5	55.5	29.5
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TCC G	35.0	55.5	46.5	29.5
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	28.5	55.5	43.5	33.0
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TCC G	<15	<15	55.5	18.0
5'- GGT <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	<15	55.5	<15	18.0
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CC-9-A TAT <u>A</u> <u>T</u> <u>A</u> TCC G	<15	<15	46.5	9.0
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AG-9-G C 3'- CCA TAT <u>A</u> <u>T</u> <u>A</u> TC-9-C G	<15	<15	43.5	6.0
5'- GG-9-T <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> T AGG C	<15	44.0	<15	6.5

3'- CC-9-A TAT <u>A</u> TA TC-9-C G				
5'- GGT A <u>T</u> A <u>T</u> AT AG-9-G C	<15	44.5	<15	7.0
3'- CC-9-A TAT <u>A</u> TA TC-9-C G				

Table 30
Thermal Denaturation Properties of exemplary probes
 Where T = 120Y; A = 120'W; C = 140'X and G = 140'Y

Probe	dsDNA target T _m (°C)	Upper probe strand vs DNA T _m (°C)	Lower probe strand vs DNA T _m (°C)	Probe T _m (°C)	TA (°C)
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>2</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>2</u> GGG <u>A</u> C <u>Cy3</u>	60.5	51.5	57.5	26.5	22
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>4</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>4</u> GGG <u>A</u> C <u>Cy3</u>	60.5	61.5	62.5	34.5	29
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>N</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>N</u> GGG <u>A</u> C <u>Cy3</u>	60.5	67	70	45.5	31
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>2</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC GGG <u>A</u> C <u>Cy3</u>	60.5	51.5	74	29.5	35.5
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>2</u> GGG <u>A</u> C <u>Cy3</u>	60.5	69.5	57.5	32.5	34
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>4</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC GGG <u>A</u> C <u>Cy3</u>	60.5	61.5	74	50.5	24.5
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>4</u> GGG <u>A</u> C <u>Cy3</u>	60.5	69.5	62.5	41.5	30
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG <u>N</u> CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC GGG <u>A</u> C <u>Cy3</u>	60.5	67	74	60	20.5
5'- <u>Cy3</u> <u>A</u> GC CCT <u>T</u> GTG CCC <u>TG</u> 3'- T <u>C</u> G GGA <u>C</u> AC <u>N</u> GGG <u>A</u> C <u>Cy3</u>	60.5	69.5	70	47	32

Table 30 above shows additional working examples of this embodiment. Hybridization properties and dsDNA-targeting potential of probes comprising non-pairing monomers 4 (402-4), 9 (402-9) or N (402-N) bulges designed for sexing of bovine cells are shown in Table 30 where A = 120'W, C = 140'X, G = 140'Y, and T = 120Y.

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VII. METHOD OF USING DISCLOSED PROBE EMBODIMENTS

In particular disclosed embodiments, the disclosed probe is capable of associating with a target. The disclosed probe comprises at least one pair of monomers that are arranged in a +/-n zipper arrangement, which allows the monomers to affect the thermostability of the probe. Certain zipper arrangements, i.e., -1, 0, +1 and +2 zipper arrangements but more commonly +1 zipper arrangements provides the probe with sufficient thermodynamic instability to cause the two strands of the probe duplex to dissociate in the presence of a significantly complementary double-stranded nucleic acid target, more commonly dsDNA, and thereby associate with a target nucleic acid. In particular disclosed embodiments, the two separated strands of the probe will partake in Watson-Crick base pairing with the nucleotides of the target nucleic acid, more commonly the two strands of a dsDNA. In particular disclosed embodiments, the probe may be used in diagnostic techniques, such as identification of biomarkers, oncogenes, gender-specific genes, etc., and/or direct detection of double-stranded DNA in living cells, embryos, organs and tissues and/or induction of site-specific mutagenesis, recombination or repair of genomic DNA and site-specific modulation of gene expression (i.e. up- or down regulation). The probe is not limited to use in these techniques, as this list is meant only to be exemplary and not limiting. In particular disclosed embodiments, the probe may be pre-annealed using methods known to those of ordinary skill in the art. The pre-annealed probe may then be added to a target, such as a double stranded DNA target.

Particular disclosed embodiments concern using the disclosed probe to inhibit transcription. The disclosed probe may be designed to have at least one pair of monomers. In particular disclosed embodiments, the probe is designed to have two pairs of monomers, wherein the two pairs may be identical or different, and are separated by zero or more natural or non-natural nucleotides, such as disclosed monomers herein. In particular disclosed embodiments, a pair of monomers comprises at least two unlocked or locked monomers functionalized with an intercalator and arranged in a +1 zipper arrangement.

The probe may be designed to target a particular isosequential nucleic acid target – whether synthetic or biological, such as any of those disclosed herein. In certain disclosed embodiments, the nucleic acid target may be the SP6 and T7 promoters on *PGEM-Teasy* plasmids that may or may not overlap with a transcription start site. With reference to this particular disclosed embodiment, the pGEM-T-Easy vector containing insb-cDNA was linearized with either SpeI or SacII and used for *in vitro* transcription reactions to synthesize cRNA driven by T7 or SP6 promoters, respectively (FIG. 18). The linearized plasmids were incubated with dsDNA-targeting agents as follows: either positive control (commercial Zorro LNA), targeting the SP6 promoter, or the disclosed probe selected to target the SP6 or T7 promoter (FIG. 19). Following incubation to facilitate binding of the positive

control and the probe, *in vitro* transcription was initiated by incubating with ribonucleotide triphosphates, buffer, and T7 or SP6 polymerases. cRNA products were reverse transcribed to cDNA. Primers designed to detect a 240 base insert amplicon were used in end-point PCR and the product was resolved by gel electrophoresis. The results of this particular disclosed embodiment are illustrated in FIG. 20 wherein lanes 1 and 9 illustrate the DNA ladder (100 bp increments); lane 2 illustrates the T7-driven product formed in SpeI digested plasmid in the absence of either the control or the probe; lane 3 illustrates the SacII digested plasmid, which does not yield T7-driven product; lane 4 illustrates that a particular embodiment of the probe binds to the T7 promoter and prevents formation of T7-driven product in SpeI digested plasmid; and lane 5 illustrates that the SP6-driven product is formed in SacII digested plasmid in the absence of either the positive control or the probe. Also referring to FIG. 20, lanes 6-8 illustrate that Zorro LNA (ln 6), one embodiment of the disclosed probe (ln 7) or a different embodiment of the disclosed probe (ln 8) bind to the SP6 promoter and prevent formation of SP6-driven product in SacII treated plasmid. Other targets are contemplated by the disclosed method, such as gene knockdown in live cell lines targeting chromosomal progesterone receptor, estrogen receptors, and any other biologically relevant targets. In particular disclosed embodiments, the disclosed probe may be used for animal sexing, such as sexing of ungulates, ruminates, and more particularly bovines, equines and porcines, as the probe may be designed to selectively targeting gender-specific DNA regions. Examples of gender-specific DNA regions are known in the art. See, for example, WO 2009079456 and Brown, Kim H., Irvin R. Schultz, J. G. Cloud, and James J. Nagler (2008) "Aneuploid Sperm Formation in Rainbow Trout Exposed to the Environmental Estrogen 17 α -ethynylestradiol PNAS (USA), 105:19786-19791, both of which are incorporated herein by reference. In particular disclosed embodiments, the probe may be designed to comprise one or more pairs of the disclosed monomers and have a sequence of nucleotides that is isosequential with a particular gene of a target cell that is specific for certain genetic traits, such as gender. In certain disclosed embodiments, the probe comprises one or more pairs of disclosed monomers selected from embodiments of monomers disclosed herein, such as those of Scheme 16, and may contain zero or more non-pairing monomers such as **402-4**, **402-9** or **402-9**. In particular disclosed embodiments, the probe may be used to determine the gender of animals and cells (in particular sperm cells), organs, tissues and embryos thereof. Particular embodiments enable gender determination of unadulterated early-stage embryos from animals used in food production and sport breeding.

In another disclosed embodiment of using the disclosed probe, a transfected plasmid in-cell assay was performed. Beta TC-6 cells (ATCC, CRL-11506) were co-transfected with [pGL4.10 (luc2/-374insb)] and an internal transfection control vector [pGL4.74 (hRluc/TK) (FIG. 21). A probe comprising at least one pair of locked monomers targeting the insb promotor was transfected 24h after plasmid co-transfection. In this particular disclosed embodiment, 2 μ g of the probe per well was used (6-well plate format). The cells were harvested 24h after probe addition (90-100% confluency)

and assayed for Firefly and Renilla luciferase (enzyme) activity using a dual luciferase assay system (Promega) to determine the efficacy and specificity of probe-mediated antigene activity. Firefly luciferase activity was normalized to Renilla luciferase activity to correct for transfection variation. Normalized Firefly luciferase activity is expressed relative to a scrambled control probe.

- 5 Experiments conducted in triplicate were replicated on three independent occasions. For the dose-response study, cells were transfected in triplicate with 0.1, 1.0 or 5.0 μ g the particular probe per well. With reference to this transfection study and FIG. 22, a particular embodiment of the probe (i.e. a probe comprising only one pair of locked monomers) did not show a detectable decrease in luciferase activity. Two different probes comprising more than one pair of locked monomers did
10 display an effect (10-30% reduction in luciferase activity). Also explored were dose-dependent studies, which illustrated that particular embodiments of the probe displayed efficacy at the highest dose, particular embodiments displayed dose-response effect, and other embodiments displayed evidence of inversed efficacy with increasing dose. These results are illustrated in FIG. 23.

- In particular disclosed embodiments, the probe may be used to target an isosequential
15 double-stranded DNA duplex or structured analogs hereof. For example, two strands of an isosequential target duplex may be connected through a linker, such as a polynucleotide, to produce a duplex having a hairpin configuration (FIG. 24). In exemplary embodiments, the linker comprises ten thymidines, with the stem of the hairpin target being the primary region recognized by the probe. In particular disclosed embodiments, the nucleic acid target may or may not comprise one or more
20 polypurine units. In certain disclosed embodiments, the intramolecular nature of the target duplex of the hairpin structure increases the T_m value of the duplex, rendering it a more challenging target than linear dsDNA targets. The ability of the probe to recognize and invade (or associate with) the complex may be analyzed using an electrophoretic mobility shift assay.

- In exemplary embodiments, the ability of a (+1) interstrand zipper probe comprising at least
25 one pair of monomers was used to target a hairpin target. According to FIG. 25, hairpin invasion by the disclosed probe resulted in a probe-target complex having a slower migration rate, such as that illustrated in lanes A-D of FIG. 25. As illustrated in FIG. 25, the concentration of the probe added to the target, affects the ability of the probe-target complex to be formed. In particular disclosed embodiments, an excess of about 5-fold to about 500-fold of the disclosed probe may result in probe-
30 target complex formation; even more typically, an excess of from about 5-fold to about 50-fold of the disclosed probe will result in significant probe-target complex formation. According to FIG. 25, lanes A-E represent samples in which a varying excess of the probe is used, with lane A representing a 500-fold excess of the probe, lane B representing a 100-fold excess of the probe, lane C representing a 50-fold excess of the probe, lane D representing a 10-fold excess of the probe, and lane
35 E representing a 5-fold excess of the probe. In particular disclosed embodiments, any of the disclosed monomers may be incorporated into the probe.

In exemplary embodiments, monomers **124X**, **124Y**, **126W**, **126X**, **126Y**, **126Z**, **120Q**, **120S**, **120V**, **120Y**, and **120'W** were used to form a (+1) interstrand zipper within the probe. Working examples of these embodiments are given in FIG 30. Thus, probes with +1 arrangements of the disclosed monomers, recognize a structured and digoxigenin-labeled dsDNA target comprised of an isosequential double-stranded target region, which is linked on one side by a single-stranded T₁₀ loop (**SL1**, FIG. 30b). The unimolecular nature of this target (**SL**) structures leads to extensive thermostabilization of the double-stranded region [T_m (**SL1**) = 56.0 °C vs T_m (**D1:D2**) = 29.5 °C]. Incubation of **126W2:126W5** with the structured dsDNA target **SL1** (3h at 20 °C) results in the formation of a recognition complex with lower electrophoretic mobility on 15% non-denaturing PAGE gels than **SL1** (FIG. 30c). A clear dose-response trend is observed; a trace of complex formation is observed when 5-fold excess of **126W2:126W5** is used, while a 100-fold excess of **126W2:126W5** results in ~48% recognition (FIG. 30c; Table 31, where for all except 124X and 120'W the probe is:

5'-GTG ABA TGC

3'-CAC TAB B ACG

where B is the disclosed monomer. For all 124X and 120'W the probe is:

5'-GTG BTA TGC

3'-CAC TBT ACG

where B is the disclosed monomer; FIG. 31). Similar results are observed for probe **120Q2:120Q5** (FIG 30 D, Table 31, FIG 31) and all other evaluated probes (Table 31, FIG 31), except for **120S2:120S5** and **120V2:120V5** (Table 31). This includes probes with +1 interstrand arrangements of locked thymine monomers **126X** and **126Y**, unlocked uracil-based monomers **120Y** and **120Q**, and adenine-based monomers **124X** and **120'W**. Thus, in exemplary embodiments, monomers **120S** and **120V** did not produce a probe-target complex, and monomer **124X** was found to be less efficient to invade the hairpin duplex, while the other monomers exhibited substantial probe-target complex formation. Without being limited to a single theory of operation, it is currently believed that the reactivity of monomers **120S**, **120V** and **124X** could be attributed to formation of less thermally stable probe-target duplexes. In exemplary embodiments, the hairpin invasion reached a saturation point at 25µM of the probe, wherein about 50% to about 75% of the hairpin was targeted. Additional results of the percentage of hairpin invasion using 5µM of the probe are summarized in Table 31, below.

Table 31
Efficiency of Hairpin Invasion by Various Invader Duplex at 100 Fold Excess of Probe^a

Monomer	% of invasion
126W	48
126X	40
126Y	38
120Q	45

120S	<5
120V	<5
120Y	47
124X	17
120'W	41
^a average of three independent measurements	

In particular disclosed embodiments, the ability of the disclosed probe to invade, or associate with, the target may be evaluated by comparing its reactivity with that of control probes. For example, incubation of the mixed-sequence hairpin target with an unmodified isosequential DNA duplex did not show any complex formation, even with up to a 500-fold excess of the unmodified DNA duplex. In addition, incubation of the hairpin target with the either single-stranded probe precursor that comprises a double-stranded probe did not exhibit substantial complex formation (FIGS. 26 D & E). Furthermore, the sequence specificity of the probe may be determined by methods known to those of ordinary skill in the art. In exemplary embodiments, singly and doubly mutated, but perfectly base paired hairpins were targeted. In these particular embodiments, the probe-target complex was not observed, even at a 100-fold (5uM) excess of the probe, thereby demonstrating high target specificity (FIGS. 26 A & B).

Specific examples of control experiments were performed to validate the approach and evaluate the binding specificity (FIG. 30e-h): a) addition of up to 500-fold excess of unmodified DNA duplex **D1:D2** to the structured DNA target **SL1** (3 hours, 20 °C) fails to produce a recognition complex, which underlines the critical role of +1 interstrand arrangements of the disclosed monomers for dsDNA-targeting (FIG. 30e); b) addition of 100-fold excess of single-stranded **126W2/126W5/120Q2/120Q5/120P2/120P5/120'W6/120'W8** to structured DNA target **SL1** (3 hours, 20 °C) fails to yield a recognition complex, which demonstrates that both strands of a probe enable dsDNA-recognition (FIG. 30f; FIG. 32); and c) addition of 100-fold excess of probes **126W2:126W5**, **120Q2:120Q5**, **126X2:126X5**, **120Y2:120Y5** or **124X6:124X8** to structured DNA targets **SL2** or **SL3** (3h, 20 °C) featuring fully base-paired but non-isosequential stem regions [one or two base pair deviations relative to probes; T_m (**SL2**) = 57.0 °C; T_m (**SL3**) = 64.5 °C], failed to form a recognition complex, which demonstrated that the approach is very specific (FIG. 30g and 30h; FIG. 33).

VIII. WORKING EXAMPLES

General Experimental Section: Unless otherwise noted, reagents and solvents were commercially available, of analytical grade and used without further purification. Petroleum ether of the distillation range 60-80°C was used. Solvents were dried over activated molecular sieves: acetonitrile and THF (3Å); CH₂Cl₂, 1,2-dichloroethane, *N,N'*-diisopropylethylamine and anhydrous DMSO (4Å). Water content of "anhydrous" solvents was verified on Karl-Fisher apparatus. Reactions were conducted under argon whenever anhydrous solvents were used. Reactions were

monitored by TLC using silica gel coated plates with a fluorescence indicator (SiO₂-60, F-254) which were visualized a) under UV light and/or b) by dipping in 5% conc. H₂SO₄ 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). Evaporation of solvents was carried out under reduced pressure at temperatures below 45 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried at high vacuum for at least 12h to give the obtained products in high purity (>95%) as ascertained by 1D NMR techniques. Chemical shifts of ¹H NMR (500 MHz), ¹³C NMR (125.6 MHz), and/or ³¹P NMR (121.5 MHz) are reported relative to deuterated solvent or other internal standards (80% phosphoric acid for ³¹P NMR). Exchangeable (ex) protons were detected by disappearance of signals upon D₂O addition. Assignments of NMR spectra are based on 2D spectra (HSQC, COSY) and DEPT-spectra. Quaternary carbons are not assigned in ¹³C NMR but verified from HSQC and DEPT spectra (absence of signals). MALDI-HRMS spectra of compounds were recorded on a Q-TOF mass spectrometer using 2,5-dihydroxybenzoic acid (DHB) as a matrix and polyethylene glycol (PEG 600) as an internal calibration standard.

9-[2-*O*-Acetyl-3-*O*-benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxy-methyl]- α -L-threo-pentofuranosyl-6-*N*-benzoyladenine (32). Benzoylated adenine (28.6 g, 120 mmol) and coupling sugar **30** α/β (40.6 g, 80 mmol) were co-evaporated with 1,2-DCE (2 $\times$ 150 mL), redissolved in anhydrous 1,2-DCE (270 mL), and BSA (49.2 mL, 0.20 mol) was added. The heterogeneous mixture was heated at reflux until becoming homogeneous. After cooling to rt. TMSOTf (43.1 mL, 0.24 mol) was added and the reaction mixture heated at reflux for 70h. After cooling to rt. the mixture was poured into sat. aq. NaHCO₃/crushed ice (500 mL, 1:1, v/v). To control effervescence additional crushed ice (400 mL) was added, and the mixture was stirred for 30 min during which a precipitate was formed. The precipitate was removed by filtration and the filtrate was washed with CH₂Cl₂ (1.5 L). The combined organic phase was washed with sat. aq. NaHCO₃ (2 $\times$ 500 mL) and the aqueous phase back-extracted with CH₂Cl₂ (2 $\times$ 500 mL). The organic phase was evaporated to afford a crude yellow foam, which was purified by silica gel column chromatography (0-10% i-PrOH in CH₂Cl₂, v/v) to afford target nucleoside **32** as a white foam (38.3 g, 70%). *R*_f = 0.4 (5% MeOH in CH₂Cl₂, v/v). MALDI-HRMS *m/z* 712.1380 ([M + Na]⁺, C₂₉H₃₁N₅O₁₁S₂·Na⁺ Calc. 712.1354); ¹H NMR (300 MHz, CDCl₃) δ 8.97 (s, 1H), 8.81 (s, 1H), 8.37 (s, 1H), 8.03 (d, 2H, *J* = 8.1 Hz), 7.50-7.65 (m, 5), 7.29-7.38 (m, 5H), 6.45 (d, 1H, *J* = 2.6 Hz), 5.95 (t, 1H, *J* = 2.4 Hz), 4.78-4.82 (d, *J* = 11.7 Hz, 1H), 4.63-4.67 (m, 3H), 4.38-4.43 (m, 4H), 3.28-3.32 (d, 1H, *J* = 10.6 Hz), 3.0 (s, 3H), 2.96 (s, 3H), 2.16 (s, 3H); ¹³C NMR (75.5 MHz, CDCl₃) δ 169.5, 164.6, 152.9 (A^{Bz}), 151.6, 149.7, 141.2 (A^{Bz}), 135.7, 133.4, 132.8, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.0, 127.8, 123.0, 87.3 (C1'), 86.0 (C4'), 81.3 (C3'), 79.4 (C2'), 73.3 (CH₂-Ph), 67.4 (C5'), 65.5 (C5''), 37.7 (OMs), 37.6 (OMs), 20.7 (Ac).

9-(3-*O*-Benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl- α -L-*threo*-pentofuranosyl)-6-*N*-benzoyladenine (34)

Small scale procedure using guanidinium nitrate.

A stock solution of guanidine/guanidinium nitrate (G/GHNO₃) was prepared according to the published procedure by dissolving guanidinium nitrate (4.91 g, 40.2 mmol) in a mixture of MeOH:CH₂Cl₂ (450 mL, 9:1, v/v) and adding NaOMe (0.24 g, 4.5 mmol). Fully protected nucleoside **32** (4.66 g, 6.7 mmol) was dissolved in an aliquot of the prepared G/GHNO₃ solution (450 mL) and the mixture was stirred at rt. for 30 min whereupon sat. aq. NH₄Cl (200 mL) was added, resulting in formation of a white precipitate. The formed precipitate was filtered off and washed repeatedly with CH₂Cl₂ until sugar residues were undetectable in the filtrate by TLC. The filtrate was concentrated under reduced pressure, the aqueous phase washed with CH₂Cl₂ (5 × 200 mL) and the combined organic phase evaporated, affording a yellowish solid. The crude solid was purified by silica gel column chromatography (0-4% MeOH in CH₂Cl₂, v/v) affording alcohol **34** (3.84 g, 88%) as a white foam. *R*_f: 0.5 (10% MeOH in CH₂Cl₂, v/v).

Large scale method using 1/2 sat. methanolic ammonia.

Fully protected nucleoside **32** (30.42 g, 44 mmol) was dissolved in MeOH (706 mL) and the solution cooled to 0 °C, whereupon sat. methanolic ammonia (353 mL) was added. The reaction mixture was stirred at rt. for 2.5h whereupon the reaction mixture was evaporated and the obtained residue co-evaporated with abs. EtOH:toluene (2 × 500 mL, 1:1, v/v). The solid residue was purified by silica gel column chromatography (0-2.5% MeOH in CH₂Cl₂, v/v) affording target alcohol **34** as a white foam (23.81 g, 84%). *R*_f: 0.5 (10% MeOH in CH₂Cl₂, v/v). MALDI-HRMS *m/z* 670.1234 ([M + Na]⁺, C₂₇H₂₉N₅O₁₀S₂·Na⁺ Calc. 647.1248); ¹H NMR (300 MHz, CDCl₃) δ 9.21 (s, 1H), 8.47 (s, 1H), 8.29 (s, 1H), 7.97 (d, 2H, *J* = 7.7 Hz), 7.61-7.32 (m, 9H), 6.11 (d, 1H, *J* = 6.2 Hz), 5.28 (d, 1H, *J* = 9.2 Hz), 4.96 (d, 1H, *J* = 11.7 Hz), 4.75 (d, 1H, *J* = 11.3 Hz), 4.61 (d, 1H, *J* = 10.6 Hz), 4.45-4.25 (m, 4H), 3.03 (s, 3H), 2.90 (s, 3H). ¹³C NMR (75.5 MHz, CDCl₃) δ 165.2, 152.4 (A^{Bz}), 151.1, 148.6, 141.9 (A^{Bz}), 136.8, 133.2, 133.0, 128.9, 128.6, 128.3, 127.8, 121.7, 87.6 (C1'), 83.0 (C4'), 81.9 (C3'), 78.6 (C2'), 73.2 (CH₂-Ph), 68.4 (C5'), 68.2 (C5''), 37.7 (OMs), 37.4 (OMs).

1-(2-Azido-3-*O*-benzyl-4-*C*-methanesulfonyloxymethyl-5-*O*-methanesulfonyl-2-deoxy- α -L-*erythro*-pentofuranosyl)-6-benzoyl-adenine-9-yl (36, 38): Alcohol **34** (18.6 g, 30 mmol) was co-evaporated with pyridine (50 mL), and subsequently dissolved in an. CH₂Cl₂ (200 mL). The mixture was then cooled to -78 °C, and pyridine (7.3 mL, 90 mmol) and trifluoromethanesulfonyl anhydride (9.90 mL, 60 mmol) was added. The mixture was stirred for 3h during which it was allowed to heat to rt. Hereafter crushed ice (50 mL) was added. The organic phase was washed with sat. aq. NaHCO₃ (2 × 50 mL), evaporated to dryness and co-evaporated with abs. EtOH (2 × 50 mL) affording a crude yellowish/brown solid which was used in the next step without further purification. The crude solid,

tentatively assigned as triflate derivative **36**, was dissolved in DMF (300 mL), and NaN₃ (19.6 g, 0.3 mol) and 15-crown-5 (6.0 mL, 30.2 mmol) was added. The reaction mixture was stirred at rt. for 15h, where analytical TLC showed approximately 50% conversion. Subsequently the mixture was heated to 50 °C for 8h, whereupon analytical TLC showed full conversion. After cooling to rt., the mixture was filtered to remove excess NaN₃. The filtrate was washed repeatedly with EtOAc and the combined organic phase was concentrated under reduced pressure. The concentrated mixture was taken up in brine (200 mL) and EtOAc (200 mL). The phases were separated and the aqueous phase extracted with EtOAc (4 × 100 mL). The combined organic phase was evaporated under reduced pressure affording a crude dark brown solid. The crude solid was then purified by silica gel column chromatography (0-90% EtOAc in petroleum ether, v/v) affording azide **38** (17.9 g, 89% over 2 steps) as a white solid. *R*_f: 0.4 (EtOAc). IR: 2115.6 cm⁻¹ (-N₃). MALDI-HRMS *m/z* 695.1295 ([M + Na]⁺, C₂₇H₂₈N₈O₉S₂·Na⁺ Calc. 695.1313); ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.30 (s, 1H, NH, ex), 8.79 (s, 1H, A^{Bz}), 8.54 (s, 1H, A^{Bz}), 8.05 (d, 2H, *J* = 7.0 Hz, Bz), 7.53-7.67 (m, 3H, Bz), 7.35-7.47 (m, 5H, Bn), 6.74 (d, 1H, *J* = 4.4 Hz, H-1'), 5.20 (t, 1H, *J* = 4.8 Hz, H-2'), 4.89 (d, 1H, *J* = 5.1 Hz, H-3'), 4.79-4.83 (d, 1H, *J* = 11.7 Hz, H-5'_a), 4.74-4.78 (d, 1H, *J* = 11.7 Hz, H-5'_b), 4.69-4.72 (d, 1H, *J* = 11.4 Hz, CH₂-Ph), 4.47-4.51 (d, 1H, *J* = 11.4 Hz, CH₂-Ph), 4.42 (s, 2H, H-5''), 3.28 (s, 3H, OMs), 3.24 (s, 3H, OMs). ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 151.9 (A^{Bz}), 150.4, 142.7 (A^{Bz}), 136.9, 133.2, 132.5, 128.5, 128.0, 81.9 (C1'), 81.7 (C3'), 80.1 (C2'), 73.5 (CH₂-Ph), 68.3 (C5'), 61.9 (C5''), 36.98 (OMs), 36.94 (OMs).

(1R,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-benzyloxy-1-methanesulfonyloxy-methyl-2-oxa-5-azabicyclo[2.2.1]heptane (40): Azide derivative **38** (35.18 g, 51.8 mmol) was dissolved in THF (500 mL) cooled to 0 °C and aq. NaOH (2M, 38.9 mL, 77.8 mmol) and trimethylphosphine (1M in THF, 77.8 mL, 77.8 mmol) was added. The reaction mixture was stirred for 21h during which it was allowed to heat to rt. subsequently, the reaction mixture was evaporated to dryness. The resulting crude residue was taken up in brine (200 mL) and EtOAc (200 mL), and the aqueous phase was washed with MeOH:CH₂Cl₂ (3 × 200 mL, 2:8, v/v). The combined organic phases was evaporated to dryness and the crude purified by silica gel column chromatography (0-5% MeOH in CH₂Cl₂, v/v) affording bicyclic nucleoside **40** as a yellow/brown solid (24.2 g, 85%). *R*_f: 0.3 (EtOAc). MALDI-HRMS *m/z* 573.1517 ([M + Na]⁺, C₂₆H₂₆N₆O₆S·Na⁺ Calc. 573.1527); ¹³C NMR data is not in accordance with previous data. The corrected data are given here. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.20 (s, 1H, NH, ex), 8.77 (s, 1H, A^{Bz}), 8.73 (s, 1H, A^{Bz}), 8.06 (d, 2H, *J* = 7.0 Hz, Bz), 7.53-7.67 (m, 3H, Bz), 7.29-7.47 (m, 5H, Ph), 6.52 (d, 1H, *J* = 1.8 Hz, H-1'), 4.72-4.76 (d, 1H, *J* = 11.7 Hz, CH₂Ph), 4.62-4.67 (d, 1H, *J* = 11.7 Hz, CH₂Ph), 4.57-4.60 (d, 1H, *J* = 11.7 Hz, H-5'_a'), 4.49-4.53 (d, 1H, *J* = 11.7 Hz, H-5'_b'), 4.44 (s, 1H, H-3'), 3.92 (m, 1H, H-2'), 3.24-3.30 (m, 1H, H5'_a'' overlap with H₂O) 3.23 (s, 3H, OMs), 3.10-3.13 (d, 1H, *J* = 9.89 Hz, H-5'_b'). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.5, 152.0, 151.4 (A^{Bz}), 150.0, 143.1 (A^{Bz}), 137.8, 133.3, 132.3 (Ar), 128.4 (Ar), 128.2 (Ar), 127.6

(Ar), 127.5 (Ar), 125.0, 87.1, 84.3 (C-1'), 80.3 (C-3'), 71.0 (CH₂-Ph), 66.8 (C-5'), 59.8 (C-2'), 51.1 (C-5''), 36.8 (Ms).

(1R,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-benzyloxy-1-methanesulfonyloxy-methyl-5-trifluoroacetyl-2-oxa-5-azabicyclo[2.2.1]heptane (42): Amine derivative **40** (8.68 g, 15.8 mmol) was co-evaporated with pyridine (2 × 20 mL) and dissolved in anhydrous CH₂Cl₂ (200 mL). Anhydrous pyridine (5.09 mL, 63 mmol) was added, the mixture cooled to 0 °C, and trifluoroacetic acid anhydride (4.45 mL, 31.5 mmol) was added. The reaction mixture was stirred for 2h whereupon crushed ice (50 mL) was added. The organic phase was washed with sat. aq. NaHCO₃ (2 × 50 mL), and the aqueous phase was back-extracted with CH₂Cl₂ (2 × 100 mL) and MeOH:CH₂Cl₂ (100 mL, 2:8, v/v). The combined organic phase was evaporated to dryness and co-evaporated with toluene (50 mL) and once with abs. EtOH:toluene (50 mL, 1:1, v/v). The crude residue was purified by silica gel column chromatography (0-100% EtOAc in petroleum ether, v/v) affording the fully protected nucleoside **42** as a white foam (6.27 g, 62%). *R*_f: 0.5 (10% MeOH:EtOAc, v/v). Physical data for the mixture of rotamers: MALDI-HRMS *m/z* 647.1541 ([M + H]⁺, C₂₈H₂₅F₃N₆O₇S·H Calc. 647.1530); ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.21 (s, 0.5H, NH, ex), 11.19 (s, 0.5H, NH, ex), 8.78 (s, 0.5H, A^{Bz}), 8.76 (s, 0.5H, A^{Bz}), 8.64 (s, 0.5H, A^{Bz}), 8.60 (s, 0.5H, A^{Bz}), 8.05 (d, 2H, *J* = 7.0 Hz, Bz), 7.52-7.67 (m, 3H, Bz), 7.31-7.41 (m, 5H, Ph), 6.83 (d, 0.5H, *J* = 1.1 Hz, H-1'), 6.80 (d, 0.5H, *J* = 1.1 Hz, H-1'), 5.26 (s, 0.5H, H-2'), 5.17 (s, 0.5H, H-2'), 4.84 (s, 1H, H-3'), 4.82 (s, 0.5H, H-3'), 4.62-4.79 (m, 4H, CH₂Ph, H5''), 4.53 (d, 0.5H, *J* = 10.6 Hz, H-5'), 4.35 (d, 0.5H, *J* = 12.1 Hz, H-5'), 4.07 (d, 0.5H, *J* = 10.6 Hz, H-5'), 3.90 (d, 0.5H, *J* = 12.1 Hz, H-5'), 3.28 (s, 3H, OMs). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.5, 155.2 (q, *J* = 36.6 Hz, COCF₃), 154.8 (q, *J* = 36.6 Hz, COCF₃), 151.8 (A^{Bz}), 151.6 (A^{Bz}), 151.5, 150.3, 150.26, 141.2 (A^{Bz}), 141.0 (A^{Bz}), 137.1, 137.0, 133.2, 132.4 (Ar), 128.4 (Ar), 128.38 (Ar), 128.36 (Ar), 128.32 (Ar), 127.9 (Ar), 127.8 (Ar), 127.5 (Ar), 125.4, 125.2, 115.5 (q, *J* = 288 Hz, CF₃), 115.2 (q, *J* = 288 Hz, CF₃), 86.15 (C-4'), 86.14 (C-4'), 84.9 (C-1'), 84.8 (C-1'), 83.9 (C-2'), 79.1 (C-3'), 77.4 (C-3'), 71.6 (CH₂Ph), 65.3 (C-5''), 65.0 (C-5''), 63.0 (C-2'), 61.4 (C-2'), 53.2 (C-5'), 53.07 (C-5') 53.05 (C-5'), 37.0 (OMs). ¹⁹F NMR (376 MHz, DMSO-*d*₆) δ -71.3, -72.1.

(1R,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-methanesulfonyloxy-methyl-5-trifluoroacetyl-2-oxa-5-azabicyclo[2.2.1]heptane (44): Fully protected nucleoside **42** (19.63 g, 30.4 mmol) was co-evaporated with 1,2-DCE (3 × 100 mL) and dissolved in anhydrous CH₂Cl₂ (610 mL). The solution was cooled to -78 °C, followed by addition of BCl₃ (1M solution in hexanes, 370 mL, 370 mmol). The reaction mixture was stirred for 17h during which it was allowed to heat to rt. The reaction mixture was subsequently cooled to 0 °C and crushed ice (800 mL) was added. The phases were separated and the organic phase was washed with sat. aq. NaHCO₃ (2 × 300 mL). The aqueous phase was back-extracted with EtOAc (4 × 500 mL), and the combined organic phase was evaporated under reduced pressure. The resulting residue was purified using silica gel column

- chromatography (0-20% MeOH in CH₂Cl₂, v/v) affording target alcohol **44** as a white solid (14.65 g, 87%). *R*_f: 0.3 (50% acetone in CH₂Cl₂, v/v). Physical data for the mixture of rotamers: MALDI-HRMS *m/z* 579.0871 ([M + Na]⁺, C₂₁H₁₉F₃N₆O₇S·Na Calc. 579.0880); ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.21 (s, 1H, NH, ex), 8.76 (s, 0.5H, A^{Bz}), 8.75 (s, 0.5H, A^{Bz}), 8.61 (s, 0.5H, A^{Bz}), 8.56 (s, 0.5H, A^{Bz}), 8.04 (d, 2H, *J* = 7.7 Hz, Bz), 7.51-7.67 (m, 3H, Bz), 6.83 (d, 0.5, *J* = 1.37 Hz, H-1'), 6.80 (d, 0.5H, *J* = 1.37 Hz, H-1'), 6.73 (d, 0.5H, *J* = 4.12 Hz, 3'-OH, ex), 6.69 (d, 0.5H, *J* = 4.12 Hz, 3'-OH, ex), 4.91 (br s, 0.5H, H-2'), 4.81 (d, 0.5H, *J* = 4.12 Hz, H-3'), 4.76 (d, 0.5H, *J* = 4.12 Hz, H-3'), 4.70 (br s, 0.5H, H-2'), 4.60-4.67 (m, 2H, H-5'), 4.46 (d, 0.5H, *J* = 10.3 Hz, H-5'), 4.26 (d, 0.5H, *J* = 11.7 Hz, H-5'), 4.03 (d, 0.5H, *J* = 10.3 Hz, H-5'), 3.86 (d, 0.5H, *J* = 11.7 Hz, H-5'), 3.29 (s, 3H, OMs); ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 165.5, 155.3 (q, *J* = 36.6 Hz, COCF₃), 155.1 (q, *J* = 36.6 Hz, COCF₃), 151.7 (A^{Bz}), 151.5 (A^{Bz}), 150.3, 150.2, 141.2 (A^{Bz}), 141.0 (A^{Bz}), 133.3, 132.4, 128.4, 125.4, 125.2, 115.5 (q, *J* = 288 Hz, CF₃), 115.2 (q, *J* = 288 Hz, CF₃), 87.0 (C-4'), 85.7 (C-4'), 84.7 (C-1'), 83.8 (C-1'), 72.5 (C-3'), 70.6 (C-3'), 65.7 (C-5'), 65.4 (C-5'), 65.3 (C-2'), 63.6 (C-2'), 52.7 (C-5'), 52.6 (C-5'), 36.97 (OMs). ¹⁹F NMR (376 MHz, DMSO-*d*₆) δ -71.1, -72.1.
- (1*S*,3*R*,4*S*,7*R*)-3-(6-Benzoyladenine-9-yl)-1-benzoylmethyl-7-hydroxy-5-trifluoro-acetyl-2-oxa-5-azabicyclo[2.2.1]heptane (46):** Alcohol **44** (5.8 g, 10.4 mmol) was dissolved in DMF (100 mL) whereupon NaOBz (2.99 g, 20.8 mmol) and 15-crown-5 (2.07 mL, 10.4 mmol) were added. The mixture was heated to 90 °C and stirred for 5h, then allowed to cool to rt. and stirred for an additional 18h. Subsequently the mixture was concentrated in-vacuo and taken up in EtOAc and brine. The phases were separated and the aqueous phase extracted with EtOAc (4 × 200 mL). The combined organic phase was evaporated under reduced pressure affording a dark brown solid material which was purified by silica gel column chromatography (0-3.5% i-PrOH in CHCl₃, v/v) to afford target benzoyl protected alcohol **46** (5.05 g, 83%) as a white foam. *R*_f = 0.4 (10% i-PrOH in CHCl₃, v/v). Physical data for the mixture of rotamers: MALDI-HRMS *m/z* 605.1337 ([M + Na]⁺, C₂₇H₂₁F₃N₆O₆·Na Calc. 605.1367); ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.18 (br s, 1.7H, NH_{a,b}, ex), 8.77 (s, 1H, A^{Bz}_a), 8.76 (s, 0.7H, A^{Bz}_b), 8.67 (s, 0.7H, A^{Bz}_b), 8.63 (s, 1H, A^{Bz}_b), 8.09-8.18 (m, 4H, Bz), 8.02-8.09 (m, 4H, Bz), 7.51-7.76 (m, 13.5H, Bz), 6.89 (d, 1H, *J* = 1.65 Hz, H1'_a), 6.86 (d, 0.7H, *J* = 1.65 Hz, H1'_b), 6.67 (d, 1H, *J* = 4.12 Hz, 3'-OH_a, ex), 6.63 (d, 0.7H, *J* = 4.12 Hz, 3'-OH_b, ex), 4.89-4.96 (m, 2.4H, H3'_{a,b}, H2'_b), 4.74-4.82 (dd, 2H, *J* = 6.04 Hz, *J* = 12.65 Hz, H5''_a), 4.68-4.72 (m, 1H, H2'_a), 4.58-4.66 (dd, 1.4H, *J* = 6.04 Hz, *J* = 12.65 Hz, H5''_b), 4.55 (d, 0.7H, *J* = 10.7 Hz, H5'_b), 4.38 (d, 1H, *J* = 11.53 Hz, H5'_a), 4.10 (d, 0.7H, *J* = 10.7 Hz, H5'_b), 3.93 (d, 1H, *J* = 11.53 Hz, H5'_a). ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 166.5, 165.3, 155.1 (q, COCF₃), 151.9 (A^{Bz}), 151.7 (A^{Bz}), 151.1, 140.3 (A^{Bz}), 140.1 (A^{Bz}), 134.5, 134.4, 133.5 (Bz), 131.9 (Bz), 129.55 (Bz), 129.54 (Bz), 129.18, 129.17, 128.7 (Bz), 128.4 (Bz), 128.2 (Bz), 125.4, 125.2, 115.5 (q, CF₃), 87.3 (C-4'), 86.0 (C-4'), 84.4 (C-1'_b), 83.6 (C-1'_a), 72.9 (C-3'_a), 71.0 (C-3'_b), 65.3 (C-2'_b), 63.6 (C-2'_a), 60.7 (C-5''_a), 60.2 (C-5''_b), 53.0 (C-5'_a), 52.9 (C-5'_b). ¹⁹F NMR (376 MHz, DMSO-*d*₆) δ -71.1, -72.0.

(1S,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-hydroxymethyl-2-oxa-5-

azabicyclo[2.2.1]heptane (48): Alcohol **46** (3.41 g, 5.85 mmol) was dissolved in 1,4-dioxane:H₂O (225 mL, 2:1, v/v) and cooled to 0 °C, whereafter aq. NaOH (2M, 17.5 mL, 35 mmol) was added.

- 5 The reaction mixture was stirred for 2h, whereupon sat. aq. NH₄Cl (25 mL) was added, and the mixture was then evaporated to dryness. The resulting crude was adsorbed on silica gel and purified by silica gel column chromatography (0-20% MeOH in CH₂Cl₂, v/v) to afford target amino diol **48** as a white solid (1.33 g, 60%). *R*_f = 0.4 (20% MeOH in CH₂Cl₂, v/v). MALDI-HRMS *m/z* 405.1294 ([M + Na]⁺, C₁₈H₁₈N₆O₄·Na⁺ Calc. 405.1282); ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.17 (br s, 1H, NH, ex), 8.73 (s, 1H, A^{Bz}), 8.71 (s, 1H, A^{Bz}), 8.05 (d, 2H, *J* = 8.1 Hz, Bz), 7.49-7.67 (m, 3H, Bz), 6.44 (d, 1H, *J* = 1.92 Hz, H-1'), 5.70 (d, 1H, *J* = 4.0 Hz, 3'-OH, ex), 4.82 (t, 1H, *J* = 5.5 Hz, 5'-OH, ex), 4.30 (d, 1H, *J* = 4.0 Hz, H-3'), 3.69 (d, 2H, *J* = 5.5 Hz, H-5'), 3.51 (s, 1H, H-2'), 3.15 (d, 1H, *J* = 10.6 Hz, H_a-5''), 2.96 (d, 1H, *J* = 10.6 Hz, H_b-5''); ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 165.5 (C=O), 152.0, 151.2 (A^{Bz}), 149.8, 143.2 (A^{Bz}), 133.3, 132.3 (Bz), 128.4 (Bz), 125.2, 91.3 (C-4'), 83.9 (C-1'), 73.4 (C-3'), 62.2 (C-5''), 58.3 (C-2'), 50.6 (C-5').

(1R,3R,4R,7R)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityloxy-methyl)-2-oxa-5-azabicyclo[2.2.1]heptane (50):

Diol **48** (2.47 g, 6.5 mmol) was co-evaporated with anhydrous pyridine (2 × 50 mL), redissolved in anhydrous pyridine (130 mL) and cooled to 0 °C. DMTrCl (3.25 g, 2.7 mmol) was added, and the reaction mixture stirred for 23h during which it was allowed to heat to rt. Subsequently MeOH (20 mL) was added, and the mixture was diluted with EtOAc (200 mL) and washed with sat. aq. NaHCO₃ (2 × 20 mL). After separating the two phases, the aqueous phase was back-extracted with EtOAc (4 × 50 mL), and the combined organic phase was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0-8% MeOH and 1% pyridine in CH₂Cl₂, v/v) and the resulting product was co-evaporated with abs. EtOH:Toluene (2 × 50 mL, 1:1, v/v) affording nucleoside **50** as a white solid (1.51 g, 34%). *R*_f: 0.2 (7% MeOH in CH₂Cl₂, v/v). MALDI-HRMS *m/z* 707.2609 ([M + Na]⁺, C₃₉H₃₆N₆O₄·Na⁺ Calc. 707.2589). ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.15 (br s, 1H, NH, ex), 8.78 (s, 1H, A^{Bz}), 8.73 (s, 1H, A^{Bz}), 8.06 (d, 2H, *J* = 7 Hz, Bz), 7.52 – 7.69 (m, 3H, Bz), 7.18 – 7.46 (m, 9H, DMTr), 6.86-6.93 (m, 4H, DMTr), 6.54 (d, 1H, *J* = 1.92 Hz, H-1'), 5.66 (d, 1H, *J* = 4.67 Hz, 3'-OH, ex), 4.39 (d, 1H, *J* = 4.94 Hz, H-3'), 3.74 (s, 6H, 2 × OMe), 3.48-3.51 (m, 1H, H-2'), 3.31 (d, 1H, *J* = 10.7 Hz, H-5'_a), 3.26 (d, 1H, *J* = 10.7 Hz, H-5'_b), 3.16-3.21 (m, 2H, H-5''_{a,b}), 2.89 (br s, 1H, 2'-NH, ex). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.5, 158.0, 152.2, 151.3 (A^{Bz}), 149.9, 144.8, 143.3 (A^{Bz}), 135.5, 135.4, 133.4, 132.3 (Ar), 129.7 (Ar), 129.68 (Ar), 128.4 (Ar), 128.39 (Ar), 127.8 (Ar), 127.7 (Ar), 126.5 (Ar), 125.1, 113.1 (Ar), 89.6 (C4'), 85.1 (C1'), 84.0, 74.0 (C-3'), 62.2 (C-2'), 61.2 (C5''), 54.9 (OMe), 51.2 (C5').

*A trace impurity of pyridine was identified in ¹³C NMR (149.5 ppm).

(1S,3R,4S,7R)-3-(6-benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityl-oxy-methyl)-5-(9'-fluorenylmethoxycarbonyl)-2-oxa-5-azabicyclo[2.2.1]heptane **51W**: Amino Alcohol **50** (200 mg, 0.29 mmol) was co-evaporated in anhydrous pyridine (2 x 2 mL) and re-dissolved in anhydrous pyridine (1.5 mL). This was cooled to 0°C whereupon 9'-fluorenylmethyl chloroformate (100 mg, 0.38 mmol) was added and allowed to reach rt while stirring for 6h. It was then diluted with EtOAc (30 mL) and washed with sat. aq. NaHCO₃ (30 mL). The aqueous phase was back extracted with EtOAc (25 mL) and the combined organic phases were evaporated to dryness and co-evaporated with 2:1 EtOH:Toluene (2 x 6 mL). The resulting crude was purified by silica gel column chromatography (30-100% EtOAc in Petroleum Ether, v/v) to afford target nucleoside **51W** as a white foam (136 mg, 51%). R_f = 0.4 (EtOAc). Physical data for the mixture of rotamers: MALDI-HRMS *m/z* 929.3241 ([M + Na]⁺, C₅₄H₄₆N₆O₈·Na⁺ Calc. 929.3269); ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.29 (br s, 2H, ex), 8.74 (s, 1H, A^{Bz}), 8.72 (s, 1H, A^{Bz}), 8.55 (s, 1H, A^{Bz}), 8.49 (s, 1H, A^{Bz}), 8.02-8.08 (m, 4H, Bz), 7.77 – 7.88 (m, 6H, Bz), 7.09 – 7.69 (m, 40H, DMT, Fmoc), 6.90-6.95 (m, 8H, DMT), 6.80 (d, 1H, *J* = 1.65 Hz, H-1'), 6.73 (d, 1H, *J* = 1.65 Hz, H-1'), 6.25 (d, 1H, *J* = 4.39 Hz, 3'-OH, ex), 6.22 (d, 1H, *J* = 4.39 Hz, 3'-OH, ex), 4.50 (br s, 1H, H-2'), 4.45 (br s, 1H, H-2'), 4.22 (d, 1H, *J* = 6.86, CH₂Fmoc), 4.15 (d, 1H, *J* = 6.86, CH₂Fmoc), 3.87 (d, 1H, *J* = 6.86, CH₂Fmoc), 3.83 (d, 1H, *J* = 6.86, CH₂Fmoc), 3.73-3.76 (m, 14H, OCH₃, CH (Fmoc)), 3.70 (d, 1H, *J* = 10.5 Hz, H-5'), 3.62 (d, 1H, *J* = 10.5 Hz, H-5'), 3.34-3.43 (m, 6H, H-5'', H-5'). ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 165.6, 158.1, 154.7, 154.6, 151.8 (A^{Bz}), 151.5 (A^{Bz}), 150.3, 150.2, 144.7, 143.7, 143.6, 143.5, 142.8, 141.3 (A^{Bz}), 141.2 (A^{Bz}), 140.6, 140.4, 140.3, 135.3, 135.2, 133.3, 132.4, 129.7, 128.8, 128.4, 127.9, 127.7, 127.6, 127.5, 127.2, 127.1, 126.9, 126.7, 125.4, 125.2, 125.1, 124.9, 124.6, 121.3, 119.98, 113.2, 88.9 (C-4'), 88.4 (C-4'), 85.4 (C-(Ph)₃, DMT), 84.9 (C-1'), 84.7 (C-1'), 72.5 (C-3'), 72.0 (C-3'), 66.7 (CH₂-fmoc), 63.9 (C-2'), 63.4 (C-2'), 60.6 (C-5''), 60.56 (C-5''), 54.96 (OCH₃), 52.7 (C-5'), 52.6 (C-5'), 46.4 (CH, fmoc), 45.9 (CH, fmoc).

Fmoc protected nucleoside phosphoramidite 52W: Nucleoside **51W** (225 mg, 0.25 mmol) was co-evaporated with an. 1,2-DCE (2 x 4 mL) and re-dissolved in anhydrous CH₂Cl₂ (3.5 mL). Anhydrous DIPEA (195 μL, 1.12 mmol) and N-methylimidazole (16 μL, 0.20 mmol) were added followed by dropwise addition of 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite (111 μL, 0.50 mmol). The reaction mixture stirred at rt. for 4h whereupon the crude was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (0-2% MeOH in CH₂Cl₂, v/v, initially built in 0.5% Et₃N) and precipitated from CH₂Cl₂/Petroleum Ether to afford target amidite **52W** as a white foam (126 mg, 46%). Physical data for the mixture of rotamers: R_f = 0.5 (5% MeOH in CH₂Cl₂, v/v); HiResESI *m/z* 1129.4356 ([M + Na]⁺, C₆₃H₆₃N₈O₉P·Na⁺ Calc. 1129.4348); ³¹P NMR (121 MHz, CDCl₃) δ 150.32, 150.26, 150.12, 149.48.

(1R,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityl-oxy-methyl)-5-

(pyren-1-yl)methyl-2-oxa-5-azabicyclo[2.2.1]heptane (51X): Nucleoside **50** (300 mg, 0.44 mmol) was co-evaporated with anhydrous 1,2-DCE (2 × 10 mL), suspended in anhydrous CH₂Cl₂ (4.4 mL), and pyrenecarboxaldehyde (171 mg, 0.74 mmol) and NaBH(OAc)₃ (186 mg, 0.88 mmol) was added.

- 5 The resulting suspension was stirred at rt. for 17h whereupon sat. aq. NaHCO₃ (4 mL) was added and the mixture subsequently diluted with CH₂Cl₂ (20 mL). The two phases were separated and the organic phase was washed with sat. aq. NaHCO₃ (20 mL). The resulting aqueous phase was back-extracted with EtOAc (2 × 20 mL). The combined organic phase was evaporated to dryness, and the resulting residue was purified by silica gel column chromatography (0-99% EtOAc and 1% pyridine
- 10 in petroleum ether, v/v) affording target nucleoside **51X** as a white foam (268 mg, 68%). *R*_f = 0.4 (EtOAc). MALDI-HRMS *m/z* 921.3326 ([M + Na]⁺, C₅₆H₄₆N₆O₆·Na⁺ Calc. 921.3371). ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.13 (s, 1H, NH, ex), 8.46 (s, 1H, A^{Bz}), 8.36 (s, 1H, A^{Bz}), 7.89-8.16 (m, 12H, Ar), 7.51-7.76 (m, 9H, Ar), 7.12-7.36 (m, 14H, Ar), 6.82 (s, 3H, DMT), 6.78 (s, 3H, DMT), 6.42 (s, 1H, H-1'), 6.07 (s, 1H, 3'-OH, ex), 4.66 (d, 1H, *J* = 12.5 Hz, CH₂Py), 4.51 (s, 1H, H-3'), 4.45 (d, 1H, *J* = 12.5 Hz, CH₂Py), 3.64 (s, 6H, 2 × OMe), 3.57 (s, 1H, H-2'), 3.42 (d, 1H, *J* = 9.6 Hz, H-5'), 3.17
- 15 (d, 1H, *J* = 9.6 Hz, H-5'). The signals from H-5''_{A+B} were not identifiable due to signal overlap with water; ¹³C NMR (75.5 MHz, CDCl₃) δ 164.1, 158.6, 151.3 (A^{Bz}), 150.5, 149.6, 148.3, 144.4, 141.8 (A^{Bz}), 136.1, 135.5, 135.4, 133.7, 132.6, 132.3, 131.1, 130.7, 130.5, 130.0, 129.1, 128.7, 128.0, 127.8, 127.6, 127.2, 127.1, 127.0, 126.8, 125.7, 125.0, 124.8, 124.6, 124.4, 124.1, 123.8, 122.9,
- 20 122.6, 113.2 (DMT), 90.2 (C-4') 86.5 (C-(Ph)₃ DMT), 86.1 (C-1'), 65.2 (C-3'), 61.2 (C-2'), 59.2 (C-5''), 57.6 (C-5'), 55.2 (OCH₃), 29.6 (CH₂Py).

(1S,3R,4R,7S)-3-(6-Benzoyladenine-9-yl)-7-[2-cyanothoxy(diisopropylamino)-phosphinoxy]-1-

(4,4'-dimethoxytrityloxymethyl)-5-(pyren-1-yl)methyl-2-oxa-5-azabicyclo[2.2.1]heptane (52X):

- 25 Nucleoside **51X** (220 mg, 0.24 mmol) was co-evaporated with 1,2-DCE (2 × 10 mL), dissolved in 20% DIPEA in anhydrous CH₂Cl₂ (4.4 mL, v/v) and 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite (0.12 mL, 0.54 mmol) was added. The reaction mixture was stirred at rt. for 21h, whereupon additional 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite (0.05 mL, 0.06 mmol) was added. The reaction mixture was then stirred for 4h followed by addition of abs. EtOH (2
- 30 mL). Subsequently the mixture was diluted with CH₂Cl₂ (10 mL) and the organic phase was washed sequentially with sat. aq. NaHCO₃ (10 mL) and brine (10 mL). After separation of the two phases the organic phase was evaporated to dryness and the resulting residue purified by silica gel column chromatography (0-60% EtOAc and 1% pyridine in petroleum ether, v/v). The resulting product was co-evaporated with abs. EtOH:toluene (2 × 10 mL, 1:1, v/v) affording a white solid which was then
- 35 precipitated from HPLC-grade EtOAc/n-hexane affording pure amidite **52X** as a white solid (136 mg, 51%). *R*_f: 0.7 (5% MeOH in CH₂Cl₂, v/v). HiResESI *m/z* 1099.4642 ([M + Na]⁺, C₆₅H₆₃N₈O₇P·Na⁺ Calc. 1099.4630). ³¹P NMR (121 MHz, CDCl₃) δ 151.3, 149.0.

(1*S*,3*R*,4*S*,7*R*)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityl-oxy-methyl)-5-(pyren-1-yl)carbonyl-2-oxa-5-azabicyclo[2.2.1]heptane (**51Y**): Amino alcohol **50** (407 mg, 0.59 mmol) was co-evaporated with anhydrous 1,2-DCE (2 × 10 mL), dissolved in anhydrous CH₂Cl₂ (11.9 mL), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochlorid (EDC·HCl, 226 mg, 1.19 mmol) and 1-pyrenylcarboxylic acid (293 mg, 1.19 mmol) was added. The reaction mixture was stirred at rt. for 45h, whereafter it was diluted with CH₂Cl₂ (50 mL) and washed with water (20 mL). The two phases were separated and the aqueous phase back-extracted with CH₂Cl₂ (3 × 50 mL). The combined organic phase was evaporated under reduced pressure and the resulting residue was purified by silica gel column chromatography (0-99% EtOAc and 1% pyridine in petroleum, v/v). The resulting product was co-evaporated with abs. EtOH:toluene (2 × 50 ml, 1:1, v/v) affording target nucleoside **51Y** as a yellow solid (343 mg, 64%). Physical data for the mixture of rotamers: *R*_f: 0.5 (5% MeOH: CH₂Cl₂, v/v); MALDI-HRMS *m/z* 935.3138 ([M + Na]⁺, C₅₆H₄₄N₆O₇·Na⁺ Calc. 935.3164). Unknown impurity is identified at 6.22 ppm in ¹H-NMR. ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.21 (s, 1H, NH, ex), 11.20 (s, 1H, NH, ex), 8.55 (s, 1H, A^{Bz}), 8.53 (s, 1H, A^{Bz}), 8.45 (s, 1H, A^{Bz}), 8.40 (s, 1H, A^{Bz}), 7.97 – 8.23 (m, 10H, Ar), 7.56 – 7.84 (m, 7H, Ar), 7.18 – 7.44 (m, 10H, Ar), 7.09 (s, 1H, Ar), 7.06 (s, 1H, Ar), 6.82 – 6.90 (m, 4H, DMTr), 6.50 (d, 1H, *J* = 2.2 Hz, H-1'), 6.40 (d, 1H, *J* = 1.8 Hz, H-1'), 6.15 (d, 1H, *J* = 4.0 Hz, 3'-OH, ex), 6.07 (d, 1H, *J* = 3.7 Hz, 3'-OH, ex), 4.42 – 4.77 (m, 4H, H-3', H-5'), 3.73 (s, 12H, 2 × OMe), 3.66 (s, 2H, H-2'), 3.41 – 3.44 (d, 1H, *J* = 13.6 Hz, H-5''_a), 3.15 – 3.20 (d, 2H, *J* = 13.6 Hz, H-5''_b); ¹³C NMR (75.5 MHz, DMSO-*d*₆) δ 165.5, 157.9, 157.7, 151.7, 150.98, 149.8, 148.2, 144.7, 142.8, 140.1, 135.4, 135.3, 133.5, 133.1, 132.3, 130.5, 130.2, 129.9, 129.6, 128.8, 128.6, 128.4, 127.8, 127.6, 127.5, 127.3, 127.2, 126.9, 126.5, 126.3, 125.9, 125.5, 124.9, 124.8, 124.78, 124.2, 123.8, 123.7, 123.1, 113.1, 112.7, 92.0, 90.3, 85.1, 84.7, 79.8, 75.3, 74.7, 66.4, 61.2, 59.1, 58.6, 58.3, 58.2, 54.6.

(1*S*,3*R*,4*S*,7*R*)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityl-oxy-methyl)-5-(pyren-1-yl)carbonyl-2-oxa-5-azabicyclo[2.2.1]heptanes (**51Y**): Pyrene-1-carboxylic acid (127 mg, 0.50 mmol), 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 166 mg, 0.44 mmol), and diisopropylethylamine (DIPEA, 0.15 mL, 0.87 mmol) were dissolved in anhydrous DMF (3 mL) and allowed to stir at rt for 1h then cooled to 0°C. Amino alcohol **50** (230 mg, 0.34 mmol) was co-evaporated with anhydrous 1,2-DCE (2 × 3 mL) and redissolved in anhydrous DMF (3 mL) and added to the cooled reaction mixture. After 5 h stirring, the mixture was diluted with EtOAc (30 mL) and washed with water (3 x 20 mL). The organic layer was evaporated to dryness and the resulting crude purified by silica gel column chromatography (0 – 4% MeOH in CH₂Cl₂, v/v) affording compound **51Y** as a white solid (227 mg, 74%). Physical data for the mixture of rotamers: *R*_f: 0.5 (5% MeOH: CH₂Cl₂, v/v); MALDI-HRMS *m/z* 935.3138 ([M + Na]⁺, C₅₆H₄₄N₆O₇·Na⁺ Calc. 935.3164). ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.32 (br s, 1.3H, NH,

ex), 8.85 (s, 0.3H, A^{Bz}), 8.79 (s, 1.3H, A^{Bz}), 8.75 (s, 0.3H, A^{Bz}), 8.02-8.39 (m, 13.7H, Ar), 7.82 (m, 0.6H, Ar), 7.49-7.75 (m, 6H, Ar), 7.10-7.42 (m, 9.7H, Ar), 6.93-6.98 (m, 4H, DMT_a), 6.92 (d, 0.3H, *J* = 1.92 Hz, H-1'_b), 6.72-6.78 (m, 1.2H, DMT_b), 6.54 (d, 0.3H, *J* = 3.57 Hz, 3'-OH_b, ex), 6.48 (d, 1H, *J* = 4.94 Hz, 3'-OH_a, ex), 6.44 (s, 1H, H-1'_a), 5.22 (s, 0.3H, H-2'_b), 4.87 (d, 0.3H, *J* = 3.56 Hz, H-3'_b), 4.61 (d, 1H, *J* = 5.21 Hz, H-3'_a), 4.36 (d, 1H, *J* = 12.08 Hz, H-5'_a) 4.02 (d, 1H, *J* = 12.08 Hz, H-5'_a), 3.77 (s, 7H, 2 x (OCH₃)_a, H-2'_a), 3.69 (d, 0.3H, *J* = 10.43 Hz, H-5'_b), 3.66 (s, 1H, (OCH₃)_b), 3.64 (s, 1H, (OCH₃)_b), 3.47 (s, 2H, H5''_a), 3.39 (d, 0.3H, *J* = 10.43 Hz, H-5'_b), 3.18 (s, 0.6H, H-5''_b). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 169.5, 169.1, 165.8, 165.6, 158.0, 157.9, 152.0, 151.6, 151.23, 151.21, 150.3, 144.7, 144.5, 141.5 (A^{Bz}), 141.4 (A^{Bz}), 135.4, 135.2, 135.15, 135.11, 133.48, 132.5 (Ar), 132.4 (Ar), 131.3, 130.9, 130.6, 130.5, 130.4, 130.1, 129.9 (Ar), 129.8 (Ar), 129.7 (Ar), 129.6 (Ar), 128.8 (Ar), 128.7, 128.6 (Ar), 128.5 (Ar), 128.49 (Ar), 128.47, 128.2 (Ar), 128.1 (Ar), 127.9 (Ar), 127.8 (Ar), 127.7 (Ar), 127.6 (Ar), 127.1 (Ar), 126.9 (Ar), 126.7 (Ar), 126.6 (Ar), 126.5 (Ar), 125.9 (Ar), 125.8 (Ar), 125.6 (Ar), 125.5 (Ar), 124.8 (Ar), 124.2 (Ar), 124.0, 123.9 (Ar), 123.5, 123.3, 123.1, 113.2 (DMT), 113.06 (DMT), 113.04 (DMT), 88.7 (C-4'_a), 88.5 (C-4'_b), 85.4 (C-(Ph)₃, DMT_a), 85.3 (C-(Ph)₃, DMT_b), 85.0 (C-1'_b), 84.7 (C-1'_a), 72.5 (C-3'_a), 71.8 (C-3'_b), 65.6 (C-2'_b), 62.8 (C-2'_a), 60.5 (C-5''_a), 60.0 (C-5''_b), 55.0 (OCH₃), 54.8 (OCH₃), 54.0 (C-5'_b), 52.2 (C-5'_a).

(1*S*,3*R*,4*S*,7*R*)-3-(6-Benzoyladenine-9-yl)-7-[2-cyanothoxy(diisopropylamino)-phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-5-(pyrene-1-yl)carbonyl-2-oxa-5-azabicyclo[2.2.1]heptane (52Y): Nucleoside **51Y** (300 mg, 0.33 mmol) was co-evaporated with 1,2-DCE (2 × 5 mL), dissolved in anhydrous CH₂Cl₂ (2.7 mL) and anhydrous DIPEA (0.66 mL), and 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite (0.11 mL, 0.5 mmol) was added. The reaction mixture stirred at rt. for 22h. whereafter abs. EtOH (2 mL) was added. The mixture was subsequently taken up in CH₂Cl₂ (50 mL) and washed with sat. aq. NaHCO₃ (50 mL) and brine (50 mL). The combined aqueous phase was back-extracted with CH₂Cl₂ (2 × 20 mL) and the combined organic phase was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography (0-99% EtOAc and 1% pyridine in petroleum ether, v/v) and the resulting product co-evaporated with abs. EtOH:toluene (2 × 50 mL, 1:1, v/v) and precipitated from HPLC-grade EtOAc/n-hexane to afford target amidite **52Y** as a white foam (247 mg, 67%). Physical data for the mixture of rotamers: *R*_f = 0.5 (EtOAc); HiResESI *m/z* 1113.4462 ([M + Na]⁺, C₆₅H₆₁N₈O₈P·Na⁺ Calc.1113.4422); ³¹P NMR (121 MHz, CDCl₃) δ 152.3, 151.8, 150.2.

(1*S*,3*R*,4*S*,7*R*)-3-(6-Benzoyladenine-9-yl)-7-hydroxy-1-(4,4'-dimethoxytrityl-oxy-methyl)-5-(pyrene-1-yl)acetyl-2-oxa-5-azabicyclo[2.2.1]heptane 51Z: Amino alcohol **50** (250 mg, 0.37 mmol) was co-evaporated with anhydrous 1,2-DCE (2 × 5 mL), dissolved in anhydrous CH₂Cl₂ (10 mL), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochlorid (EDC·HCl, 108 mg, 0.55 mmol) and 1-pyreneacetic acid (145 mg, 0.55 mmol) was added. The reaction mixture was stirred at rt. for

2.5h, whereafter it was diluted with CH₂Cl₂ (20 mL) and washed with water (2 x 10 mL). The two phases were separated and the aqueous phase back-extracted with CH₂Cl₂ (10 mL). The combined organic phase was evaporated under reduced pressure and the resulting residue was purified by silica gel column chromatography (0-4% MeOH in CH₂Cl₂, v/v) affording target nucleoside **51Z** as a white solid (266 mg, 79%). Physical data for the mixture of rotamers: *R*_f: 0.5 (10% MeOH:CH₂Cl₂, v/v); MALDI-HRMS *m/z* 949.3321 ([M + Na]⁺, C₅₇H₄₆N₆O₇·Na⁺ Calc. 949.3320). ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.29 (br s, 1.5H, NH, ex), 8.96 (s, 1H, A^{Bz}_a), 8.73 (s, 0.5H, A^{Bz}_b), 8.71 (s, 1H, A^{Bz}_a), 8.55 (s, 0.5H, A^{Bz}_b), 7.92-8.33 (m, 16H, Py, Bz), 7.83 (m, 1H, Py, Bz), 7.60-7.79 (m, 4.5H, Py, Bz), 7.44-7.54 (m, 3.5H, DMT), 7.20-7.40 (m, 10.5H, DMT), 6.88-6.98 (m, 6H, DMT), 6.82 (d, 0.5H, *J* = 1.65 Hz, H-1'_b), 6.79 (d, 1H, *J* = 1.37 Hz, H-1'_a), 6.31 (d, 0.5H, *J* = 4.39 Hz, 3'-OH_b, ex), 6.19 (d, 1H, *J* = 4.39 Hz, 3'-OH_a, ex), 5.07 (s, 0.5H, H-2'_b), 4.71-4.74 (m, 1.5H, H-2'_a, H-3'_b), 4.67 (d, 1H, *J* = 10.43, H-5'_{1a}), 4.63 (d, 1H, *J* = 4.39 Hz, H-3'_a), 4.57 (d, 1H, *J* = 16.2 Hz, CH₂Py_a), 4.38 (d, 1H, *J* = 10.2 Hz, CH₂Py_a), 4.11 (d, 0.5H, *J* = 16.2 Hz, CH₂Py_b), 3.95-4.04 (m, 1.5H, H-5'_{1b}, H-5'_{2a}), 3.66-3.77 (m, 9.5H, OCH₃, H-5'_{2b}), 3.34-3.51 (m, 3.5H, H-5''_{a,b}, CH₂Py_b). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 172.7, 169.8, 169.6, 165.5, 165.4, 158.1, 158.0, 151.9, 151.7 (A^{Bz}), 151.5, 151.4 (A^{Bz}), 150.5, 150.2, 144.7, 144.6, 141.7 (A^{Bz}), 141.5 (A^{Bz}), 135.5, 135.2, 133.5, 133.4, 132.5, 130.8, 130.7, 130.6, 130.3, 130.2, 130.1, 129.9, 129.88, 129.82, 129.78, 129.77, 129.74, 129.68, 129.67, 129.66, 129.62, 129.4, 129.2, 128.9, 128.8, 128.7, 128.6, 128.5, 128.5, 128.2, 127.8, 127.77, 127.72, 127.4, 127.3, 127.27, 127.25, 127.1, 126.9, 126.8, 126.7, 126.6, 126.2, 126.0, 125.9, 125.4, 125.3, 125.2, 125.0, 124.9, 124.8, 124.76, 124.72, 124.6, 124.5, 124.4, 124.3, 124.0, 123.9, 123.8, 123.78, 123.76, 123.72, 123.6, 123.2, 113.2 (DMT), 89.0 (C-4'), 88.4 (C-4'), 85.47 (C-(Ph)₃), 85.40 (C-(Ph)₃), 84.7 (C-1'_a), 84.6 (C-1'_b), 72.9 (C-3'_b), 72.0 (C-3'_a), 64.5 (C-2'_b), 61.8 (C-2'_a), 60.9 (C-5''_a), 60.8 (C-5''_b), 55.0 (OCH₃), 54.8 (OCH₃), 52.8 (C-5'_a), 52.3 (C-5'_b), 38.7, 37.8 (CH₂Py_a), 37.7 (CH₂Py_b). *Trace impurities of residual 1-pyreneacetic acid were identified in ¹³CNMR (C=O at 172 ppm, CH₂ at 38.7 ppm, plus extra pyrene peaks, couples to impurities in HNMR (4.35 ppm is extra CH₂Py peak that couples to 38.7 in HSQC).

(1S,3R,4S,7R)-3-(6-Benzoyladenine-9-yl)-7-[2-cyanothoxy(diisopropylamino)-phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-5-(pyren-1-yl)acetyl-2-oxa-5-azabicyclo[2.2.1]heptane (52Z):

Nucleoside **51Z** (235 mg, 0.25 mmol) was co-evaporated with an. 1,2-DCE (2 x 5 mL) and re-dissolved in anhydrous CH₂Cl₂ (5 mL). Anhydrous DIPEA (220 μL, 1.27 mmol) was added followed by dropwise addition of 2-cyanoethyl-*N,N'*-(diisopropyl)-phosphoramidochloridite (115 μL, 0.51 mmol). The reaction mixture stirred at rt. for 22h. whereafter abs. EtOH (1 mL) was added. The crude was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (0-2% MeOH in CH₂Cl₂, v/v) and precipitated from CH₂Cl₂/Petroleum Ether to afford target amidite **52Z** as a white foam (203 mg, 71%). Physical data for the mixture of rotamers:

$R_f = 0.6$ (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 1149.4358 ($[\text{M} + \text{Na}]^+$, $\text{C}_{66}\text{H}_{63}\text{N}_8\text{O}_8\text{P}\cdot\text{Na}^+$ Calc. 1149.4399); ^{31}P NMR (121 MHz, CDCl_3) δ 150.39, 150.31, 150.26, 148.09.

Synthesis of probes with locked monomers 124W-124Y: Syntheses of probes containing incorporations of locked phosphoramidites **52W**, **52X**, and **52Y** were performed on an automated DNA synthesizer (0.2 μmol scale) using the following hand coupling conditions (activator; coupling time; approximate coupling yield): Monomer **124W** (pyridinium hydrochloride; 30 min; ~82%), monomers **124X** and **124Y** (pyridinium hydrochloride; 15 min; ~95%). The probes were deprotected using 32% aq. NH_3 at 55 °C for 2h. Purification of probes (till at least 75% purity) was performed by RP-HPLC (DMT-ON), followed by detritylation (80% aq. AcOH, 20 min) and precipitation (abs. EtOH, -18 °C, 12 h). RP-HPLC purification of oligonucleotides was performed using a Waters Prep LC 4000 system equipped with an Xterra MS C18-column (10 μm , 300 mm $\times$ 7.8 mm). A representative RP-HPLC gradient protocol for purification of oligonucleotides with DMT-ON is to use an isocratic hold of 100% A-buffer for 5 min followed by a linear gradient to 55% B-buffer over 75 min at a flow rate of 1.0 mL/min (A-buffer: 95% 0.1 M NH_4HCO_3 , 5% CH_3CN ; B-buffer: 25% 0.1 M NH_4HCO_3 , 75% CH_3CN). The composition of the probes was verified by MALDI-MS analysis (Table 32) whereas the purity (>80%, unless stated otherwise) was verified by ion-exchange HPLC using a LaChrom L-7000 system (VWR International) equipped with a Gen-Pak Fax column (100 mm $\times$ 4.6 mm). A representative protocol involves the use of an isocratic hold of 95% A-buffer for 5 min, followed by a linear gradient to 70% B-buffer over 41 min at a flow rate of 0.75 mL/min (A-buffer: 25 mM Tris-Cl, 1 mM EDTA, pH 8.0; B-buffer: 1 M NaCl).

Table 32
MS-data of representative single-stranded probe modified with adenine monomers
124X (= K) or 124Y (= L).^a

ONs	Sequence	Calc. m/z	Found m/z
124X6	5'-GTG <u>K</u> TA TGC	2995	2995
124X7	5'-GTG AT <u>K</u> TGC	2995	2993
124X8	3'-CAC T <u>K</u> T ACG	2924	2923
124X9	3'-CAC TAT <u>K</u> CG	2924	2923
124Y6	5'-GTG <u>L</u> TA TGC	3009	3008
124Y7	5'-GTG AT <u>L</u> TGC	3009	3009
124Y8	3'-CAC T <u>L</u> T ACG	2937	2937
124Y9	3'-CAC TAT <u>L</u> CG	2937	2936

Thermal Affinity of probes modified with monomers 124W/X/Y: The thermal affinity of probes toward complementary DNA or RNA targets and of probe duplexes was evaluated via UV thermal denaturation experiments ($[\text{Na}^+] = 110 \text{ mM}$, Tables 33 and 34. Changes in thermal denaturation temperatures (T_m -values) of modified duplexes are discussed relative to T_m -values of unmodified reference duplexes, unless otherwise stated. Single-stranded probes that are modified with monomer **124W** show similar thermal affinity toward single-stranded DNA as toward single-stranded RNA targets. Single-stranded probes that are modified with **124X** and **124Y** show higher thermal affinity toward single-stranded DNA than toward single-stranded RNA targets (Tables 33 and 34). This DNA selectivity makes these monomers useful as probes for targeting double stranded DNA.

Table 33

 T_m -Values of Duplexes Between B6-B11 and Complementary DNA Targets^a

ON	Duplex	B=	T_m [$\Delta T_m/\text{mod}$] ($^{\circ}\text{C}$)			
			T	124W	124X	124Y
B6	5'-GTG <u>B</u> TA		27.5	27.0	32.5	38.5
D2	TGC			[-0.5]	[+5.0]	[+11.0]
	3'-CAC TAT					
	ACG					
B7	5'-GTG AT <u>B</u>		27.5	28.0	34.5	41.5
D2	TGC			[+0.5]	[+7.0]	[+14.0]
	3'-CAC TAT					
	ACG					
B10	5'-GTG <u>B</u> T <u>B</u>		27.5	26.5	32.5	41.0
D2	TGC			[-0.5]	[+2.5]	[+6.8]
	3'-CAC TAT					
	ACG					
D1	5'-GTG ATA		27.5	27.5	34.0	39.0
B8	TGC			[+0.0]	[+6.5]	[+11.5]
	3'-CAC T <u>B</u> T					
	ACG					
D1	5'-GTG ATA		27.5	27.5	33.0	29.5
B9	TGC			[+0.0]	[+5.5]	[+12.0]
	3'-CAC TAT					
	<u>B</u> CG					
D1	5'-GTG ATA		27.5	25.5	32.5	41.0
B11	TGC			[-1.0]	[+2.5]	[+6.8]
	3'-CAC T <u>B</u> T					
	<u>B</u> CG					

^a ΔT_m = change in T_m 's relative to unmodified reference duplex; T_m 's determined as the maximum of the first derivative of melting 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 $1.0 \mu\text{M}$ of each strand. T_m 's are averages of at least two measurements within $1.0 \text{ }^{\circ}\text{C}$; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer. ND = not determined.

Table 34

 T_m -Values of Duplexes Between B6-B11 and Complementary RNA Targets^a

ON	Duplex	B=	T_m [ΔT_m /mod] (°C)			
			T	124W	124X	124Y
B6 R2	5'-GTG <u>B</u> TA TGC 3'-CAC UAU ACG		26.0	27.0 [+1.0]	25.0 [-1.0]	27.5 [+1.5]
B7 R2	5'-GTG AT <u>B</u> TGC 3'-CAC UAU ACG		26.0	27.0 [+1.0]	27.0 [+1.0]	31.0 [+5.0]
B10 R2	5'-GTG <u>B</u> T <u>B</u> TGC 3'-CAC UAU ACG		26.0	27.5 [+0.8]	24.5 [-0.8]	30.5 [+2.3]
R1 B8	5'-GUG AUA UGC 3'-CAC T <u>B</u> T ACG		26.0	25.5 [-0.5]	24.0 [-2.0]	27.0 [+1.0]
R1 B9	5'-GUG AUA UGC 3'-CAC TAT <u>B</u> CG		26.0	28.0 [+2.0]	27.0 [+1.0]	31.0 [+5.0]
R1 B11	5'-GUG AUA UGC 3'-CAC T <u>B</u> T <u>B</u> CG		26.0	28.0 [+1.0]	24.5 [-0.8]	31.5 [+2.8]

^aFor conditions of thermal denaturation experiments, see Table 33.

- 5 The Watson-Crick specificity of single-stranded probes modified with monomers **124W**, **124X** and **124Y** (B8-series) was evaluated using DNA (Table 35) or RNA targets (Table 36) that contain mismatched nucleobases opposite to the modification sites. All monomers show excellent discrimination in terms of T_m .

Table 35

10 Discrimination of Mismatched DNA Targets By Probes Modified with Monomers 124W-Y^a

ON	Sequence	B=	DNA: 5'-GTG <u>A</u> <u>B</u> A TGC			
			T_m [°C]	ΔT_m [°C]		
			T	A	C	G
D2	3'-CAC TAT ACG		27.5	-21.0	-16.5	-7.5
124W8	3'-CAC T <u>124W</u> T ACG		27.5	-20.0	-17.0	-16.0
124X8	3'-CAC T <u>124X</u> T ACG		34.0	-15.5	-7.0	-14.5
124Y8	3'-CAC T <u>124Y</u> T ACG		39.0	-21.0	-11.0	-13.5

^aFor conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:DNA duplex.

Table 36
Discrimination of Mismatched RNA Targets by Probes Modified with Monomers 124W-Y.^a

		RNA: 5'-GUG <u>A</u> BA UGC				
ON	Sequence	<u>B</u> =	T_m [°C]	ΔT_m [°C]		
			T	A	C	G
D1	3'-CAC TAT ACG		26.0	-16.0	-15.5	-11.0
124W8	3'-CAC T <u>124W</u> T ACG		25.5	-13.5	-13.0	-11.5
124X8	3'-CAC T <u>124X</u> T ACG		24.0	-10.5	-8.0	-10.0
124Y8	3'-CAC T <u>124Y</u> T ACG		27.0	-11.0	-11.0	-8.5

^aFor conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched RNA:DNA duplex.

5

The specificity of doubly-modified single-stranded probes against centrally mismatched DNA targets was also evaluated (Table 37). Decreased mismatch discrimination compared to unmodified reference strand **D1** was observed.

10

Table 37
Discrimination of Mismatched DNA Targets by Probes Modified with Monomers 124X/Y^A

		DNA : 3'-CAC T <u>B</u> T ACG				
ON	Sequence	<u>B</u> =	T_m [°C]	ΔT_m [°C]		
			A	T	C	G
D1	5'-GTG ATA TGC		27.5	-16.5	-16.5	-8.0
124X10	5'-GTG <u>124X</u> T <u>124X</u> TGC		32.5	+2.0	+2.0	+0.5
124Y10	5'-GTG <u>124Y</u> T <u>124Y</u> TGC		41.0	-2.0	-5.0	-2.0

^a For conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:DNA duplex. ND = not determined

15

Absorption maxima in the 300-400 nm region for single-stranded probes modified with monomers **124X** and **124Y** in the presence or absence of complementary DNA/RNA targets are given below (Table 38).

20

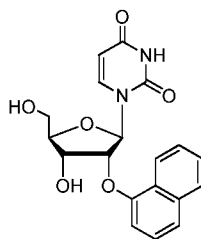
Table 38
Absorption Maxima in The 300-400 Nm Region for Single-Stranded Probes Modified with Monomers 124X And 124Y in The Presence or Absence of Complementary DNA/RNA Targets.^a

		<u>B</u> =	$\lambda_{\text{max}}[\Delta\lambda_{\text{max}}]$ (nm)					
			124X			124Y		
ON	Sequence		ss	+DNA	+RNA	ss	+DNA	+RNA
B6	5'-GTG B TA TGC	348	350 [+2]	350 [+2]	349	352 [+3]	351 [+2]	
B7	5'-GTG AT B TGC	348	350 [+2]	349 [+1]	349	353 [+4]	351 [+2]	
B10	5'-GTG B TB TGC	347	349 [+2]	348 [+1]	349	351 [+2]	351 [+2]	

B8	3'-CAC TBT ACG	348	350 [+2]	348 [+0]	347	353 [+6]	352 [+5]
B9	3'-CAC TAT BCG	348	350 [+2]	350 [+2]	350	351 [+1]	350 [+0]
B11	3'-CAC TBT BCG	348	350 [+2]	348 [+0]	348	352 [+4]	353 [+5]

^aMeasurements were performed using a spectrophotometer and quartz optical cells with a 1.0 cm path length. Buffer conditions are as for thermal denaturation experiments.

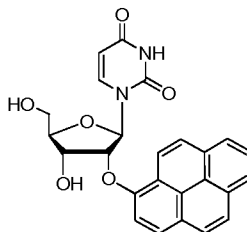
- 5 **General protocol for coupling between 1 and phenols (ArOH) to prepare 72W/72X (description for ~1.33 mmol scale):** The appropriate phenol and 2,2'-anhydrouridine **70** were placed in a sealed pressure tube (specific quantities of substrates and reagents given below) and heated (165 °C for **72W**; 175 °C for **72X**) until analytical TLC indicated full conversion (~2h). The resulting crude was purified by silica gel column chromatography (2-4% MeOH in CH₂Cl₂, v/v) to afford nucleoside
- 10 **72W/72X** (yields specified below).



72W

- 2'-O-(Naphth-2-yl)uridine (72W):** A mixture of 2,2'-anhydrouridine **70** (1.00 g, 4.42 mmol) and 2-naphthol (2.40 g, 22.1 mmol) were reacted and purified as described above to afford nucleoside **72W**
- 15 (0.41 g, 25 %) as a light brown solid. *R*_f: 0.4 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 393.1039 ([M+Na]⁺, C₁₉H₁₈N₂O₆Na⁺, Calc. 393.1057); ¹H NMR (DMSO-*d*₆) δ 11.34 (s, 1H, ex, NH), 8.03 (1H, d, *J* = 8.1 Hz, H6), 7.82-7.85 (ap d, 2H, Nap), 7.73-7.75 (1H, d, *J* = 8.3 Hz, Nap), 7.44-7.48 (ap t, 1H, Nap), 7.41 (d, 1H, *J* = 2.5 Hz, Nap), 7.34-7.37 (ap t, 1H, Nap), 7.23-7.25 (dd, 1H, *J* = 9.1 Hz, *J* = 2.5 Hz, Nap), 6.14 (d, 1H, *J* = 4.9 Hz, H1'), 5.68 (d, 1H, *J* = 8.1 Hz, H5), 5.46 (d,
- 20 1H, ex, *J* = 6.4 Hz, 3'-OH), 5.25 (t, 1H, ex, *J* = 5.2 Hz, 5'-OH), 5.02 (ap t, 1H, H2'), 4.43-4.46 (m, 1H, H3'), 4.02-4.05 (m, 1H, H4'), 3.74-3.78 (m, 1H, H5'), 3.65-3.69 (m, 1H, H5'); ¹³C NMR (DMSO-*d*₆) δ 162.9, 155.4, 150.5, 140.4 (C6), 133.9, 129.2 (Nap), 128.7, 127.4 (Nap), 126.6 (Nap), 126.4 (Nap), 123.8 (Nap), 118.9 (Nap), 108.9 (Nap), 102.0 (C5), 86.4 (C1'), 85.2 (C4'), 79.3 (C2'), 68.2 (C3'), 60.4 (C5').

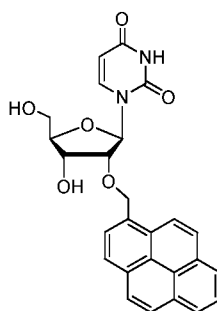
25



72X

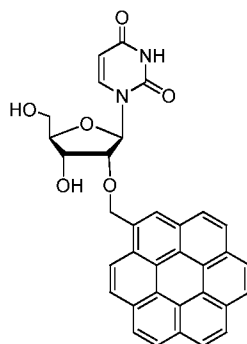
2'-O-(Pyren-1-yl)uridine (72X): A mixture of 2,2'-anhydrouridine **70** (0.30 g, 1.33 mmol) and 1-pyrenol¹⁹ (0.86 g, 3.97 mmol) were reacted and purified as described above to afford nucleoside **72X** (0.26 g, 44 %) as a pale yellow solid. *R*_f: 0.4 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 467.1217 ([M+Na]⁺, C₂₅H₂₀N₂O₆Na⁺, Calc. 467.1214); ¹H NMR (DMSO-*d*₆) δ 11.36 (s, 1H, ex, NH), 8.45 (d, 1H, *J* = 9.3 Hz, Py), 8.20-8.24 (m, 3H, Py), 8.14 (d, 1H, *J* = 9.3 Hz, Py), 7.99-8.09 (m, 4H, H₆, Py), 7.86 (1H, d, *J* = 8.5 Hz, Py), 6.35 (d, 1H, *J* = 4.6 Hz, H1'), 5.68 (d, 1H, *J* = 8.2 Hz, H5), 5.63 (br s, 1H, ex, 3'-OH), 5.30 (br s, 1H, ex, 5'-OH), 5.22-5.25 (ap t, 1H, H2'), 4.55-4.56 (m, 1H, H3'), 4.19-4.21 (m, 1H, H4'), 3.81-3.84 (ap d, 1H, H5'), 3.72-3.75 (ap d, 1H, H5'); ¹³C NMR (DMSO-*d*₆) δ 162.9, 151.7, 150.5, 140.3 (C6), 131.0, 130.9, 127.1 (Py), 126.4 (Py), 125.6 (Py), 125.2, 125.0 (Py), 124.8 (Py), 124.5 (Py), 124.3 (Py), 123.9, 121.1 (Py), 120.1, 111.9 (Py), 102.0 (C5), 86.6 (C1'), 85.4 (C4'), 80.9 (C2'), 68.5 (C3'), 60.4 (C5').

General protocol for coupling between 1 and arylmethyl alcohol (ArCH₂OH) for the preparation of 72Y/72Z (description for ~44.2 mmol scale): The appropriate aromatic alcohol (ArCH₂OH), NaHCO₃ and 1.0M BH₃ in THF were placed in a pressure tube, suspended in anhydrous DMSO and stirred under an argon atmosphere at rt until effervescence ceased (~10 min). At this point, 2,2'-anhydrouridine **70** was added (specific quantities of substrates and reagents given below), the pressure tube was purged with argon and sealed, and the reaction was heated at ~160 °C until analytical TLC indicated full conversion (~3h). At this point, the reaction mixture was poured into water (200 mL), stirred for 30 min and diluted with EtOAc (500 mL). The organic phase was washed with water (4 × 200 mL), evaporated to dryness, and the resulting crude purified by silica gel column chromatography (2-4%, MeOH in CH₂Cl₂, v/v) to afford a residue, which was precipitated from cold acetone to obtain nucleosides **72Y/72Z** (yields specified below).

**72Y**

2'-O-(Pyren-1-yl-methyl)uridine (72Y): 2,2'-Anhydrouridine **70** (10.00 g, 44.2 mmol), pyren-1-ylmethanol (20.5 g, 88.4 mmol), NaHCO₃ (0.73 g, 8.80 mmol), 1.0 M BH₃ in THF (24.5 mL, 22.0 mmol) and anhydrous DMSO (40 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **72Y** (5.04 g, 25 %) as a white solid. *R*_f: 0.4 (10 % MeOH in CH₂Cl₂, v/v);

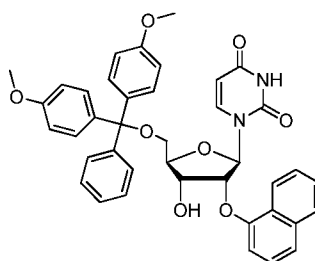
MALDI-HRMS m/z 458.1480 ($[M]^+$, $C_{26}H_{22}N_2O_6^+$, Calc. 458.1472); 1H NMR (DMSO- d_6): δ 11.29 (s, 1H, ex, NH), 8.37-8.39 (d, 1H, J = 9.3 Hz, Py), 8.29-8.31 (m, 2H, Py), 8.24-8.26 (d, 1H, J = 7.7 Hz, Py), 8.17-8.19 (m, 3H, Py), 8.06-8.12 (m, 2H, Py), 7.82 (d, 1H, J = 8.4 Hz, H6), 6.04 (d, 1H, J = 5.1 Hz, H1'), 5.43-5.50 (m, 2H, H5, CH₂Py), 5.37 (d, 1H, ex, J = 5.7 Hz, 3'-OH), 5.28-5.30 (d, 1H, J = 12.0 Hz, CH₂Py), 5.11 (t, 1H, ex, J = 4.9 Hz, 5'-OH), 4.26-4.31 (m, 1H, H3'), 4.18-4.21 (m, 1H, H2'), 3.96-3.97 (m, 1H, H4'), 3.64-3.68 (m, 1H, H5'), 3.59-3.62 (m, 1H, H5'); ^{13}C NMR (DMSO- d_6) δ 162.9, 150.6, 140.1 (C6), 131.4, 130.7, 130.2, 128.7, 127.4 (Py), 127.3 (Py), 127.0 (Py), 126.2 (Py), 125.3 (Py), 124.5 (Py), 124.0 (Py), 123.8, 123.5 (Py), 101.7 (C5), 86.2 (C1'), 85.4 (C4'), 80.9 (C2'), 69.8 (CH₂Py), 68.5 (C3'), 60.6 (C5').

**72Z**

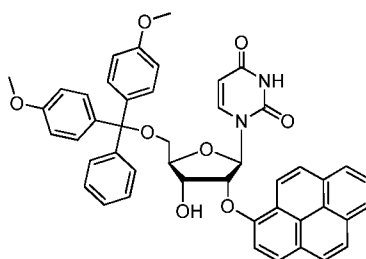
2'-O-(Coronen-1-yl-methyl)uridine (72Z): 2,2'-Anhydrouridine **70** (1.40g, 6.19 mmol), coronen-1-ylmethanol (4.08 g, 12.4 mmol), NaHCO₃ (0.104 g, 1.24 mmol), 1.0 M BH₃ in THF (3.5 mL, 3.1 mmol) and anhydrous DMSO (40 mL) were mixed, reacted, worked up and purified as described above with a minor modification. The precipitate that formed upon pouring the reaction mixture into water was collected by filtration, washed with water (3 × 100 mL) and purified by column chromatography to afford nucleoside **72Z** (450 mg, 13 %) as a pale yellow solid. R_f : 0.4 (10 % MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 579.1537 ($[M+Na]^+$, $C_{34}H_{24}N_2O_6 \cdot Na^+$, Calc. 579.1532); 1H NMR (DMSO- d_6) δ 11.30 (br d, ex, 1H, J = 1.9 Hz, NH), 9.13-9.15 (d, 1H, J = 8.7 Hz, Cor), 8.93-9.03 (m, 10H, Cor), 7.82 (d, 1H, J = 8.0 Hz, H6), 6.18 (d, 1H, J = 4.9 Hz, H1'), 5.85-5.88 (d, 1H, J = 12.1 Hz, CH₂Cor), 5.67-5.69 (d, 1H, J = 12.1 Hz, CH₂Cor), 5.52 (d, 1H, ex, J = 5.5 Hz, 3'-OH), 5.38 (dd, 1H, J = 8.0 Hz, 1.9 Hz, H5), 5.11 (ap t, 1H, ex, 5'-OH), 4.37-4.43 (m, 2H, H2', H3'), 4.03-4.06 (m, 1H, H4'), 3.63-3.71 (m, 2H, H5'); ^{13}C NMR (DMSO- d_6) δ 162.9, 150.6, 140.1 (C6), 132.1, 128.2, 128.1, 127.9, 127.4, 126.5, 126.23 (Cor), 126.20 (Cor), 126.1 (Cor), 126.0 (Cor), 122.5 (Cor), 121.8, 121.5, 121.4, 121.23, 121.17, 101.7 (C5), 86.3 (C1'), 85.5 (C4'), 81.1 (C2'), 70.7 (CH₂Cor), 68.6 (C3'), 60.6 (C5').

General DMTr-protection protocol for the preparation of 74W-74Z (description for ~2.2 mmol scale): The appropriate nucleoside **72** (specific quantities given below) was co-evaporated twice

with anhydrous pyridine (15 mL) and redissolved in anhydrous pyridine. To this was added 4,4'-dimethoxytritylchloride (DMTrCl) and *N,N*-dimethyl-4-aminopyridine (DMAP), and the reaction mixture was stirred at rt until TLC indicated complete conversion (~14h). The reaction mixture was diluted with CH₂Cl₂ (70 mL) and the organic phase sequentially washed with water (2 × 70 mL) and sat. aq. NaHCO₃ (2 × 100 mL). The organic phase was evaporated to near dryness and the resulting crude co-evaporated with absolute EtOH and toluene (2:1, v/v, 3 × 6 mL) and purified by silica gel column chromatography (0-5%, MeOH in CH₂Cl₂, v/v) to afford nucleoside **74** (yields specified below).

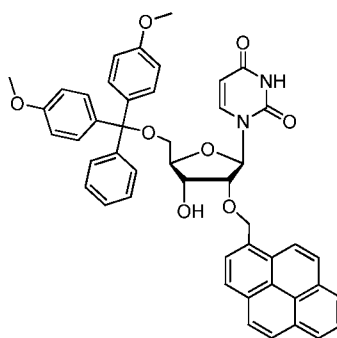
**74W**

2'-O-(Naphth-2-yl)-5'-O-(4,4'-dimethoxytrityl)uridine (74W): Nucleoside **72W** (150 mg, 0.40 mmol), DMTrCl (240 mg, 0.60 mmol) and DMAP (~6 mg) in anhydrous pyridine (7 mL) were mixed, reacted, worked up and purified as described above to afford nucleoside **74W** (120 mg, 47%) as a pale yellow foam. *R*_f: 0.6 (5%, MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 695.2379 ([M+Na]⁺, C₄₀H₃₆N₂O₈Na⁺, Calc. 695.2364); ¹H NMR (DMSO-*d*₆) δ 11.40 (d, 1H, ex, *J* = 2.1 Hz, NH), 7.83-7.86 (m, 3H, H6, Nap), 7.68 (d, 1H, *J* = 8.2 Hz, Nap), 7.24-7.45 (m, 13H, DMTr, Nap), 6.90-6.92 (d, 4H, *J* = 7.1 Hz, DMTr), 6.06 (d, 1H, *J* = 3.2 Hz, H1'), 5.51 (d, 1H, ex, *J* = 7.1 Hz, 3'-OH), 5.38-5.40 (m, 1H, H5), 5.12-5.14 (m, 1H, H2'), 4.51-4.55 (m, 1H, H3'), 4.14-4.17 (m, 1H, H4'), 3.75 (s, 6H, 2 × CH₃O), 3.32-3.41 (m, 2H, H5'); ¹³C NMR (DMSO-*d*₆) δ 162.9, 158.1, 155.5, 150.3, 144.6, 140.6 (C6), 135.4, 135.2, 133.9, 129.8 (Ar), 129.1 (Nap), 128.8, 127.9 (Ar), 127.7 (Ar), 127.5 (Nap), 126.8 (Ar), 126.6 (Nap), 126.4 (Ar), 123.8 (Ar), 119.0 (Ar), 113.2 (Ar), 109.0 (Ar), 101.8 (C5), 87.6 (C1'), 85.9, 82.6 (C4'), 79.1 (C2'), 68.5 (C3'), 62.7 (C5'), 55.0 (CH₃O).

**74X**

2'-O-(Pyren-1-yl)-5'-O-(4,4'-dimethoxytrityl)uridine (74X): Nucleoside **72X** (230 mg, 0.52 mmol), DMTrCl (0.30 g, 0.78 mmol) and DMAP (~9 mg) in anhydrous pyridine (8 mL) were mixed,

reacted, worked up and purified as described above to afford nucleoside **74X** (0.30 g, 78%) as a light yellow foam. R_f : 0.6 (5%, MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 769.2504 ($[\text{M}+\text{Na}]^+$, $\text{C}_{46}\text{H}_{38}\text{N}_2\text{O}_8\text{Na}^+$, Calc. 769.2520); ^1H NMR ($\text{DMSO}-d_6$): δ 11.32 (s, 1H, ex, NH), 8.49 (d, 1H, $J = 9.2$ Hz, Py), 8.20-8.24 (m, 3H, Py), 8.14 (d, 1H, $J = 9.2$ Hz, Py), 8.08-8.10 (d, 1H, $J = 9.1$ Hz, Py), 7.99-8.04 (m, 2H, Py), 7.86-7.89 (m, 2H, H6, Py), 7.43-7.45 (m, 2H, DMTr), 7.24-7.36 (m, 7H, DMTr), 6.90-6.92 (m, 4H, DMTr), 6.25 (d, 1H, $J = 3.2$ Hz, H1'), 5.69 (d, 1H, ex, $J = 6.8$ Hz, 3'-OH), 5.38 (d, 1H, $J = 8.2$ Hz, H5), 5.35-5.37 (m, 1H, H2'), 4.61-4.65 (m, 1H, H3'), 4.34-4.37 (m, 1H, H4'), 3.74 (s, 6H, $2 \times \text{CH}_3\text{O}$), 3.46-3.50 (m, 1H, H5'), 3.38-3.41 (m, 1H, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 162.9, 158.1, 151.7, 150.3, 144.6, 140.5 (C6), 135.4, 135.1, 131.1, 131.0, 129.78 (DMTr), 129.76 (DMTr), 127.9 (DMTr), 127.1 (Py), 126.8 (DMTr), 126.4 (Py), 125.6 (Py), 125.3, 125.1 (Py), 124.9, 124.5 (Py), 124.3 (Py), 124.0, 121.2 (Py), 120.1, 113.2 (DMTr), 112.3 (Py), 101.7 (C5), 87.8 (C1'), 85.9, 82.7 (C4'), 80.7 (C2'), 68.7 (C3'), 62.7 (C5'), 55.0 (CH_3O).

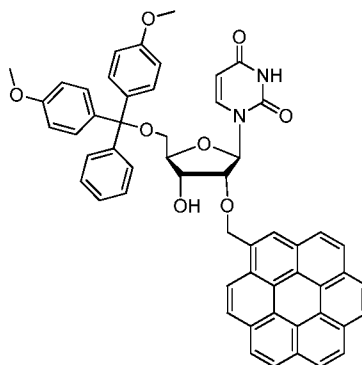


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74Y

2'-O-(Pyren-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)uridine (74Y): Nucleoside **72Y** (1.02 g, 2.20 mmol), DMTrCl (1.29 g, 3.30 mmol) and DMAP (~18 mg) in anhydrous pyridine (20 mL) were mixed, reacted, worked up and purified as described above to afford **74Y** (1.20 g, 72 %) as pale yellow foam. R_f : 0.6 (5%, MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 783.2698 ($[\text{M}+\text{Na}]^+$, $\text{C}_{47}\text{H}_{40}\text{N}_2\text{O}_8\text{Na}^+$, Calc. 783.2677); ^1H NMR ($\text{DMSO}-d_6$) δ 11.36 (d, 1H, ex, $J = 1.9$ Hz, NH), 8.41-8.43 (d, 1H, $J = 9.3$ Hz, Py), 8.30-8.32 (m, 2H, Py), 8.14-8.25 (m, 5H, Py), 8.07-8.10 (t, 1H, $J = 7.7$ Hz, Py), 7.63 (d, 1H, $J = 8.1$ Hz, H6), 7.17-7.34 (m, 9H, DMTr), 6.82-6.86 (m, 4H, DMTr), 6.02 (d, 1H, $J = 3.9$ Hz, H1'), 5.48-5.50 (d, 1H, $J = 12.1$ Hz, CH_2Py), 5.45 (d, 1H, ex, $J = 6.3$ Hz, 3'-OH), 5.36-5.38 (d, 1H, $J = 12.1$ Hz, CH_2Py), 5.13 (dd, 1H, $J = 8.1$ Hz, 1.9 Hz, H5), 4.34-4.38 (m, 1H, H3'), 4.21-4.24 (m, 1H, H2'), 4.05-4.09 (m, 1H, H4'), 3.71 (s, 3H, CH_3O), 3.69 (s, 3H, CH_3O), 3.20-3.24 (m, 2H, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 162.8, 158.08, 158.06, 150.4, 144.5, 140.0 (C6), 135.3, 135.0, 131.3, 130.7, 130.2, 129.71 (DMTr), 129.66 (DMTr), 128.7, 127.8 (DMTr), 127.6 (DMTr), 127.4 (Py), 127.3 (Py), 127.0 (Py), 126.7 (DMTr), 126.2 (Py), 125.3 (Py), 124.5 (Py), 124.0, 123.8, 123.4 (Py), 113.20 (DMTr), 113.17 (DMTr), 101.4 (C5), 87.1 (C1'), 85.9, 83.1 (C4'), 80.6 (C2'), 69.9 (CH_2Py), 68.7 (C3'), 62.8 (C5'), 55.0 (CH_3O).

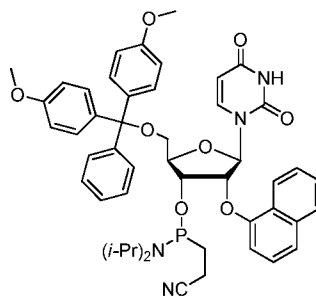
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**74Z**

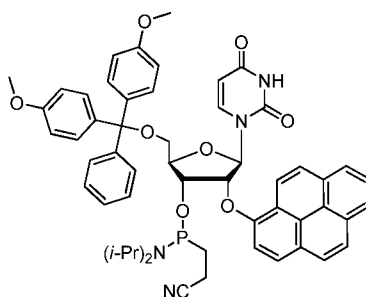
2'-O-(Coronen-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)uridine (74Z): Nucleoside **72Z** (250 mg, 0.45 mmol), DMTrCl (262 mg, 0.67 mmol) and DMAP (~15 mg) in anhydrous pyridine (6 mL) were mixed, reacted, worked up and purified as described above to afford nucleoside **74Z** (245 mg 63%) as a yellow foam. R_f : 0.6 (5%, MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 881.2824 ($[\text{M}+\text{Na}]^+$, $\text{C}_{55}\text{H}_{42}\text{N}_2\text{O}_8\text{Na}^+$, Calc. 881.2839); ^1H NMR ($\text{DMSO}-d_6$) δ 11.40 (br d, 1H, ex, $J = 1.9$ Hz, NH), 9.14-9.16 (d, 1H, $J = 8.8$ Hz, Cor), 8.96-9.02 (m, 9H, Cor), 8.88-8.90 (d, 1H, $J = 8.5$ Hz, Cor), 7.63 (d, 1H, $J = 8.1$ Hz, H6), 7.29-7.31 (d, 2H, $J = 7.4$ Hz, DMTr), 7.11-7.22 (m, 7H, DMTr), 6.74-6.79 (m, 4H, DMTr), 6.18 (d, 1H, $J = 4.1$ Hz, H1'), 5.87-5.90 (d, 1H, $J = 12.6$ Hz, CH_2Cor), 5.75-5.78 (d, 1H, $J = 12.6$ Hz, CH_2Cor), 5.59 (d, 1H, ex, $J = 6.3$ Hz, 3'-OH), 5.06 (dd, 1H, $J = 8.1$ Hz, 1.9 Hz, H5), 4.46-4.50 (m, 1H, H3'), 4.40-4.43 (m, 1H, H2'), 4.15-4.18 (m, 1H, H4'), 3.63 (s, 3H, CH_3O), 3.58 (s, 3H, CH_3O), 3.32-3.34 (m, 1H, H5'), 3.25-3.27 (m, 1H, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 162.8, 158.02, 157.97, 150.4, 144.4, 140.0 (C6), 135.3, 135.0, 132.2, 129.7 (DMTr), 129.6 (DMTr), 128.3, 128.2, 128.0, 127.7 (DMTr), 127.59 (DMTr), 127.56, 126.61 (DMTr), 126.59, 126.41 (Cor), 126.36 (Cor), 126.3 (Cor), 126.23 (Cor), 126.21, 126.19, 126.1 (Cor), 122.6 (Cor), 121.9, 121.6, 121.5, 121.4, 121.35, 121.28, 113.13 (DMTr), 113.09 (DMTr), 101.4 (C5), 87.1 (C1'), 85.9, 83.2 (C4'), 80.7 (C2'), 70.7 (CH_2Cor), 68.8 (C3'), 62.8 (C5'), 54.9 (CH_3O), 54.8 (CH_3O).

20 General phosphitylation protocol for the preparation of 4W-4Z (description for ~1 mmol scale):

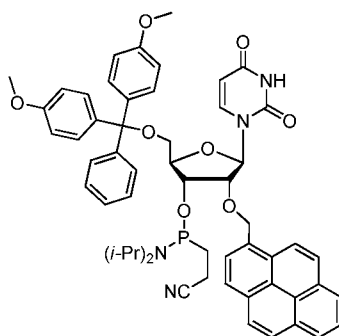
The appropriate nucleoside **74** (specific quantities of substrates and reagents given below) was co-evaporated with anhydrous 1,2-dichloroethane (4 mL) and redissolved in anhydrous CH_2Cl_2 . To this was added *N,N*-diisopropylethylamine (DIPEA) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI-reagent) and the reaction mixture was stirred at rt until TLC indicated complete conversion (~3h), whereupon abs. EtOH (2 mL) and CH_2Cl_2 (20 mL) were sequentially added to the solution. The organic phase was washed with sat. aq. NaHCO_3 (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 corresponding phosphoramidite **76** (yields specified below).

**76W**

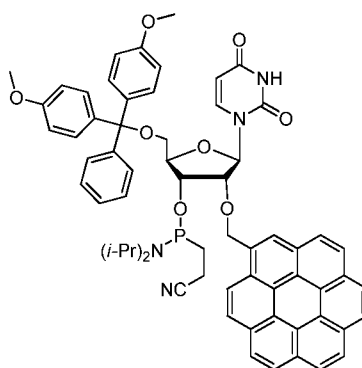
2'-O-(Naphth-2-yl)-3'-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)uridine (76W): Nucleoside **74W** (100 mg, 0.15 mmol), DIPEA (106 μ L, 0.59 mmol) and PCl-reagent (66 μ L, 0.23 mmol) in anhydrous CH_2Cl_2 (1.5 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **76W** (95 mg, 74 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 895.3462 ($[\text{M}+\text{Na}]^+$, $\text{C}_{49}\text{H}_{53}\text{N}_4\text{O}_9\text{PNa}^+$, Calc. 895.3448); ^{31}P NMR (CDCl_3) δ 151.0, 150.9.

**76X**

3'-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-2'-O-(pyren-1-yl)-5'-O-(4,4'-dimethoxytrityl)uridine (76X): Nucleoside **74X** (0.28 g, 0.38 mmol), DIPEA (268 μ L, 1.50 mmol) and PCl-reagent (167 μ L, 0.75 mmol) in anhydrous CH_2Cl_2 (2.5 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **76X** (0.27 g, 76 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 969.3608 ($[\text{M}+\text{Na}]^+$, $\text{C}_{55}\text{H}_{55}\text{N}_4\text{O}_9\text{PNa}^+$, Calc. 969.3604); ^{31}P NMR (CDCl_3) δ 149.8, 149.4.

**76Y**

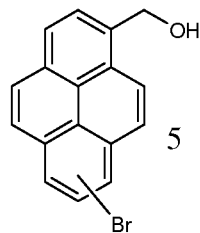
- 3'-*O*-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphinyl)-2'-*O*-(pyren-1-yl-methyl)-5'-*O*-(4,4'-dimethoxytrityl)uridine (**76Y**): Nucleoside **74Y** (0.58 g, 0.76 mmol), DIPEA (0.53 mL, 3.05 mmol) and PCl-reagent (340 μ L, 1.53 mmol) in anhydrous CH_2Cl_2 (5 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **76Y** (0.56 g, 76 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 983.3767 ($[\text{M}+\text{Na}]^+$, $\text{C}_{56}\text{H}_{57}\text{N}_4\text{O}_9\text{PNa}^+$, Calc. 983.3761); ^{31}P NMR (CDCl_3) δ 150.3, 150.2.

**76Z**

- 2'-*O*-(Coronen-1-yl-methyl)-3'-*O*-(*N,N*-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-*O*-(4,4'-dimethoxytrityl)uridine (**76Z**): Nucleoside **74Z** (240 mg, 0.28 mmol), DIPEA (200 μ L, 1.11 mmol) and PCl-reagent (125 μ L, 0.56 mmol) in anhydrous CH_2Cl_2 (6 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **76Z** (230 mg, 78 %) as a light yellow foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 1081.3864 ($[\text{M}+\text{Na}]^+$, $\text{C}_{64}\text{H}_{59}\text{N}_4\text{O}_9\text{PNa}^+$, Calc. 1081.3917); ^{31}P NMR (CDCl_3) δ 150.2.

- 7-neopentylpyrene-1-ylmethyl alcohol:** Pyrene (2.0 g, 9.9 mmol) was added into anhydrous CH_2Cl_2 containing a mixture of anhydrous AlCl_3 (1.3 g, 9.0 mmol) and 2,2,2-trimethylacetylchloride (0.97 mL, 7.9 mmol) at 0°C . The yellow crude product was purified using silica gel column chromatography to afford 7-(2,2,2-trimethylacetyl)pyrene (1.4 g, 50%) as a bright yellow solid. This intermediate was then reduced to 7-neopentylpyrene (1.4 g, 98%) in the presence of trifluoroacetic acid (3.6 mL, 49 mmol) and triethylsilane (4.7 mL, 29.0 mmol). R_f : 0.8 (10% EtOAc in petroleum ether, v/v). To a stirred solution of 7-neopentylpyrene (0.9 g, 3.3 mmol) and dichloromethylmethylether (0.4 mL, 4.3 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0°C was added a solution of titanium tetrachloride (0.7 mL, 6.3 mmol). The mixture was poured into a large amount of ice water and extracted with CH_2Cl_2 . The crude was purified by silica gel column chromatography (40% benzene in petroleum ether, v/v) to afford 7-neopentylpyrene-1-carboxaldehyde (0.9 g, 91%) as a yellow solid. This intermediate was then reduced to 7-neopentylpyrene-1-ylmethylalcohol (0.9 g, 99%) using sodium borohydride (0.1 g, 2.66 mmol) in tetrahydrofuran (50 mL).

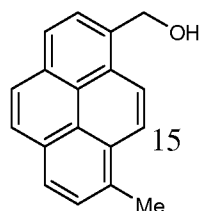
6/8-bromopyrene-1-ylmethylalcohol: To a solution of 1-bromopyrene (2.6 g, 9.25 mmol) and dichloromethylmethylether (1.08 mL, 12.0 mmol) in anhydrous CH_2Cl_2 (60 mL) at 0°C was added a solution of titaniumtetrachloride (1.98 mL, 17.5 mmol). The mixture was poured into a large amount of ice water and extracted with CH_2Cl_2 . The crude was purified by silica gel column chromatography (40% benzene in petroleum ether, v/v) to afford a mixture of 6- and 8-bromopyrene-1-carboxaldehyde (1.4 g, 49%) as a yellow solid.



This intermediate was then reduced to a mixture of 6- and 8-bromopyrene-1-ylmethylalcohol (1.4 g, 99%) as using sodium borohydride (0.17 g, 4.53 mmol) in tetrahydrofuran (80 mL).

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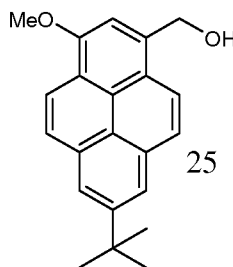
8-methylpyrene-1-ylmethylalcohol: To a stirred solution of 1-methylpyrene (4.3 g, 19.8 mmol) and dichloromethylmethylether (2.34 mL, 25.8 mmol) in anhydrous CH_2Cl_2 (150 mL) at 0°C was added a solution of titaniumtetrachloride (4.26 mL, 37.7 mmol). The mixture was poured into a large amount of ice water and extracted with CH_2Cl_2 . The crude was purified by silica gel column chromatography (40% benzene in petroleum ether, v/v) to afford 8-methylpyrene-1-carboxaldehyde (4.0 g, 83%) as a yellow solid. This



intermediate was then reduced to 8-methylpyrene-1-ylmethylalcohol (4.0 g, 99%) in the presence of sodium borohydride (0.74 g, 19.6 mmol) in tetrahydrofuran (100 mL).

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7-tert-Butyl-3-methoxypyrene-1-ylmethylalcohol: 7-tert-butyl-3-methoxypyrene-1-carboxaldehyde (obtained as described in: Yamoto et al. *Organic Preparation and Procedure International*. **1997**, 29, 3) was reduced to 7-tert-butyl-3-methoxypyrene-1-ylmethylalcohol (2.1 g, 99%) using sodium borohydride (0.26 g, 6.94 mmol) in tetrahydrofuran (30 mL).



General protocol for coupling between O2,O2'-anhydronucleoside 70 and arylmethyl alcohol (ArCH₂OH) for the preparation of 200W-Z (description for ~44.2 mmol scale). The appropriate aromatic alcohol (ArCH₂OH), NaHCO₃ and 1.0M BH₃ in THF were placed in a pressure tube, suspended in anhydrous DMSO and stirred under an argon atmosphere at room temperature until effervescence ceased (~10 min). At this point, nucleoside **70** was added (specific quantities of substrates and reagents given below), the pressure tube was purged with argon and sealed, and the reaction was heated at ~160 $^\circ\text{C}$ until analytical TLC indicated full conversion (~3h). At this point, the reaction mixture was poured into water (200 mL), stirred for 30 min and diluted with EtOAc (500 mL). The organic phase was washed with water (4 $\times$ 200 mL), evaporated to dryness, and the resulting crude purified by silica gel column chromatography (2-4%, MeOH in CH_2Cl_2 , v/v) to afford

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a residue, which was precipitated from cold acetone to obtain nucleosides **200** (yields specified below).

- 5 **2'-O-(7-neopentylpyrene-1-ylmethyl)uridine (200W).** Nucleoside **70** (0.40 g, 1.8 mmol), 7-neopentylpyrene-1-ylmethanol (1.07 g, 3.5 mmol), NaHCO₃ (0.03 g, 0.35 mmol), 1.0 M BH₃ in THF (0.9 mL, 0.9 mmol) and anhydrous DMSO (3 mL) were mixed, reacted, worked up, and purified as described above to nucleoside **200W** (0.18 g, 20 %) as a white solid. *R*_F: 0.4 (10 % MeOH in CH₂Cl₂, v/v).
- 10 **2'-O-(6/8-bromopyrene-1-ylmethyl)uridine (200X).** Nucleoside **70** (0.7 g, 3.1 mmol), 6/8-bromopyrene-1-ylmethanol (1.9 g, 6.2 mmol), NaHCO₃ (0.05 g, 0.62 mmol), 1.0 M BH₃ in THF (1.6 mL, 1.55 mmol) and anhydrous DMSO (10 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **200X** (0.28 g, 17 %) as a white solid. *R*_F: 0.4 (10 % MeOH in CH₂Cl₂, v/v).
- 15 **2'-O-(8-methylpyrene-1-ylmethyl)uridine (200Y).** Nucleoside **70** (2.0 g, 8.84 mmol), 8-methylpyrene-1-ylmethanol (4.3 g, 17.7 mmol), NaHCO₃ (0.15 g, 1.77 mmol), 1.0 M BH₃ in THF (4.9 mL, 4.42 mmol) and anhydrous DMSO (10 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **200Y** (0.87 g, 20 %) as a white solid. *R*_F: 0.4 (10 % MeOH in CH₂Cl₂, v/v).
- 20 **2'-O-(3-methoxy-7-tert-butylpyrene-1-ylmethyl)uridine (200Z).** Nucleoside **70** (0.36 g, 1.57 mmol), 3-methoxy-7-tert-butylpyrene-1-ylmethanol (1.0 g, 3.14 mmol), NaHCO₃ (26.0 mg, 0.31 mmol), 1.0 M BH₃ in THF (0.8 mL, 0.78 mmol) and anhydrous DMSO (8 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **200Z** (0.2 g, 21 %) as a white solid.
- 25 *R*_F: 0.4 (10 % MeOH in CH₂Cl₂, v/v).

- General DMTr-protection protocol for the preparation of 202V-Z (description for ~2.2 mmol scale).** The appropriate nucleoside **200** (specific quantities of substrates and reagents given below) was co-evaporated twice with anhydrous pyridine (15 mL) and redissolved in anhydrous pyridine. To this was added 4,4'-dimethoxytritylchloride (DMTrCl) and *N,N*-dimethyl-4-aminopyridine (DMAP), and the reaction mixture was stirred at room temperature until TLC indicated complete conversion (~14h). The reaction mixture was diluted with CH₂Cl₂ (70 mL) and the organic phase sequentially washed with water (2 × 70 mL) and sat. aq. NaHCO₃ (2 × 100 mL). The organic phase was evaporated to near dryness and co-evaporated with absolute EtOH and toluene (2:1, v/v, 3 × 6 mL) and the resulting crude purified by silica gel column chromatography (0-5%, MeOH in CH₂Cl₂, v/v) to afford nucleosides **202** (yields specified below).
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5'-O-(4,4'-dimethoxytrityl)-2'-O-(7-neopentylpyrene-1-ylmethyl)uridine (202W). Nucleoside **200W** (0.13 g, 0.25 mmol), DMTrCl (0.14 g, 0.37 mmol) and DMAP (~1 mg) in anhydrous pyridine (3 mL) were mixed, reacted, worked up and purified as described above to afford **202W** (0.13 g, 64 %) as pale yellow foam. R_f : 0.6 (5%, MeOH in CH₂Cl₂, v/v).

5 **5'-O-(4,4'-dimethoxytrityl)-2'-O-(6/8-bromopyrene-1-ylmethyl)uridine (202X).** Nucleoside **200X** (0.21 g, 0.39 mmol), DMTrCl (0.23 g, 0.58 mmol) and DMAP (~2 mg) in anhydrous pyridine (7 mL) were mixed, reacted, worked up and purified as described above to afford **202X** (0.28 g, 85 %) as pale yellow foam. R_f : 0.6 (5%, MeOH in CH₂Cl₂, v/v).

10 **5'-O-(4,4'-dimethoxytrityl)-2'-O-(8-methylpyrene-1-ylmethyl)uridine (202Y).** Nucleoside **200Y** (0.7 g, 1.48 mmol), DMTrCl (0.86 g, 2.20 mmol) and DMAP (~4 mg) in anhydrous pyridine (10 mL) were mixed, reacted, worked up and purified as described above to afford **202Y** (0.9 g, 78 %) as pale yellow foam. R_f : 0.6 (5%, MeOH in CH₂Cl₂, v/v).

15 **5'-O-(4,4'-dimethoxytrityl)-2'-O-(pyrene-1-ylmethyl)uridine (202Z).** Nucleoside **200Z** (0.18 g, 0.33 mmol), DMTrCl (0.19 g, 0.49 mmol) and DMAP (~2 mg) in anhydrous pyridine (7 mL) were mixed, reacted, worked up and purified as described above to afford **202Z** (0.2 g, 70 %) as pale yellow foam. R_f : 0.6 (5%, MeOH in CH₂Cl₂, v/v).

General phosphitylation protocol for the preparation of 204V-Z (description for ~1 mmol scale). The appropriate nucleoside **202** (specific quantities of substrates and reagents are given below) was co-evaporated with anhydrous 1,2-dichloroethane (4 mL) and redissolved in anhydrous CH₂Cl₂. To this was added DIPEA and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (PCI-reagent) and the reaction mixture was stirred at rt until TLC indicated complete conversion (~3h), whereupon abs. EtOH (2 mL) and CH₂Cl₂ (20 mL) were sequentially added to the solution. 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 corresponding phosphoramidite **204** (yields specified below)

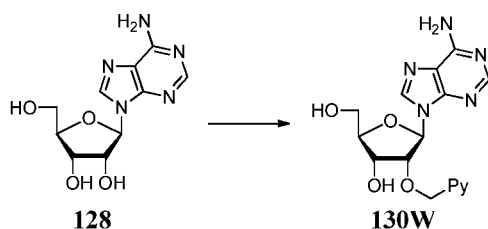
30 **5'-O-(4,4'-dimethoxytrityl)-2'-O-(7-neopentylpyrene-1-ylmethyl)uridine-3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (204W).** Nucleoside **202W** (0.11 g, 0.13 mmol), DIPEA (0.1 mL, 0.53 mmol) and PCI-reagent (60 µL, 0.26 mmol) in anhydrous CH₂Cl₂ (3 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **204W** (0.11 g, 81 %) as a white foam. R_f : 0.8 (5% MeOH in CH₂Cl₂, v/v).

35 **5'-O-(4,4'-dimethoxytrityl)-2'-O-(6/8-bromopyrene-1-ylmethyl)uridine-3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (204X).** Nucleoside **202X** (0.25 g, 0.29 mmol), DIPEA (0.21 mL, 1.19 mmol) and PCI-reagent (133 µL, 0.59 mmol) in anhydrous CH₂Cl₂ (5 mL) were mixed, reacted,

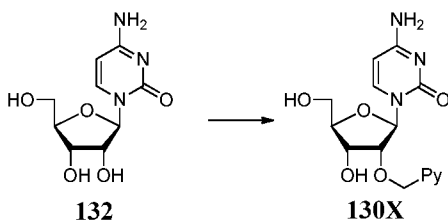
worked up and purified as described above to afford phosphoramidite **204X** (0.26 g, 82 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v).

5'-O-(4,4'-dimethoxytrityl)-2'-O-(8-methylpyrene-1-ylmethyl)uridine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (204Y). Nucleoside **202Y** (0.65 g, 0.846 mmol), DIPEA (0.59 mL, 3.35 mmol) and PCI-reagent (374 μL , 1.77 mmol) in anhydrous CH_2Cl_2 (10 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **204Y** (0.6 g, 78 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v)

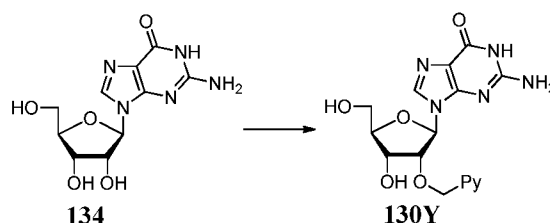
5'-O-(4,4'-dimethoxytrityl)-2'-O-(3-methoxy-7-tertiarybutylpyrene-1-ylmethyl)uridine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (204Z). Nucleoside **202Z** (0.15 g, 0.18 mmol), DIPEA (126 μL , 0.71 mmol) and PCI-reagent (79 μL , 0.35 mmol) in anhydrous CH_2Cl_2 (5 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **204Z** (0.15 g, 80 %) as a white foam. R_f : 0.8 (5% MeOH in CH_2Cl_2 , v/v).



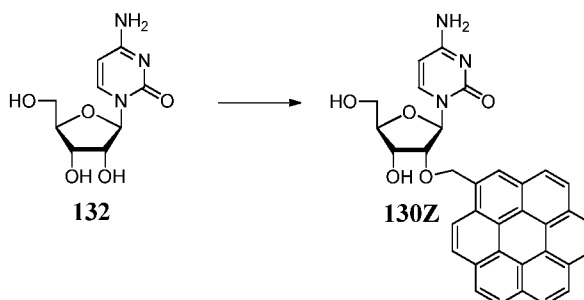
2'-O-(Pyren-1-yl-methyl)adenosine (130W): An adenosine-functionalized nucleoside **128** (1.60 g, 5.98 mmol), pyren-1-ylmethylchloride (1.0 g, 3.9 mmol), NaH (0.36 g, 14.9 mmol), and anhydrous DMSO (30 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **2W** (0.48 g, 17%) as a pale yellow solid. R_f 0.29 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 504.1649 ($[\text{M}+\text{Na}]^+$, $\text{C}_{27}\text{H}_{23}\text{N}_5\text{O}_4\cdot\text{Na}^+$, Calcd 504.1642); ^1H NMR ($\text{DMSO}-d_6$) δ 8.32(s, 1H, adenine), 7.95–8.29 (m, 10H, pyrene, adenine), 7.27 (s, 2H, NH_2), 6.14 (d, 1H, $\text{H}1'$, $J = 6.3$ Hz), 5.48 (d, 1H, ex, $J = 5.1$ Hz, 3'-OH), 5.39–5.45 (m, 2H, $\text{CH}_2\text{-Py}$, 5'-OH (ex)), 5.17–5.20 (d, 1H, $J = 11.7$ Hz, CH_2Py), 4.81 (t, 1H, $J = 5.1$ Hz, 11.1 Hz, $\text{H}2'$), 4.50–4.54 (m, 1H, $\text{H}3'$), 4.08–4.11 (m, 1H, $\text{H}4'$), 3.68–3.75 (m, 1H, $\text{H}5'$), 3.57–3.65 (m, 1H, $\text{H}5'$); ^{13}C NMR ($\text{DMSO}-d_6$) δ 156.1, 152.3, 148.9, 139.6, 131.2, 130.6, 130.6, 130.1, 128.7, 127.2, 127.2, 127.0, 126.1, 125.2, 125.2, 124.3, 123.8, 123.7, 123.3, 119.3, 86.7, 86.1, 80.8, 69.9, 69.1, 61.5.



2'-O-(Pyren-1-yl-methyl)cytidine (130X): Cytidine **132** (10.0 g, 41.1 mmol), pyren-1-ylmethylchloride (6.8 g, 27.1 mmol), NaH (2.47 g, 102.8 mmol), and anhydrous DMSO (150 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **130X** (3.5 g, 19%) as a pale yellow solid. R_f 0.3 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 480.1532 ($[\text{M}+\text{Na}]^+$, $\text{C}_{26}\text{H}_{23}\text{N}_3\text{O}_5\cdot\text{Na}^+$, Calcd 480.1529); ^1H NMR ($\text{DMSO}-d_6$) δ 8.06-8.49 (m, 9H, Py), 7.90 (d, 1H, cytosine), 7.15 (s, 2H, NH_2), 6.08 (d, 1H, $\text{H}1'$), 5.67 (d, 1H, cytosine), 5.41-5.43 (d, 1H, CH_2Py), 5.24 (d, 1H, CH_2Py), 5.22 (d, 1H, $3'-\text{OH}$), 5.07-5.11 (m, 1H, $5'-\text{OH}$), 4.18-4.22 (m, 1H, $\text{H}3'$), 4.06-4.11 (m, 1H, $\text{H}2'$), 3.93-3.95 (m, 1H, $\text{H}4'$), 3.53-3.74 (m, 2H, $\text{H}5'$); ^{13}C NMR ($\text{DMSO}-d_6$) δ 165.6, 155.1, 141.0, 131.6, 130.7, 130.5, 130.2, 128.6, 127.4, 127.3, 127.2, 126.9, 126.1, 125.2, 124.5, 123.9, 123.8, 123.6, 93.8, 89.7, 87.5, 84.2, 81.7, 77.2, 72.9, 69.7, 68.2, 60.1.

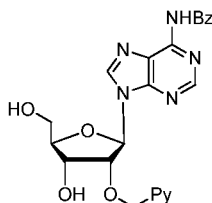


2'-O-(Pyren-1-yl-methyl)guanosine (130Y): Guanosine **134** (3.0 g, 10.6 mmol), pyren-1-ylmethylchloride (1.86 g, 7.4 mmol), NaH (0.64 g, 26.5 mmol), and anhydrous DMSO (50 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **130Y** (0.90 g, 17%) as a white solid. R_f 0.29 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 520.1608 ($[\text{M}+\text{Na}]^+$, $\text{C}_{27}\text{H}_{23}\text{N}_5\text{O}_5\cdot\text{Na}^+$, Calcd 520.1591); ^1H NMR ($\text{DMSO}-d_6$) 10.6 (s, 1H, NH), 8.0-8.35 (m, 9H, Py), 7.97 (s, 1H, $\text{H}7$ -adenine), 6.37 (s, 2H, NH_2), 5.97 (d, 1H, $\text{H}1'$), 5.40-5.43 (dd, 2H, CH_2Py), 5.20 (d, 1H, ex, $3'-\text{OH}$), 5.08 (t, 1H, ex, $5'-\text{OH}$), 4.61-4.63 (m, 1H, $\text{H}2'$), 4.44-4.46 (m, 1H, $\text{H}3'$), 4.00-4.02 (m, 1H, $\text{H}4'$), 3.60-3.68 (m, 2H, $\text{H}5'$); ^{13}C NMR ($\text{DMSO}-d_6$) δ 156.6, 153.6, 151.1, 135.2, 131.2, 130.6, 130.2, 128.7, 127.3, 127.0, 126.2, 125.2, 125.1, 124.4, 123.8, 123.7, 123.5, 116.7, 86.1, 84.5, 81.3, 69.8, 69.0, 61.3.

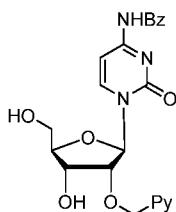


2'-O-(Coronen-1-yl-methyl)cytidine (130Z): Cytidine **132** (0.70 g, 2.88 mmol), coronen-1-ylmethylchloride (0.66 g, 1.99 mmol), NaH (0.17 g, 7.19 mmol), and anhydrous DMSO (20 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **130Z** (0.16 g, 10%) as a pale yellow solid. R_f 0.3 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 578.1679 ($[\text{M}+\text{Na}]^+$, $\text{C}_{34}\text{H}_{25}\text{N}_3\text{O}_5\cdot\text{Na}^+$, Calcd 578.1686); ^1H -NMR ($\text{DMSO}-d_6$) δ 9.01-9.17 (m, 11H, Cor), 7.94

(d, 1H, cytosine), 7.20 (s, 2H, NH₂), 6.08 (d, 1H, H1'), 5.85 (d, 1H, cytosine), 5.68-5.78 (m, 2H, CH₂Cor), 5.22 (d, 1H, 3'-OH), 5.07-5.11 (m, 1H, 5'-OH), 4.18-4.22 (m, 1H, H3'), 4.13-4.15 (m, 1H, H2'), 3.90-3.93 (m, 1H, H4'), 3.58-3.88 (m, 2H, H5').

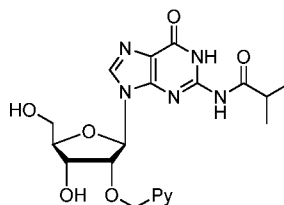
**136W**

2'-O-(Pyren-1-yl-methyl)-N-benzoyl-adenosine (136W): Nucleoside **130W** (0.40g, 0.83 mmol) was dried by co-evaporation with pyridine (5 mL) and re-dissolved in anhydrous pyridine (10 mL) followed by addition of trimethylsilyl chloride (0.42 mL, 3.32 mmol). After stirring for 30 min at rt, benzoyl chloride (0.14 mL, 1.25 mmol) was added and the mixture was stirred for additional 5h. Aqueous NH₃ (28%, 10 mL) was added and stirred for additional 1h and the reaction mixture was concentrated to near dryness. The residual material was re-dissolved in CH₂Cl₂ (150 mL) and washed with water (2 x 75 mL). The organic phase was evaporated to near dryness and the product was purified by silica gel column chromatography (1-3% MeOH in CH₂Cl₂, v/v), to afford nucleoside **136W** (0.245 g, 50%) as pale yellow solid. R_f 0.56 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 608.1910 ([M+Na]⁺, C₃₄H₂₇N₅O₅·Na⁺, calcd 608.1904); ¹H NMR (DMSO-*d*₆) δ 11.09 (s, 1H, amide), 8.62(s, 1H, adenine), 8.57(s, 1H, adenine), 7.99–8.28(m, 11H, benzoyl, Py), 7.56-7.70 (m, 3H, benzoyl), 6.25(d, 1H, H1', *J* = 6 Hz), 5.58(d, 1H, *J* = 5.1 Hz, ex, 3'-OH), 5.46-5.50 (d, 1H, *J* = 11.4 Hz, CH₂Py), 5.17–5.24 (m, 2H, 1 ex, 5'-OH, CH₂Py), 4.86 (m, 1H, H2'), 4.52-4.57 (m, 1H, H3'), 4.10-11 (m, 1H, H4'), 3.59-3.74 (m, 2H, H5'). ¹³C NMR (DMSO-*d*₆) δ 165.4, 151.8, 151.4, 150.2, 142.77, 133.4, 132.4, 131.1, 131.1, 130.7, 130.6, 130.1, 128.7, 128.4, 128.4, 128.1, 127.4, 127.3, 127.2, 127.1, 126.1, 125.6, 125.2, 125.2, 124.3, 123.8, 123.7, 123.3, 86.6, 85.9, 80.8, 70.1, 69.0, 61.3.

**136X**

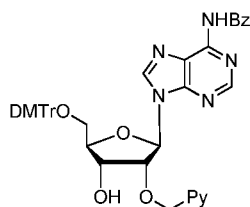
2'-O-(Pyren-1-yl-methyl)-N-benzoyl-cytidine (136X): Benzoic anhydride (1.68 g, 7.45 mmol) was added to the solution of nucleoside **130X** (3.1 g, 6.77 mmol) in anhydrous DMF (100 mL). The reaction mixture was stirred at 60°C until TLC indicated complete conversion (~6h). The reaction mixture was diluted with CH₂Cl₂ (150 mL), and the organic phase was sequentially washed with

water (2 x 60 mL) and satd aq NaHCO₃ (2 x 100 mL). The organic phase was dried over Na₂SO₄, evaporated to near dryness, and the resulting crude was purified by silica gel column chromatography (0-2% MeOH in CH₂Cl₂, v/v) to afford nucleoside **136X** (2.1 g, 55%) as pale yellow solid. R_f 0.6 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 584.1785 ([M+Na]⁺, C₃₃H₂₇N₃O₆.Na⁺, calcd 584.1790); ¹HNMR (DMSO-*d*₆) δ 11.16 (s, 1H, amide), 8.15-8.45 (m, 9H, Py), 8.00-8.03 (m, 3H, benzoyl, cytosine), 7.62-7.66 (m, 1H, benzoyl), 7.52-7.55 (m, 2H, benzoyl), 7.44 (d, 1H, cytosine), 6.13 (d, 1H, H1'), 5.43-5.53 (dd, 2H, CH₂Py), 5.30 (d, 1H, ex, 3'-OH), 5.19 (t, 1H, 5'-OH), 4.24-4.27 (m, 1H, H3'), 4.17-4.18 (m, 1H, H2'), 4.02-4.04 (m, 1H, H4'), 3.76-3.80 (m, 1H, H5'), 3.63-3.67 (m, 1H, H5'); ¹³C NMR (DMSO-*d*₆) δ 167.2, 162.9, 154.5, 144.8, 133.1, 132.7, 132.6, 131.8, 131.5, 130.6, 130.3, 130.2, 129.2, 128.7, 128.4, 127.4, 127.3, 126.2, 125.2, 124.5, 123.9, 123.7, 123.6, 96.0, 88.1, 84.7, 82.6, 81.7, 76.3, 73.0, 69.9, 67.8, 59.7

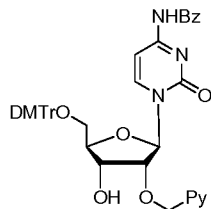
**136Y**

2'-O-(Pyren-1-yl-methyl)-N-isobutyryl-guanosine (136Y): Trimethylsilyl chloride (0.5 mL, 3.92 mmol) was added to a solution of nucleoside **130Y** (0.26 g, 0.52 mmol), which was dried by coevaporation with pyridine three times, in pyridine (6 mL). After stirring 2h at rt, isobutyrylchloride (0.15 mL, 1.57 mmol) was added drop wise at 0°C for 10 min. Water (2 mL) was added to the reaction mixture at 0°C and stirred for another 10 min. The solution was stirred for another 5 min at rt, and aq NH₃ (28%, 5 mL) was added to the solution. After stirring for another 15 min at room temperature, the solution was concentrated to near dryness. The residual material was re-dissolved in CH₂Cl₂ (100 mL) and washed with water (2 x 60 mL). The organic phase was dried over Na₂SO₄, evaporated to near dryness and the crude was purified by silica gel column chromatography (1-3% MeOH in CH₂Cl₂, v/v), to afford nucleoside **136Y** (0.220 g, 74 %) as pale yellow solid. R_f 0.56 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 590.2008 ([M+Na]⁺, C₃₁H₂₉N₅O₆.Na⁺, calcd 590.2010); ¹HNMR (DMSO-*d*₆) 11.8 (s, 1H, amide), 11.3 (s, 1H, NH), 8.01-8.27 (m, 9H, Py), 7.95 (s, 1H, H7-guanine), 5.93 (d, 1H, H1'), 5.43-5.46 (m, 2H, 1 ex, 3'-OH, CH₂Py), 5.20 (d, 1H, ex, 3'-OH), 5.06-5.16 (m, 2H, 1 ex, 5'-OH, CH₂Py), 4.65-4.67 (m, 1H, H2'), 4.45-4.48 (m, 1H, H3'), 4.01-4.03 (m, 1H, H4'), 3.61-3.66 (m, 2H, H5'), 2.56-2.61 (m, 1H, isobutyryl), 1.00-1.04 (dd, 6H, isobutyryl); ¹³C NMR (DMSO-*d*₆) δ 179.8, 154.5, 148.5, 147.8, 137.2, 131.1, 130.6, 130.1, 128.7, 127.3, 127.1, 127.0, 126.1, 125.2, 125.1, 124.3, 123.8, 123.6, 123.5, 86.5, 84.6, 81.6, 70.2, 69.2, 61.3, 34.5, 18.7, 18.6

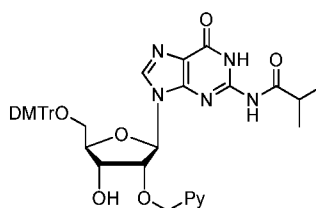
General DMTr-Protection Protocol for the preparation of 138W-138Y [Description for ~3.6 mmol Scale]: The appropriate nucleoside **136** (specific quantities given below) was coevaporated twice with anhydrous pyridine (15 mL) and redissolved in anhydrous pyridine. To this were added 4,4'-dimethoxytritylchloride (DMTrCl) and N,N-dimethyl-4-aminopyridine (DMAP), and the reaction mixture was stirred at rt until TLC indicated complete conversion (~14h). The reaction mixture was diluted with CH₂Cl₂ (100 mL), and the organic phase was sequentially washed with water (2 x 50 mL) and satd aq NaHCO₃ (2 x 100 mL). The organic phase was evaporated to near dryness, and the resulting crude coevaporated with absolute EtOH and toluene (2: 1, v/v, 3 x 10 mL) and purified by silica gel column chromatography (0-5%, MeOH in CH₂Cl₂, v/v) to afford nucleoside **138** (yields specified below).

**138W**

5'-O-(4,4'-dimethoxytrityl)-2'-O-(Pyren-1-yl-methyl)-N-benzoyl-adenosine (138W). Nucleoside **136W** (0.24 g, 0.41 mmol), DMTrCl (0.27 g, 0.70 mmol), and DMAP (~12 mg) in anhydrous pyridine (6 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **138W** (0.3 g, 71%) as a pale yellow foam. *R*_f 0.78 (10% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 910.3225 ([*M*+Na]⁺, C₅₅H₄₅N₅O₇.Na⁺, calcd 910.3211); ¹H NMR (DMSO-*d*₆) δ 11.12 (s, 1H, amide), 8.50 (s, 1H, adenine), 8.46 (s, 1H, adenine), 8.00–8.29 (m, 11H, benzoyl, Py), 7.64–7.69 (m, 1H, benzoyl), 7.55–7.60 (m, 2H, benzoyl), 7.35 (d, 2H, *J* = 9 Hz, DMTr), 7.14–7.27 (m, 7H, DMTr), 6.81 (d, 4H, *J* = 8.4 Hz, DMTr), 6.27 (d, 1H, *J* = 5.7 Hz, H1'), 5.56 (d, 1H, ex, *J* = 5.7 Hz, 3'-OH), 5.50 (d, 1H, *J* = 11.7 Hz, CH₂Py), 5.28 (d, 1H, *J* = 11.7 Hz, CH₂Py), 4.97–5.00 (m, 1H, H2'), 4.61–4.65 (m, 1H, H3'), 4.20–4.24 (m, 1H, H4'), 3.70 (s, 6H, 2 x CH₃O), 3.26–3.28 (m, 2H, H5'). ¹³C NMR (DMSO-*d*₆) δ 165.5, 158.0, 151.8, 151.3, 150.3, 144.7, 135.5, 135.4, 133.4, 132.4, 131.1, 131.1, 130.7, 130.6, 130.1, 129.6, 129.6, 128.8, 128.7, 128.4, 128.4, 128.1, 128.1, 127.7, 127.6, 127.4, 127.3, 127.2, 127.1, 126.6, 126.1, 125.7, 125.2, 124.3, 123.9, 123.7, 123.3, 113.1, 86.3, 85.5, 84.2, 79.8, 70.1, 69.2, 63.5, 54.9.

**138X**

5'-O-(4,4'-dimethoxytrityl)-2'-O-(Pyren-1-yl-methyl)-N-benzoyl-cytidine (138X). Nucleoside **136X** (2.0 g, 3.56 mmol), DMTrCl (2.07 g, 5.34 mmol), and DMAP (~20 mg) in anhydrous pyridine (50 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **138X** (2.0 g, 65%) as a pale yellow foam. R_f 0.78 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 886.3087 ($[\text{M}+\text{Na}]^+$, $\text{C}_{54}\text{H}_{45}\text{N}_3\text{O}_8\cdot\text{Na}^+$, calcd 886.3098); ^1H NMR ($\text{DMSO}-d_6$) δ 11.22 (s, 1H, amide), 847-8.49 (d, 1H, Py), 8.21-8.30 (m, 6H, H6 and Py), 8.16 (s, 2H, Py), 8.00-8.05 (m, 3H, benzoyl and Py), 7.63-7.65 (m, 1H, benzoyl), 7.52-7.55 (m, 2H, benzoyl), 7.24-7.39 (m, 9H, DMTr), 7.04 (d, 1H, H5), 6.86-6.90 (m, 4H, DMTr), 6.15 (d, 1H, H1'), 5.56 (bs, 2H, CH_2Py), 5.37 (d, 1H, ex, 3'-OH), 4.41-4.45 (m, 1H, H3'), 4.16-4.20 (m, 2H, H2' and H4'), 3.73 (bs, 6H, 2 x CH_3O), 3.38-3.41 (m, 1H, H5'), 3.29-3.34 (m, 1H, H5'); ^{13}C NMR ($\text{DMSO}-d_6$) δ 167.1, 163.1, 158.1, 154.3, 144.3, 135.5, 135.1, 133.1, 132.6, 131.4, 130.6, 130.2, 129.7, 129.6, 128.7, 128.4, 127.8, 127.7, 127.5, 127.2, 127.8, 126.1, 125.2, 124.5, 123.9, 123.8, 123.6, 113.2, 96.0, 88.7, 85.9, 82.3, 81.5, 69.9, 68.0, 61.8, 54.9.

**138Y**

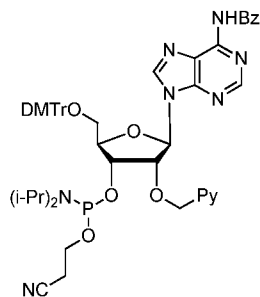
5'-O-(4,4'-dimethoxytrityl)-2'-O-(Pyren-1-yl-methyl)-N-isobutyryl-guanosine (138Y).

Nucleoside **136Y** (0.20 g, 0.35 mmol), DMTrCl (0.20 g, 0.53 mmol), and DMAP (~10 mg) in anhydrous pyridine (5 mL) were mixed, reacted, worked up, and purified as described above to afford nucleoside **138Y** (0.27 g, 89%) as a pale yellow foam. R_f 0.8 (10% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 892.3325 ($[\text{M}+\text{Na}]^+$, $\text{C}_{52}\text{H}_{47}\text{N}_5\text{O}_8\cdot\text{Na}^+$, calcd 892.3317); ^1H NMR ($\text{DMSO}-d_6$) 11.8 (s, 1H, amide), 11.3 (s, 1H, NH), 8.25-8.32 (m, 3H, Py), 7.99-8.15 (m, 7H, H7-guanine and Py), 7.32-7.34 (m, 2H, DMTr), 7.18-7.25 (m, 7H, DMTr), 6.79-6.82 (m, 4H, DMTr), 5.97 (d, 1H, H1'), 5.46-5.51 (m, 2H, 1 ex, 3'-OH, CH_2Py), 5.22-5.25 (d, 1H, CH_2Py), 4.71-4.73 (m, 1H, H2'), 4.45-4.48 (m, 1H, H3'), 4.11-4.13 (m, 1H, H4'), 3.70-3.71 (bd, 6H, 2 x CH_3O), 3.18-3.28 (m, 2H, H5'), 2.59-2.65 (m, 1H, isobutyryl), 1.04-1.06 (dd, 6H, isobutyryl); ^{13}C NMR ($\text{DMSO}-d_6$) δ 179.8, 158.0, 154.5, 148.5, 147.8, 144.6, 137.1, 135.4, 135.3, 131.12, 130.7, 130.6, 130.1, 129.6, 128.7, 127.7, 127.6, 127.3, 127.2, 127.1, 127.0, 126.6, 126.1, 125.2, 125.1, 124.4, 123.8, 123.7, 123.4, 120.1, 113.1, 85.6, 85.0, 84.2, 80.8, 70.3, 69.3, 63.9, 54.9, 34.6, 18.7, 18.6.

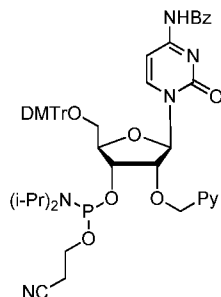
30 General Phosphitylation Protocol: [Description for ~2.3 mmol Scale]

The appropriate nucleoside (specific quantities of substrates and reagents given below) was co-evaporated twice with anhydrous 1,2-dichloroethane (20 mL) and redissolved in anhydrous CH_2Cl_2 (25 mL). To this was added *N,N*-diisopropylethylamine (DIPEA) and 2-cyanoethyl-*N,N*-

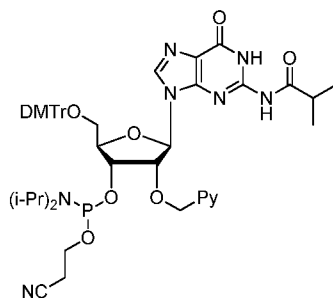
diisopropylchlorophosphoramidite (PCI-reagent) and the reaction mixture was stirred at rt until TLC indicated complete conversion (~3h), whereupon abs. EtOH (4 mL) and CH₂Cl₂ (50 mL) were sequentially added to the solution. The organic phase was washed with sat. aq. NaHCO₃ (20 mL), evaporated to near dryness, and the resulting residue purified by silica gel column chromatography (40-70% EtOAc in petroleum ether, v/v) to afford the corresponding phosphoramidite (yields specified below).

**76'W**

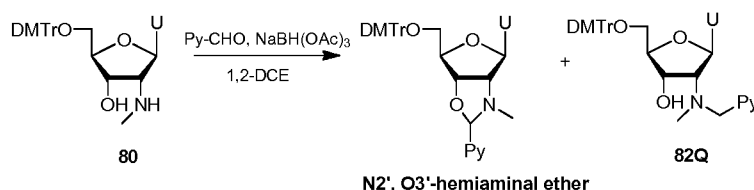
3'-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-2'-O-(pyren-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)-N-benzoyl-adenosine (76'W). Nucleoside **138W** (0.25 g, 0.28 mmol), DIPEA (74 μ L, 0.43 mmol) and PCI-reagent (95 μ L, 0.43 mmol) in anhydrous CH₂Cl₂ (6 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **76'W** (0.26 g, 82 %) as a white foam. *R*_f: 0.75 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1110.4295 ([M+Na]⁺, C₆₄H₆₂N₇O₈PNa⁺, Calcd 1110.4289); ³¹P NMR (CDCl₃) δ 150.7, 150.8.

**140X**

5 **3'-O-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphinyl)-2'-O-(pyren-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)-N-benzoyl-cytidine (140X).** Nucleoside **138X** (2.0 g, 2.31 mmol), DIPEA (1.65 mL, 9.26 mmol) and PCI-reagent (1.03 mL, 4.63 mmol) in anhydrous CH₂Cl₂ (25 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **140X** (2.3 g, 93 %) as a white foam. *R*_f: 0.67 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1086.4183 ([M+Na]⁺, C₆₃H₆₂N₅O₉PNa⁺, Calcd 1086.4177); ³¹P NMR (CDCl₃) δ 149.7, 150.1.

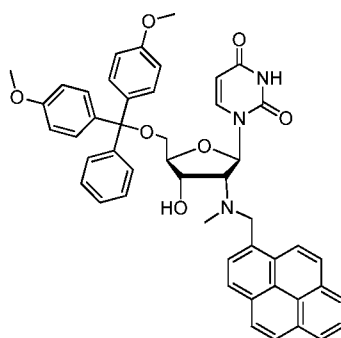
**140Y**

10 **3'-O-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphinyl)-2'-O-(pyren-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)-N-isobutyryl-guanosine (140Y).** Nucleoside **138Y** (0.26 g, 0.29 mmol), DIPEA (213 μL, 1.19 mmol) and PCI-reagent (133 μL, 0.59 mmol) in anhydrous CH₂Cl₂ (7 mL) were mixed, reacted, worked up and purified as described above to afford phosphoramidite **140Y** (0.25 g, 78 %) as a white foam. *R*_f: 0.7 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1092.4389 ([M+Na]⁺, C₆₁H₆₄N₇O₉PNa⁺, Calcd 1092.4395); ³¹P NMR (CDCl₃) δ 150.3, 150.4.



Scheme 15

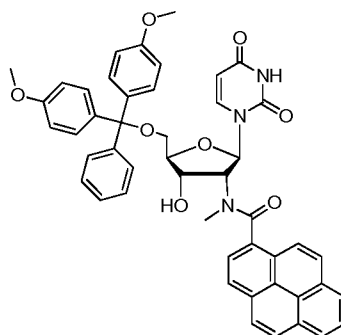
Cyclic N2',O3'-hemiaminal ether: The structure of the cyclic N2',O3'-hemiaminal ether byproduct (Scheme 15) is supported by the following ^1H NMR observations (500 MHz, $\text{DMSO}-d_6$, results not shown): a) appearance of the hemiaminal ether proton as a singlet at 6.07 ppm; b) absence of an exchangeable signal corresponding to a 3'-OH group, c) absence of a signal corresponding to a CH_2 -group linking the O2'-position and the pyrene moiety, and, d) appearance of the H1'-signal as a singlet at 6.13 ppm indicating formation of a restricted North type furanose conformation unlike what is observed for **6Q** (H1' signal appearing as a doublet at 6.43 ppm, $J = 8.2$ Hz). Despite formation of a new stereocenter, we only observed one set of signals, suggesting that only one of the diastereomers of the cyclic N2',O3'-hemiaminal ether byproduct is formed.

**82Q**

2'-Amino-2'-deoxy-2'-N-methyl-2'-N-(pyren-1-yl-methyl)-5'-O-(4,4'-dimethoxytrityl)uridine (82Q): Nucleoside **80** (200 mg, 0.36 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2×4 mL) and redissolved in anhydrous THF (5 mL). Pyrene-1-ylmethylchloride (205 mg, 0.37 mmol) and triethylamine (0.52 mL, 3.73 mmol) were added and the reaction mixture was heated at reflux for two days, whereupon the solvent was evaporated off. The crude residue was taken up in a mixture CHCl_3 and sat. aq. NaHCO_3 (50 mL, 3:2, v/v) and the layers were separated. The aqueous phase was extracted with CHCl_3 (2×20 mL) and the combined organic phase was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0-1.25 %

$\text{MeOH}/\text{CH}_2\text{Cl}_2$, v/v) to afford nucleoside **82Q** as a yellow foam (129 mg, 46 %). R_f : 0.5 (60%, EtOAc in petroleum ether, v/v); MALDI-HRMS m/z 774.3156 ($[\text{M}+\text{H}]^+$ $\text{C}_{48}\text{H}_{43}\text{N}_3\text{O}_7\text{H}^+$, Calc 774.3174); ^1H NMR ($\text{DMSO}-d_6$) δ 11.41 (d, 1H, ex, $J = 1.7$ Hz, NH), 8.50 (d, 1H, $J = 9.1$ Hz, Py), 8.01-8.29 (m,

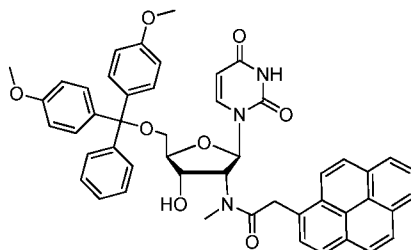
8H, Py), 7.63 (d, 1H, $J = 8.2$ Hz, H6), 7.20-7.38 (m, 9H, DMTr), 6.85-6.89 (m, 4H, DMTr), 6.43 (d, 1H, $J = 8.2$ Hz, H1'), 5.56 (d, 1H, ex, $J = 5.2$ Hz, 3'-OH), 5.43 (dd, 1H, $J = 8.3$ Hz, 1.7 Hz, H5), 4.41-4.50 (m, 3H, CH₂Py, H3'), 4.06-4.08 (m, 1H, H4'), 3.71 (s, 3H, CH₃O), 3.70 (s, 3H, CH₃O), 3.44-3.48 (dd, 1H, $J = 8.3$ Hz, 8.1 Hz, H2'), 3.28-3.31 (m, 1H, H5', overlap with H₂O), 3.16-3.20 (dd, 1H, $J = 10.6$ Hz, 3.6 Hz, H5'), 2.33 (s, 3H, CH₃N); ¹³C NMR (DMSO-*d*₆) δ 162.7, 158.08, 158.07, 150.6, 144.5, 140.2 (C6), 135.4, 135.1, 132.7, 130.7, 130.3, 130.2, 129.71 (DMTr), 129.67 (DMTr), 129.2, 128.0 (Py), 127.8 (DMTr), 127.6 (DMTr), 127.3 (Py), 126.9 (Py), 126.8 (Py), 126.7 (DMTr), 126.1 (Py), 125.01, 124.98 (Py), 124.4 (Py), 124.1, 124.0 (Py), 123.9, 113.20 (DMTr), 113.17 (DMTr), 102.1 (C5), 85.9, 85.1 (C4'), 83.4 (C1'), 71.3 (C3'), 67.8 (C2'), 64.1 (C5'), 57.4 (CH₂Py), 55.0 (CH₃O), 38.6 (CH₃N).



82S

2'-Amino-2'-deoxy-2'-N-methyl-2'-N-(pyren-1-yl-carbonyl)-5'-O-(4,4'-dimethoxytrityl)uridine (82S): Nucleoside **80** (150 mg, 0.27 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2 × 5 mL), dissolved in anhydrous DMF (4.5 mL) and added to a pre-stirred (1h at rt) solution of 1-pyrenecarboxylic acid (100 mg, 0.40 mmol), *O*-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 125 mg, 0.32 mmol) and DIPEA (0.12 mL, 0.70 mmol) in anhydrous DMF (4.5 mL). The reaction mixture was stirred for 17 h, whereupon it was diluted with EtOAc (50 mL) and sequentially washed with sat. aq. NaHCO₃ (20 mL) and H₂O (2 × 20 mL). The organic phase was evaporated to dryness and the resulting residue purified by silica gel column chromatography (0-2% MeOH in CH₂Cl₂, v/v) to afford nucleoside **82S** as a white foam (164 mg, 78 %). $R_f = 0.4$ (5% MeOH in CH₂Cl₂, v/v). MALDI-HRMS m/z 788.2985 ($[M+H]^+$, C₄₈H₄₁N₃O₈·H⁺, calc. 788.2966); ¹H NMR (CDCl₃) δ 8.65 (br s, 1H, NH, ex), 7.97-8.28 (m, 9H, Py), 7.85 (d, 1H, $J = 8.3$ Hz, H6), 7.20-7.44 (m, 9H, DMTr), 6.80-6.90 (m, 4H, DMTr), 6.78 (d, 1H, $J = 6.0$ Hz, H1'), 5.42-5.48 (m, 1H, H5), 4.80-4.90 (m, 2H, H2', H3'), 4.28-4.43 (m, 1H, H4'), 3.77 (s, 6H, CH₃O), 3.48-3.63 (m, 2H, H5'), 2.98 (s, 3H, NCH₃), traces of a second rotamer are observed; ¹³C NMR (CDCl₃) δ 174.4, 162.9, 159.0, 150.5, 144.5, 140.0 (C6), 135.6, 135.5, 132.3, 131.4, 131.0, 130.44 (DMTr), 130.42 (DMTr), 129.4 (Py), 128.7 (Py), 128.5 (DMTr), 128.3 (DMTr), 127.4, 126.7 (Py), 126.1 (Py), 126.0 (Py), 125.0 (Py), 124.8, 124.7, 124.3 (Py), 113.6 (DMTr), 113.3 (DMTr),

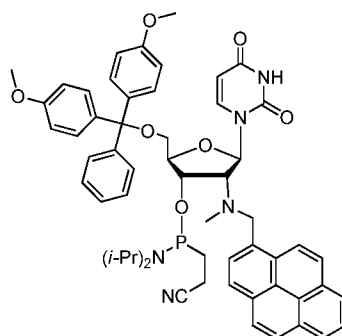
103.2 (C5), 87.3, 86.6 (C4'), 85.3 (C1'), 71.8 (C3'/C2'), 65.8 (C2'/C3'), 63.0 (C5'), 55.5 (CH₃O), 38.5 (NCH₃). A trace impurity of grease was observed at 29.9 ppm.



82V

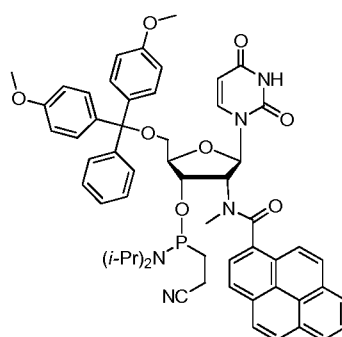
- 5 **2'-Amino-2'-deoxy-2'-N-methyl-2'-N-(pyren-1-yl-methylcarbonyl)-5'-O-(4,4'-dimethoxytrityl)uridine (82V):** Nucleoside **80** (158 mg, 0.28 mmol) was co-evaporated with 1,2-dichloroethane (2 × 5 mL) and subsequently dissolved in anhydrous CH₂Cl₂ (8 mL). To this was added 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC·HCl, 73 mg, 0.38 mmol) and pyrene-1-ylacetic acid (108 mg, 0.41 mmol). The reaction mixture was stirred under argon
- 10 at rt for 3h whereupon the reaction mixture was diluted with CH₂Cl₂ (30 mL) and sequentially washed with sat. aq. NaHCO₃ (20 mL) and H₂O (3 × 15 mL). The organic phase was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (0-3 % *i*-PrOH/CH₂Cl₂, v/v) to afford a rotameric mixture (2:5 by ¹HNMR) of nucleoside **82V** (187 mg, 83 %) as a brown foam. *R*_f: 0.5 (5%, *i*-PrOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 801.3078 ([M]⁺, C₄₉H₄₃N₃O₈, calc. 801.3045); ¹H NMR (DMSO-*d*₆) δ 11.52 (d, 1H, *J* = 1.7 Hz, NH_(A)), 11.46 (d, 0.4H, *J* = 1.7 Hz, NH_(B)), 8.05-8.31 (m, 11.2H, Py_(A) + Py_(B)), 7.89 (d, 1H, *J* = 7.8 Hz, Py_(A)), 7.82 (d, 0.4H, *J* = 7.8 Hz, Py_(B)), 7.67-7.70 (m, 1.4H, H6_(A) + H6_(B)), 7.13-7.43 (m, 12.6H, DMT_(A+B)), 6.78-6.87 (m, 5.6H, DMT_(A+B)), 6.42 (d, 1H, *J* = 8.0 Hz, H1'_(A)), 6.31 (d, 0.4H, *J* = 5.5 Hz, H1'_(B)), 5.92 (d, 0.4H, ex, *J* = 4.9 Hz, 3'-OH_(B)), 5.76 (d, 1H, ex, *J* = 4.9 Hz, 3'-OH_(A)), 5.38 (dd, 1H, *J* = 8.2 Hz, 1.7 Hz, H5_(A)), 5.33 (dd, 0.4H, *J* = 8.2 Hz, 1.7 Hz, H5_(B)), 5.11-5.17 (m, 1H, H2'_(A)), 4.80-4.86 (m, 0.4H, H2'_(B)), 4.58-4.63 (d, 1H, *J* = 16.5 Hz, CH₂Py_(A)), 4.51-4.56 (d, 0.4H, *J* = 16.5 Hz, CH₂Py_(B)), 4.37-4.49 (m, 2.4H, 1 × CH₂Py_(A), H3'_(A), H3'_(B)), 4.30-4.37 (d, 0.4H, *J* = 16.5 Hz, CH₂Py_(B)), 4.10-4.16 (m, 1.4H, H4'_(A) + H4'_(B)), 3.68 (s, 2.4H, CH₃O_(B)), 3.64 (s, 3H, CH₃O_(A)), 3.62 (s, 3H, CH₃O_(A)), 3.35-3.37 (m, 0.4H, H5'_(B)), 3.32 (s, 3H, CH₃N_(A)), 3.29-3.32 (m, 1H, H5'_(A), overlap with H₂O), 3.23-3.27 (dd, 0.4H, *J* = 10.6 Hz, 2.6 Hz, H5'_(B)), 3.15-3.19 (dd, 1H, *J* = 10.6 Hz, 2.6 Hz, H5'_(A)), 3.03 (s, 1.2H, CH₃N_(B)); ¹³C NMR (DMSO-*d*₆) δ 172.2, 172.1, 162.81, 162.78, 158.06, 158.03, 158.02, 150.5, 150.4, 144.6, 144.3, 140.2 (C6_(B)), 140.1 (C6_(A)), 135.4, 135.3, 135.2, 135.0, 130.7, 130.62, 130.56, 130.3, 130.2, 129.71 (DMTr), 129.69 (DMTr), 129.1, 128.1 (Py-CH_A), 127.9 (Py-CH_B), 127.8 (DMTr), 127.73 (DMTr), 127.70 (DMTr), 127.3 (Py), 127.14 (Py), 127.10 (Py), 126.73 (Py), 126.66 (DMTr), 126.1 (Py), 125.1 (Py), 125.0 (Py), 124.9 (Py), 124.5 (Py), 124.1, 124.0, 123.85 (Py), 123.76 (Py), 113.2 (DMTr), 113.1 (DMTr), 102.2 (C5_(A)), 102.0 (C5_(B)), 85.9, 85.7, 85.5 (C1'_(B)), 85.2 (C4'_(A)), 84.8 (C4'_(B)), 83.2 (C1'_(A)), 70.5 (C3'_(A)), 69.3 (C3'_(B)), 63.8
- 20
- 25
- 30

(C5' _(A)), 63.7 (C5' _(B)), 62.1 (C2' _(B)), 59.0 (C2' _(A)), 54.92 (CH₃O _(B)), 54.87 (CH₃O _(B)), 54.85 (CH₃O _(A)), 54.82 (CH₃O _(A)), 38.1 (CH₂Py _(A)), 37.8 (CH₂-Py _(B)), 34.2 (CH₃N _(A)), 31.4 (CH₃N _(B)).

**84Q**

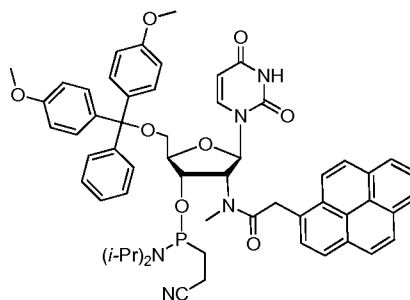
5 **2'-Amino-2'-deoxy-2'-N-methyl-2'-N-(pyren-1-yl-methyl)-3'-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)uridine (84Q):** Nucleoside **82Q** (135 mg, 0.18 mmol) was co-evaporated with CH₃CN (2 × 4 mL) and redissolved in anhydrous CH₃CN (2.5 mL). To this was added DIPEA (153 μL, 0.87 mmol) and PCI-reagent (78 μL, 0.35 mmol). The reaction mixture was stirred at rt for 4h, whereupon it was cooled on an ice bath and abs. EtOH (3 mL) was added. The solvent was evaporated off and the resulting residue purified by silica gel column chromatography (0-40 % EtOAc in petroleum ether, v/v; column built in 0.5 % Et₃N) to afford nucleoside **84Q** as a white foam (97 mg, 57 %). *R*_f: 0.3 (40% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 996.4046 ([M+Na]⁺, C₅₇H₆₀N₅O₈PNa⁺, Calc. 996.4077); ³¹P NMR (CDCl₃) δ 151.0, 149.8.

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**84S**

20 **2'-Amino-2'-deoxy-2'-N-methyl-3'-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-2'-N-(pyren-1-yl-carbonyl)-5'-O-(4,4'-dimethoxytrityl)uridine (84S):** Nucleoside **82S** (219 mg, 0.28 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2 × 2 mL) and redissolved in anhydrous CH₂Cl₂ (2 mL). To this was added DIPEA (58 μL, 0.33 mmol) followed by dropwise addition of PCI-reagent (74 μL, 0.33 mmol). After stirring at rt for 2h, CH₂Cl₂ (10 mL) was added and the reaction mixture stirred for additional 10 min whereupon the solvent was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography (1st

column: 0-40 % EtOAc in petroleum ether, v/v; 2nd column: 0-4 % MeOH in CH₂Cl₂, v/v) to afford a rotameric mixture of phosphoramidite **84S** as a white foam (138 mg, 49 %). *R*_f: 0.8 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1010.3865 ([M+Na]⁺, C₅₇H₅₈N₅O₉PNa⁺, Calc. 1010.3870); ³¹P NMR (CDCl₃) δ 151.8, 151.2, 150.8.

**84V**

2'-Amino-2'-deoxy-2'-N-methyl-3'-O-(N,N-diisopropylamino-2-cyanoethoxyphosphinyl)-2'-N-(pyren-1-yl-methylcarbonyl)-5'-O-(4,4'-dimethoxytrityl)uridine (84V): Nucleoside **82V** (0.30 g, 0.37 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2 × 3 mL) and redissolved in anhydrous CH₂Cl₂ (4 mL). To this was added DIPEA (0.32 mL, 1.84 mmol) followed by dropwise addition of PCl-reagent (0.16 mL, 0.74 mmol). After stirring for 1.5 h at rt, CH₂Cl₂ (10 mL) was added and the reaction mixture stirred for additional 10 min whereupon the solvent was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography (0-60 % EtOAc in petroleum ether, v/v) to afford phosphoramidite **84V** as a bright yellow foam (153 mg, 42 %). *R*_f: 0.4 (60% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 1024.4037 ([M+Na]⁺, C₅₈H₆₀N₅O₉PNa⁺, Calc. 1024.4026); ³¹P NMR (CDCl₃) δ 150.6, 150.5.

General procedure for **222**: Coevaporated starting material (1 mmol) in anhydrous 1,2-dichloroethane (2 x 5 mL) and redissolved in anhydrous 1,2-dichloroethane (5 mL). To this was added NaBH(OAc)₃ (1.5-7 eq) and RCHO (1.5 eq). Allowed to stir at rt under an argon atmosphere for 5-22 h whereupon the reaction mixture was diluted with CH₂Cl₂ (50 mL) washed with sat. aq. NaHCO₃ (2 x 30 mL) and H₂O (30 mL). Back-extracted aqueous phase with CH₂Cl₂ (2 x 20 mL), dried over Na₂SO₄ and evaporated to dryness. The crude was then purified via silica gel column chromatography isolate products (43-95%).

General procedure for synthesis of **224**: Coevaporated starting material (1 mmol) in anhydrous 1,2-dichloroethane (2 x 10 mL) and redissolved in anhydrous 1,2-dichloroethane (10 mL). To this was added NaBH(OAc)₃ (10 eq) and CH₂O (1.5 eq) and was stirred at rt under argon atmosphere for 4-7 h whereupon the reaction mixture was diluted with CH₂Cl₂ (50 mL) washed with sat. aq. NaHCO₃ (2 x 30 mL) and the aqueous back-extracted with CH₂Cl₂ (2 x 20 mL), dried over Na₂SO₄ and evaporated

to dryness. The crude was then purified via silica gel column chromatography isolate products (89-99%).

General procedure for synthesis of **226**: Coevaporated starting material (1 mmol) in anhydrous 1,2-dichloroethane (10 mL) and redissolved in an. CH₂Cl₂ (10 mL). To this was added DIPEA (4-5 eq) followed by dropwise addition of "PCI" reagent (2-3 eq) and allowed to stir under argon atmosphere at rt for 2-4 h whereupon the reaction mixture was quenched with cold EtOH (1 mL) and evaporated to dryness. The crude was purified by silica gel column chromatography and precipitated in CH₂Cl₂ and petroleum ether to afford the desired phosphoramidite (62-90%).

Synthesis of **240**. Naphthy-2-methylthiol (616 mg, 3.53 mmol) and sodium hydride (42 mg, 1.77 mmol) were dissolved in an. DMA (3 mL) and stirred under argon atm for 10 min whereupon O₂,O₂'-anhydrouridine **70** (200 mg, 0.88 mmol) was added. After stirring for 48 h, the reaction mixture was diluted with EtOAc (30 mL), neutralized with acetic acid (1 drop) and washed with H₂O (2 x 15 mL). The aqueous was back-extracted with CH₂Cl₂ (3 x 15 mL) and the organic layers evaporated to dryness. The crude product was purified by silica gel column chromatography (0-7% MeOH/CH₂Cl₂, v/v) to give nucleoside **240** (311 mg, 88%). R_f = 0.3 (10% MeOH in CH₂Cl₂); ¹H NMR (DMSO-d₆) 11.33 (br s, 1H, NH, ex), 7.77-7.89 (m, 3H, Ar), 7.61-7.68 (m, 2H, Ar), 7.39-7.54 (m, 3H, Ar, H₆), 6.09 (d, 1H, *J* = 8.23 Hz, H1'), 5.65 (d, 1H, *J* = 5.21 Hz, ex, 3'-OH), 5.34 (d, 1H, *J* = 7.96 Hz, H₅), 5.05 (t, 1H, *J* = 5.21, ex, 5'-OH), 4.15-4.20 (m, 1H, H₃'), 3.81-3.94 (m, 3H, SCH₂, H₄'), 3.50-3.58 (m, 2H, H₅'), 3.36 (dd, 1H, *J* = 8.5 Hz, 5.5 Hz, H₂').

Synthesis and purification of single-stranded probes modified with monomers 120W/X/Y/Z, 120Q/S/V, 120'W, 120'X, 140'X, 140'Y, 208W/X/Y/Z, 228Y/Z and 244: Synthesis of modified probes was performed on a DNA synthesizer using 0.2 μmol scale succinyl linked LCAA-CPG (long chain alkyl amine controlled pore glass) columns with a pore size of 500Å. Standard protocols for incorporation of DNA phosphoramidites were used. A ~50-fold molar excess of modified phosphoramidites in anhydrous acetonitrile (at 0.05 M) was typically used during hand-couplings (performed to conserve material) except with **76Z** (~70-fold molar excess in anhydrous CH₂Cl₂, at 0.07M). Moreover, extended oxidation (45s) and coupling times were typically used (0.01 M 4,5-dicyanoimidazole as activator, 15 min for monomers **84V/76W/76Y**, 35 min for monomer **76Z**; 0.01 M 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole [Activator 42], 15 min for monomers **84Q/84S/84V**). Probes modified with monomers **120'W, 120'X, 140'X, 140'Y, 208W/X/Y/Z, 228Y/Z** and **244**, were made in an equivalent manner. Cleavage from solid support and removal of protecting groups was typically accomplished upon treatment with 32% aq. ammonia (55 °C, 24 h). Purification of all modified oligonucleotides was performed to minimum 80% purity using either of two methods: a) overall synthesis yield >80%: cleavage of DMT using 80% aq. AcOH, followed by

precipitation from acetone (-18 °C for 12-16 h) and washing with acetone, or b) overall synthesis yield <80%; purification of oligonucleotides by RP-HPLC as described below, followed by detritylation and precipitation.

Purification of the crude oligonucleotides was performed on a HPLC system equipped with an XTerra MS C18 pre-column (10 µm, 7.8 x 10 mm) and a XTerra MS C18 column (10µm, 7.8 x 150 mm). The identity of synthesized oligonucleotides was established through MALDI-MS/MS analysis (Table 35-36) recorded in positive ions mode on a Quadrupole Time-Of-Flight Tandem Mass Spectrometer equipped with a MALDI source using anthranilic acid as a matrix (Tables 39-51), while purity (>80%) was verified by RP-HPLC running in analytical mode.

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Table 39
MALDI-MS of Representative Single-Stranded Probes Modified with Monomers 120W/X/Y/Z

ONs	Sequence	Calc. <i>m/z</i> [M]	Found <i>m/z</i> [M+H]
120W1	5'-G 120W G ATA TGC	2880	2881
120W2	5'-GTG A 120W A TGC	2880	2881
120W3	5'-GTG ATA 120W G C	2880	2881
120W4	3'-CAC 120W AT ACG	2809	2810
120W5	3'-CAC TA 120W ACG	2809	2810
120X1	5'-G 120X G ATA TGC	2954	2955
120X2	5'-GTG A 120X A TGC	2954	2955
120X3	5'-GTG ATA 120X G C	2954	2955
120X4	3'-CAC 120X AT ACG	2883	2884
120X5	3'-CAC TA 120X ACG	2883	2884
120X6	3'-CAC 120X A 120X ACG	3085	3086
120X7	5'-G 120X G A 120X A 120X G C	3358	3359
120Y1	5'-G 120Y G ATA TGC	2968	2969
120Y2	5'-GTG A 120Y A TGC	2968	2969
120Y3	5'-GTG ATA 120Y G C	2968	2969
120Y4	3'-CAC 120Y AT ACG	2897	2898
120Y5	3'-CAC TA 120Y ACG	2897	2898
120Y6	3'-CAC 120Y A 120Y ACG	3113	3114
120Y7	5'-G 120Y G A 120Y A 120Y G C	3400	3401
120Z1	5'-G 120Z G ATA TGC	3066	3067
120Z2	5'-GTG A 120Z A TGC	3066	3067
120Z3	5'-GTG ATA 120Z G C	3066	3067
120Z4	3'-CAC 120Z AT ACG	2995	2996
120Z5	3'-CAC TA 120Z ACG	2995	2996
120Z6	3'-CAC 120Z A 120Z ACG	3309	3310
120Z7	5'-G 120Z G A 120Z A 120Z G C	3694	3695

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Table 40
MALDI-MS of Representative Single-Stranded Probes Modified with Monomers 120Q/S/V

ONs	Sequence	Calc. m/z [M]	Found m/z [M+H]
120Q1	5'-G 120Q G ATA TGC	2982	2983
120Q2	5'-GTG A 120Q A TGC	2982	2983
120Q4	3'-CAC 120Q AT ACG	2911	2912
120Q5	3'-CAC TA 120Q ACG	2911	2912
120Q6	3'-CAC 120QA120Q ACG	3140	3141
120S1	5'-G 120S G ATA TGC	2996	2997
120S2	5'-GTG A 120S A TGC	2996	2997
120S4	3'-CAC 120S AT ACG	2925	2926
120S5	3'-CAC TA 120S ACG	2925	2926
120S6	3'-CAC 120SA120S ACG	3168	3169
120V1	5'-G 120V G ATA TGC	3009	3010
120V2	5'-GTG A 120V A TGC	3009	3010
120V4	3'-CAC 120V AT ACG	2938	2939
120V5	3'-CAC TA 120V ACG	2938	2939
120V6	3'-CAC 120VA120V ACG	3195	3196

5

Table 41
MS-Data of Representative Single-Stranded Probes Modified with Monomer 120'W (= M).^a

ONs	Sequence	Calc. m/z	Found m/z
120'W6	5'-GTG M TA TGC	2984	2984
120'W7	5'-GTG AT M TGC	2984	2984
120'W8	3'-CAC T M T ACG	2913	2913
120'W9	3'-CAC TAT M CG	2913	2914

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Table 42
MS-Data of Representative Single-Stranded Probes Modified with Monomer 208W/X/Y/Z^a

ONs	expected mass	observed mass [M+H]
5'-GTG A(208W)A TGC	3038	3039
3'-CAC TA(208W) ACG	2967	2968
5'-GTG A(208X)A TGC	3046	3046
3'-CAC TA(208X) ACG	2975	2975
5'-GTG A(208Y)A TGC	2982	2983
3'-CAC TA(208Y) ACG	2911	2912
5'-GTG A(208Z)A TGC	3054	3056
3'-CAC TA(208Z) ACG	2983	2984

Table 43
MS-Data of Representative Single-Stranded Probes Modified with Monomer 228Y/Z^a

Probe	Calc [M]	Found [M+1]
5' – GTG A(228Y)A TGC	3031.6	3032.4
3' – CAC TA(228Y) ACG	2960.6	2961.4
5' – GTG A(228Z)A TGC	3079.6	3079.6
3' – CAC TA(228Z) ACG	3008.6	3010.5

Table 44
MS-Data of Additional Single-Stranded Probes Modified with Monomer 120Y

Probes	Expected mass	Observed [M+H]
5'-GG(120Y) ATA TAT AGG C 3'-CCA (120Y)AT ATA TCC G	4227 4107	4228 4108
5'-GGT A(120Y)A TAT AGG C 3'-CCA TA(120Y) ATA TCC G	4227 4107	4228 4108
5'-GGT ATA (120Y)AT AGG C 3'-CCA TAT A(120Y)A TCC G	4227 4107	4228 4108
5'-GGT ATA TA(120Y) AGG C 3'-CCA TAT ATA (120Y)CC G	4227 4107	4228 4108
5'-GG(120Y) A(120Y)A TAT AGG C 3'-CCA (120Y)A(120Y) ATA TCC G	4443 4323	4444 4324
5'-GG(120Y) ATA (120Y)AT AGG C 3'-CCA (120Y)AT A(120Y)A TCC G	4443 4323	4444 4324
5'-GG(120Y) ATA TA(120Y) AGG C 3'-CCA (120Y)AT ATA (120Y)CC G	4443 4323	4444 4324
5'-GGT A(120Y)A (120Y)AT AGG C 3'-CCA TA(120Y) A(120Y)A TCC G	4443 4323	4444 4324
5'-GGT ATA (120Y)A(120Y) AGG C 3'-CCA TAT A(120Y)A (120Y)CC G	4443 4323	4444 4324
5'-GG(120Y) A(120Y)A (120Y)A(120Y) AGG C 3'-CCA (120Y)A(120Y) A(120Y)A (120Y)CC G	4875 4755	4876 4757

Table 45
MS-Data of Additional Single-Stranded Probes Modified with Monomer 120'W

5		Probe	Expected mass	Observed Mass [M+H]
		3'-CC(120'W) TAT ATA TCC G	4121	4122
		3'-CCA T(120'W)T ATA TCC G	4121	4122
10		3'-CCA TAT (120'W)TA TCC G	4121	4122
		3'-CCA TAT AT(120'W) TCC G	4121	4123
15		3'-CC(120'W) T(120'W)T ATA TCC G	4351	4352
		3'-CC(120'W) TAT (120'W)TA TCC G	4351	4352
		3'-CC(120'W) TAT AT(120'W) TCC G	4351	4353
20		3'-CCA T(120'W)T (120'W)TA TCC G	4351	4353
		3'-CCA TAT (120'W)T(120'W) TCC G	4351	4352
25		3'-CC(120'W) T(120'W)T (120'W)T(120'W) TCC G	4812	4813

Table 46
MS-Data of Even Further Additional Single-Stranded Probes Modified with Monomer 120Y

30		Probe	Expected mass	Observed Mass [M+H]
		5'-G-G(120Y)A-TAT-AAG-CAG-CAC-A	5440	5441
		3'-C-CA(120Y)-ATA-TTC-GTC-GTG-T	5364	5365
		5'-G-G(120Y)A-(120Y)AT-AAG-CAG-CAC-A	5657	5658
		3'-C-CA(120Y)-A(120Y)A-TTC-GTC-GTG-T	5580	5582
		5'-AGG-AAG-G(120Y)A-(120Y)AT-AAG-CA	5721	5722
		3'-TCC-TTC-CA(120Y)-A(120Y)A-TTC-GT	5515	5516
		5'-G-G(120Y)A-(120Y)AT-AAG-CAG-C	4741	4742
		3'-C-CA(120Y)-A(120Y)A-TTC-GTC-G	4643	4684
		5'-AC(120Y)-A(120Y)A-GAA-TAC-TCA-AG	5616	5617
		3'-TGA-(120Y)A(120Y)-CTT-ATG-AGT-TC	5620	5621
		5'-AC(120Y)-A(120Y)A-GAA-TAC-TC	4660	4661
		3'-TGA-(120Y)A(120Y)-CTT-ATG-AG	4722	4723

Table 47
MS-Data of Even Further Single-Stranded Probes Modified with Monomer 120Q

Probe	Calc [M]	Found [M+H]
5' - GG(120Q) ATA TAT AGG C	4240.8	4242.5
3' - CCA (120Q)AT ATA TCC G	4120.8	4122.6
5' - GG(120Q) A(120Q)A TAT AGG C	4469.9	4471.5
3' - CCA (120Q)A(120Q) ATA TCC G	4349.9	4351.6
5' - GGT A(120Q)A (120Q)AT AGG C	4469.9	4471.9
3' - CCA TA(120Q) A(120Q)A TCC G	4349.9	4351.4
5' - GG(120Q) A(120Q)A (120Q)A(120Q) AGG C	4928.1	4929.7
3' - CCA (120Q)A(120Q) A(120Q)A (120Q)CC G	4808.1	4810.5

5

Table 48
MS-Data of Yet Even Further Additional Single-Stranded Probes Modified with Monomer 120Q

Probe	Expected mass	Observed Mass [M+H]
5'-G-G(120Q)A-TAT-AAG-CAG-CAC-A 3'-C-CA(120Q)-ATA-TTC-GTC-GTG-T	5454 5377	5455 5378
5'-G-G(120Q)A-(120Q)AT-AAG-CAG-CAC-A 3'-C-CA(120Q)-A(120Q)A-TTC-GTC-GTG-T	5683 5607	5684 5608
5'-AGG-AAG-G(120Q)A-(120Q)AT-AAG-CA 3'-TCC-TTC-CA(120Q)-A(120Q)A-TTC-GT	5747 5542	5749 5543
5'-AC(120Q)-A(120Q)A-GAA-TAC-TCA-AG 3'-TGA-(120Q)A(120Q)-CTT-ATG-AGT-TC	5642 5646	5646 5648

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Table 49
MS-Data of Even Further Additional Single-Stranded Probes Modified with Monomer 120Y, 120'W and 140'X

Probe	Expected mass	Observed Mass [M+H]
5'-CC(140'X) ACG T(120Y)A GCA GTT 3'-GGG (120Y)GC AA(120Y) CGT CAA	4971 5046	4973 5047
5'-CCC ACG T(120Y)A G(140'X)A GTT 3'-GGG TGC AA(120Y) CG(120Y) CAA	4971 5046	4972 5047
5'-AGA CAA AA(140'X) AC(140'X) AGT 3'-TCT GTT TTG (120Y)GG (120Y)CA	5020 5009	5021 5010
5'-AGA (140'X)AA AA(140'X) ACC AGT 3'-TCT G(120Y)T TTG (120Y)GG TCA	5020 5009	5021 5011

5'-CTA (140'X)AT (120Y)GT CTC GCC 3'-GAT G(120Y)A A(140'X)A GAG CGG	4922 5109	4923 5110
5'-CTA C(120'W)T (120Y)GT CTC GCC 3'-GAT GT(120'W) A(140'X)A GAG CGG	4922 5123	4923 5124
5'-CGT (140'X)AT CG(120Y) GCT CGC 3'-GCA G(120Y)A GCA (140'X)GA GCG	4963 5070	4965 5071

5 **Table 50**
MS-Data of Yet Even Further Additional Single-Stranded Probes Modified with Monomers
120Y, 120'W, 140'X and 140'Y

Probes	Expected mass	Observed Mass [M+H]
5'-CGG ACC ACG (120Y)G(120Y) GTG 3'-GCC TGG TGC A(140'X)A (140'X)AC	5038 4995	5040 4996
5'-CGG AC(140'X) ACG TG(120Y) GTG 3'-GCC TGG (120Y)GC ACA (140'X)AC	5052 4981	5053 4982
5'-GT(140'X) AG(120Y) GGG CGT TGC 3'-CAG (120Y)CA (140'X)CC GCA ACG	5083 4950	5084 4951
5'-GT(140'X) AG(120Y) GGG CG(120Y) TGC 3'-CAG (120Y)CA (140'X)CC GCA (120'W)CG	5299 5181	5299 5182
5'-CCT C(120Y)A (120Y)AA AAG CGG 3'-GGA GA(120Y) A(120Y)T TTC GCC	4990 5012	4991 5013
5'-CC(120Y) C(120Y)A (120Y)AA AAG CGG 3'-GGA (140'Y)A(120Y) A(120Y)T TTC GCC	5206 5242	5208 5244

Table 51
MS-Data of Yet Even Further More Additional Single-Stranded Probes
Modified with Monomer 120Y, 120'W, 140'X and 140'Y

5'-Cy3 labeled probes	Expected mass	Observed mass [M+H]
5'-AGC CC(120Y) G(120Y)G CCC TG 3'-TCG GGA (140'X)A(140'X) GGG AC	5522 5495	5523 5496
5'-CC(120Y) G(120Y)G CCC TG 3'-GGA (140'X)A(140'X) GGG AC	5390 6075	5391 6076
5'-CC(120Y) GTG CC(140'X) TG 3'-GGA (140'X)AC GGG (120'W)C	5713 5922	5714 5923
5'-(120'W)GC CC(120Y) GTG CC(140'X) TG 3'-T(140'X)G GGA (140'X)AC GGG (120'W)C	5508 6520	5509 5621
5'-C(120Y)G (120'W)GC CC(120Y) G(120Y)G CCC (120Y)G-3' 3'-GA(140'Y) T(140'X)G GGA (140'X)A(140'X) GGG A(140'X)-5'	6697 6940	6696 6941
5'-(120'W)GC CC(120Y) G(120Y)G CCC (120Y)G-3' 3'-T(140'X)G GGA (140'X)A(140'X) GGG A(140'X)-5'	5599 5739	5600 5740

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Thermal Denaturation Studies involving probes modified with 120W/X/Y/Z, 120Q/S/V, 120'W, 120'X, 140'X, 140'Y, 208W/X/Y/Z, 228Y/Z and 244: Concentrations of oligonucleotides were estimated using the following extinction coefficients for DNA (OD/ μ mol): G (12.01), A (15.20), T (8.40), C (7.05); for RNA (OD/ μ mol): G (13.70), A (15.40), U (10.00), C (9.00); for fluorophores (OD/ μ mol): naphthalene (3.75), pyrene (22.4), and coronene (36.0). Each strand was thoroughly 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 values [°C]) of duplexes (1.0 μ M final concentration of each strand) were measured on a 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 1°C). A temperature ramp of 0.5 °C/min was used in all experiments. Reported T_m -values are averages of two experiments within ± 1.0 °C.

In particular disclosed embodiments, single-stranded probes modified with monomers **120W/X/Y/Z** or **120 Q/S/V**, display varying trends in thermal affinity toward complementary RNA ($\Delta T_m/\text{mod} = -12.0$ °C to +10.5 °C, Table 52 Substantial DNA-selectivity, defined as $\Delta\Delta T_m$ (DNA-

RNA) = ΔT_m (vs DNA) - ΔT_m (vs RNA) > 0 °C, is observed for these probes (Table 53). The DNA-selectivity of probes modified with unlocked monomers **120Y** and **120Z** is especially pronounced and comparable to that of probes modified with locked monomer **126W** (Table 53). This suggests that interesting DNA-targeting applications are possible. DNA-selective hybridization is typically observed for oligonucleotides modified with intercalating moieties, since intercalation favors the less compressed helix geometry of DNA:DNA duplexes. In contrast, the majority of probes modified with monomers **120X** or **120W** display much lower DNA selectivity, indicating that intercalative binding modes are less prominent than in probes modified with monomer **120Y** and **120Z** (Table 53).

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Table 52
 T_m -Values of Duplexes between Probe Modified with Monomers 120W/X/Y/Z/Q/S/V Or 126W and Complementary RNA Targets

ON	Duplex	<u>B</u> =	T_m [ΔT_m /mod] (°C)							
			T	120W	120X	120Y	120Z	120Q	120S	120V 126W
B1	5'-G <u>B</u> G ATA TGC		26.5	21.0	22.5	24.5	21.5	22.0	14.0	20.0 27.0
R2	3'-CAC UAU ACG			[-5.5]	[-4.0]	[-2.0]	[-5.0]	[-4.5]	[-12.5]	[-6.5] [+0.5]
B2	5'-GTG A <u>B</u> A TGC		26.5	16.0	22.5	30.5	37.0	29.0	18.0	28.0 31.5
R2	3'-CAC UAU ACG			[-10.5]	[-4.0]	[+4.0]	[+10.5]	[+2.5]	[-8.5]	[+1.5] [+5.0]
B3	5'-GTG ATA <u>B</u> GC		26.5	18.0	14.5	26.5	30.5	ND	ND	ND 28.0
R2	3'-CAC UAU ACG			[-8.5]	[-12.0]	[±0.0]	[+4.0]			
R1	5'-GUG AUA UGC		24.5	16.5	16.5	20.0	22.5	17.5	9.0	16.0 23.5
B4	3'-CAC <u>B</u> AT ACG			[-8.0]	[-8.0]	[-4.5]	[-2.0]	[-7.0]	[-15.5]	[-8.5] [-1.0]
R1	5'-GUG AUA UGC		24.5	19.5	21.5	27.0	30.5	27.0	16.0	26.5 32.0
B5	3'-CAC TA <u>B</u> ACG			[-5.0]	[-3.0]	[+2.5]	[+6.0]	[+2.5]	[-8.5]	[+2.0] [+7.5]
R1	5'-GUG AUA UGC		24.5	ND	14.5	24.0	11.0	20.0	ND	19.0 ND
B6	3'-CAC <u>B</u> A <u>B</u> ACG				[-5.0]	[-0.3]	[-6.8]	[-2.3]		[-2.8]
B7	5'-G <u>B</u> G A <u>B</u> A <u>B</u> GC		26.5	ND	15.5	27.5	ND	ND	ND	ND ND
D2	3'-CAC UAU ACG				[-3.7]	[+0.3]				

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Table 53
DNA-Selectivity of Probes Modified With Monomers 120W/X/Y/Z/Q/S/V or 126W

ON	Duplex	<u>B</u> =	$\Delta\Delta T_m$ (DNA-RNA) [°C]						
			120W	120X	120Y	120Z	120Q	120S	120V 126W
B1	5'-G <u>B</u> G ATA TGC		-2.5	+1.0	+7.0	+9.5	+9.5	+6.5	+ 6.0 +6.5
D2	3'-CAC TAT ACG								
B2	5'-GTG A <u>B</u> A TGC		+5.5	+8.0	+8.5	+9.5	+11.5	+11.5	+4.5 +9.0
D2	3'-CAC TAT ACG								
B3	5'-GTG ATA <u>B</u> GC		+3.5	+8.5	+8.0	+7.0	ND	ND	ND +9.0
D2	3'-CAC TAT ACG								
D1	5'-GTG ATA TGC		+5.0	+4.5	+8.0	+8.5	+8.5	+9.5	+9.5 +7.5
B4	3'-CAC <u>B</u> AT ACG								

D1	5'-GTG ATA TGC								
B5	3'-CAC TAB <u>B</u> ACG	0	+4.0	+9.0	+10.0	+10.5	+12.5	+4.5	+8.0
D1	5'-GTG ATA TGC								
B6	3'-CAC <u>B</u> AB <u>B</u> ACG	ND	+6.0	+14.5	+31.0	+18.5	ND	+13.0	ND
B7	5'-G <u>B</u> G A <u>B</u> A <u>B</u> G C								
D2	3'-CAC TAT ACG	ND	+7.0	+20.5	ND	ND	ND	ND	ND

^aDNA selectivity defined as $\Delta\Delta T_m(\text{DNA-RNA}) = \Delta T_m(\text{vs DNA}) - \Delta T_m(\text{vs RNA})$.

The Watson–Crick specificity the disclosed probes may also be determined. In particular disclosed embodiments, modified oligonucleotides may be evaluated using nucleic acid targets with mismatched nucleotides opposite of the monomer-incorporation site. Certain disclosed embodiments concern using single-stranded probes and their ability to associate with DNA (Table 54) or RNA targets (Table 55) comprising mismatched nucleotides opposite of the incorporation site.

The **B2**-series probes generally displayed reduced DNA target specificity relative to reference strand **D1**. For example, **120Y2** and **120Z2** discriminate dT-mismatches very poorly relative to **D1** (compare ΔT_m -values for **D1/120Y2/120Z2** against targets with a dT-mismatch, Table X) while dC- and dG-mismatches almost are as efficiently discriminated as with **D1**. Particular disclosed embodiments of oligonucleotides comprising locked monomers had a similar specificity profile but discriminated the dT-mismatch even more poorly. Interestingly, **120Q2** displays substantially better discrimination of dC- and dT-mismatches than **120Y2**, **120Z2** and **126W2** rendering it as the most specific of the high-affinity DNA-targeting modifications. In particular disclosed embodiments, even minor changes in linker chemistry and length have marked influence on target specificity. For example, **120S2** displays similar DNA target specificity as **120Y2/120Z2/126W2**, while **120V2** discriminates DNA mismatches more poorly. Similarly, **120W2** exhibits very poor target specificity, while **120X2** displays much higher target specificity than **120W2** or **120Y2** (Table 54).

Table 54
Discrimination of Mismatched DNA Targets by the B2-series of Single-stranded Probes.^a

ON	Sequence	B =	DNA: 3'-CAC T <u>B</u> T ACG			
			T_m [°C]		ΔT_m [°C]	
			A	C	G	T
D1	5'-GTG ATA TGC		29.5	-16.5	-9.5	-17.0
120W2	5'-GTG A(120W)A TGC		24.5	-11.0	+2.0	-3.5
120X2	5'-GTG A(120X)A TGC		33.5	<-23.5	-7.0	-13.0
120Y2	5'-GTG A(120Y)A TGC		42.0	-13.0	-5.0	-6.5
120Z2	5'-GTG A(120Z)A TGC		49.0	-13.5	-6.0	-7.0
120Q2	5'-GTG A(120Q)A TGC		43.5	-22.0	-3.5	-12.0
120S2	5'-GTG A(120S)A TGC		32.5	-11.5	-9.0	-8.5
120V2	5'-GTG A(120V)A TGC		35.5	-11.5	+1.5	-3.5
126W2	5'-GTG A(126W)A TGC		43.5	-12.5	-5.5	-3.5

^aFor conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:DNA duplex.

Table 55
Discrimination of Mismatched RNA Targets by the B2-Series of Single-Stranded Probes^a

ON	Probe	<u>B</u> =	RNA: 3'-CAC U <u>B</u> U ACG			
			T_m [°C]		ΔT_m [°C]	
			A	C	G	U
D1	5'-GTG ATA TGC		26.5	<-16.5	-4.5	<-16.5
120W2	5'-GTG A(120W)A TGC		16.0	-1.0	+0.5	+6.0
120X2	5'-GTG A(120X)A TGC		22.5	-11.5	-5.0	<-12.5
120Y2	5'-GTG A(120Y)A TGC		31.0	-17.5	-3.5	-9.5
120Z2	5'-GTG A(120Z)A TGC		37.0	-12.0	-9.0	-13.0
120Q2	5'-GTG A(120Q)A TGC		29.0	-16.5	-0.5	-13.0
120S2	5'-GTG A(120S)A TGC		18.0	<-8.5	-6.0	-6.0
120V2	5'-GTG A(120V)A TGC		28.0	-16.5	-1.0	-7.5
126W2	5'-GTG A(126W)A TGC		31.5	-12.0	-1.0	-4.5

5 ^aFor conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:RNA duplex.

In other disclosed embodiments, the specificity of oligonucleotides with two modifications positioned as next-nearest neighbors (**B6-series**) were evaluated against nucleic acid (e.g. DNA) targets with a single central mismatched nucleotide opposite of the central 2'-deoxyadenosine residue (Table 56). Interestingly, this probe design generally resulted in improved mismatch discrimination relative to reference strand **D2**; A- and G-mismatches are particularly efficiently discriminated.

Table 56
Discrimination of Mismatched DNA Targets by B6-Series and Reference Strands^a

ON	Sequence	<u>B</u> =	DNA : 5'-GTG A <u>B</u> A ACG			
			T_m [°C]		ΔT_m [°C]	
			T	A	C	G
D2	3'-CAC TAT ACG		29.5	-17.0	-15.5	-9.0
120X6	3'-CAC (120X)A(120X) ACG		25.5	<-15.5	-14.0	-14.5
120Y6	3'-CAC (120Y)A(120Y) ACG		43.5	-24.0	-17.0	-14.0
120Z6	3'-CAC (120Z)A(120Z) ACG		47.0	-19.5	-13.0	-11.0
120Q6	3'-CAC (120Q)A(120Q) ACG		43.5	-21.5	-10.5	-13.5
120V6	3'-CAC (120V)A(120V) ACG		37.0	-14.5	-13.5	-11.0

15 ^a For conditions of thermal denaturation experiments, see Table 33 above. T_m -values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:DNA duplex.

In particular disclosed embodiments, the fluorescence characteristics of the disclosed probe can be examined, wherein an observed optical signal provides information regarding the probe and/or target. In particular disclosed embodiments, absorption and steady state fluorescence emission spectra of a probe comprising one or more of the disclosed monomers, such as monomers

- 5 **120Q/120S/120V/120X/120Y**, in the presence or absence of complementary or mismatched nucleic acid targets (e.g. DNA/RNA) were recorded to gain additional insight into the binding modes of the intercalator (e.g. pyrene) moieties and/or presence of a matched or mismatched target. A person of ordinary skill in the art will recognize that intercalation of pyrene moieties is known to induce bathochromic shifts of pyrene absorption peaks. Particular embodiments of the disclosed probe, such as those modified with monomers **120Q/120S/120Y**, display significant bathochromic shifts upon hybridization with nucleic acid (e.g. DNA) targets (average $\Delta\lambda_{\max} = 2.8$ -5.0 nm vs 2.5 nm, respectively, Table 58 and FIG 8. Hybridization with complementary RNA leads to smaller bathochromic shifts (average $\Delta\lambda_{\max} = 2.0$ -4.5 nm, Table 58). Probes **120X1-120X5** display distinctly smaller hybridization-induced bathochromic shifts than the other pyrene-functionalized oligonucleotides (average $\Delta\lambda_{\max} \sim 0.6$ nm, Table 57 and FIG 7), which substantiates the trends from thermal denaturation studies indicating that intercalation of the pyrene moiety is a less important binding mode for monomer **120X**.

20 **Table 57**
Absorption Maxima in the 320-360 Nm Region for Probes Modified with Monomers 120X-120Z
Or 126W in the Presence or Absence of Complementary DNA/RNA Targets^a

ON	Sequence	$\lambda_{\max}[\Delta\lambda_{\max}]$ (nm)											
		B=			120X			120Y			120Z		
		ss	+DNA	+RNA	ss	+DNA	+RNA	ss	+DNA	+RNA	ss	+DNA	+RNA
B1	5'-G <u>B</u> G ATA TGC	349	352[+3]	351[+2]	350	353[+3]	352[+2]	347	348[+1]	348[+1]	348	350[+2]	349[+1]
B2	5'-GTG A <u>B</u> A TGC	353	352[-1]	352[±0]	348	353[+5]	352[+4]	347	348[+1]	348[+1]	347	351[+4]	349[+2]
B3	5'-GTG ATA <u>B</u> GC	352	352[±0]	352[±0]	350	353[+3]	352[+2]	348	348[±0]	348[±0]	348	351[+3]	350[+2]
B4	3'-CAC <u>B</u> AT ACG	350	352[+2]	352[+2]	350	352[+2]	352[+2]	347	348[+1]	348[+1]	348	350[+2]	348[±0]
B5	3'-CAC TA <u>B</u> ACG	353	352[-1]	352[-1]	349	353[+4]	352[+3]	347	348[+1]	347[±0]	348	350[+2]	349[+1]

^a Measurements were performed at room temperature (monomer **126W**), 10 °C (monomer **120Z**) and 7 °C (monomer **120X** and **120Y**) using a spectrophotometer and quartz optical cells with a 1.0 cm path length. Buffer conditions are as for thermal denaturation experiments. Values in italics are for duplexes with low thermostability (partial duplex dissociation at experimental temperature possible).

Table 58
Absorption Maxima in the 320-360 Nm Region for Probes Modified with Monomers
120Q/120S/120V in the Presence or Absence of Complementary DNA/RNA Targets^a

ON	Sequence	B=	$\lambda_{\max}[\Delta\lambda_{\max}]$ (nm)											
			120Q			120S			120V			126W		
			ss	+DNA	+RNA	ss	+DNA	+RNA	ss	+DNA	+RNA	ss	+DNA	+RNA
B1	5'-G <u>B</u> G ATA TGC		349	353[+4]	351[+2]	347	351[+4]	348[+1]	348	352[+4]	352[+4]	348	350[+2]	349[+1]
B2	5'-GTG A <u>B</u> A TGC		348	353[+5]	351[+3]	346	349[+3]	349[+3]	347	352[+5]	353[+6]	347	351[+4]	349[+2]
B4	3'-CAC <u>B</u> AT ACG		349	354[+5]	349[±0]	348	350[+2]	348[±0]	349	353[+4]	352[+3]	348	350[+2]	348[±0]
B5	3'-CAC TA <u>B</u> ACG		348	354[+6]	352[+4]	346	348[+2]	350[+4]	347	352[+5]	352[+5]	348	350[+2]	349[+1]

^aMeasurements were performed at 5 °C.

- 5 **Steady-state fluorescence emission spectra:** Steady-state fluorescence emission spectra of oligonucleotides modified with pyrene-functionalized monomers **120Q/120S/120V/120X/120Y** and the corresponding duplexes with complementary DNA/RNA targets were recorded in non-deoxygenated thermal denaturation buffer (each strand 1.0 μM) and obtained as an average of five scans using an excitation wavelength of $\lambda_{\text{ex}} = 350$ nm, excitation slit 5.0 nm, emission slit 2.5 nm and
- 10 a scan speed of 600 nm/min (FIGS 9-17). Experiments were determined at 5 °C to ascertain maximal hybridization of probes to DNA/RNA targets. Solutions were heated to 80 °C followed by cooling to 5 °C over 10 min.

The steady-state fluorescence emission spectra ($\lambda_{\text{ex}} = 350$ nm; $\lambda_{\text{em}} = 360$ -600 nm; $T = 5$ °C) display the two expected vibronic bands I and III at $\lambda_{\text{em}} = 382 \pm 3$ nm and 402 ± 3 nm, respectively, as

15 well as a small shoulder at ~420 nm (FIGS. 9-17). Hybridization of oligonucleotides modified with monomers **120Q/120S/120V/120X/120Y** with complementary targets, and DNA in particular, results in reduced fluorescence emission intensity (FIGS. 9-17). This trend is indicative of pyrene intercalation as nucleobase moieties are known to quench pyrene fluorescence via photoinduced electron transfer (PET) with guanine and cytosine moieties being the strongest quenchers. In

20 agreement with this, duplexes involving the **120Y/120Q/120V**-series (intercalation, important binding mode) display far lower emission intensity than those involving the **120X**-series (intercalation, less important binding mode).

On balance, the data from the thermal denaturation and optical spectroscopy studies (DNA-selectivity; mismatch discrimination; UV-vis; fluorescence) suggest that intercalation of the attached

25 hydrocarbon is a possible binding mode for all studied monomers. Intercalation is least important for monomers **120W** and **120X**, while being a dominant binding mode for monomers **120Y**, **120Z** and **120Q**.

Additional working examples of embodiments involving probes modified with monomers 228Y/Z.

Particular embodiments involve probes modified with monomers **228Y** and **228Z**. With reference to Table 59 below, it is observed that single-stranded probes modified with monomers **228Y** and **228Z** display significantly increased thermal affinity toward nucleic acid targets, more commonly single-stranded DNA. This property will facilitate targeting of double-stranded nucleic acid targets, more commonly dsDNA, via the approach shown in FIG 1.

Table 59
Additional Examples of Thermal Denaturation Temperatures of DNA Duplexes
Modified with Monomers 228Y or 228Z

Sequence	B =	T _m (ΔT _m /mod)	
		228Y	228Z
5' – GTG ABA TGC		48.0	50.5
3' – CAC TAT ACG		(+18.5)	(+21.0)
5' – GTG ATA TGC		51.5	49.5
3' – CAC TAB ACG		(+22.0)	(+20.0)
5' – GTG ATA TGC		41.0	37.0
3' – CAC BAT ACG		(+11.5)	(+7.5)
5' – GBG ATA TGC		41.0	37.0
3' – CAC TAT ACG		(+11.5)	(+7.5)
5' – GTG ATA TBC		45.5	43.5
3' – CAC TAT ACG		(+16.0)	(+14.0)
5' – GTG ATA TGC		54.0	54.0
3' – CAC BAB ACG		(+12.3)	(+12.3)

Particular embodiments involve double-stranded probes with certain interstrand zipper arrangements of monomers **228Y** and/or **228Z**. With reference to Table 60 below, it is observed that double-stranded probes +1 and +2 interstrand zipper arrangements display relatively low thermostability, which coupled with the high DNA affinity that each probe strands displays (Table 59), results in pronounced potential for targeting of double-stranded nucleic acid targets, more commonly dsDNA via the method outlined in FIG 1.

Table 60
Additional Examples of Double-Stranded Probes with Various
Interstrand Zipper Arrangements of Monomer 228Y or 228Z

Sequence	B =	T _m (ΔT _m /mod)	
		228Y	228Z
5' – GTG ABA TGC		32.5	41.0
3' – CAC TAB ACG		(+1.5)	(+5.8)
5' – GTG ABA TGC		29.5	62.5
3' – CAC BAT ACG		(±0)	(+16.5)
5' – GBG ATA TGC		34.5	43.5
3' – CAC BAT ACG		(+2.5)	(+6.8)
5' – GBG ATA TGC		54.0	61.5
3' – CAC TAB ACG		(+12.3)	(+16.0)

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General click reaction protocol for preparation of 104V-104Z (description for ~6 mmol scale):

5'-O-Dimethoxytrityl-2'-azido-2'-deoxyuridine **102** and the appropriate alkyne **92-100** were added to a mixture of THF/*t*-BuOH/H₂O (3:1:1, v/v/v) along with sodium ascorbate and CuSO₄·5H₂O (reagent quantities, and solvent volumes are specified below). The reaction mixture was stirred under a nitrogen atmosphere until analytical TLC indicated full conversion (reaction times and temperatures specified below) whereupon it was diluted with EtOAc (10 mL). The organic phase was successively washed with sat. aq. NaHCO₃ (20 mL) and brine (20 mL), dried over anhydrous Na₂SO₄ and evaporated to dryness. The resulting crude was purified by silica column chromatography (eluent specified below) to afford the corresponding nucleoside **2** (yield specified below).

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1-(Pyren-1-yl)-prop-2-yn-1-ol (90): Trimethylsilylacetylene (1.0 mL, 7.00 mmol) was added to MeMgBr in THF (1M, 4.0 mL, 4.00 mmol) under an argon atmosphere and stirred at rt for 1h. At this point, pyrene-1-carboxaldehyde (0.70 g, 3.00 mmol) was added and the reaction mixture was stirred at rt for another 2h. Sat. aq. NH₄Cl (~1 mL) was added and the mixture was extracted with EtOAc (2 x 20 mL). The combined organic phase was dried over Na₂SO₄ and evaporated to dryness. The resulting crude [assumed to be 1-(pyren-1-yl)-3-trimethylsilyl-prop-2-yn-1-ol] was dissolved in CH₂Cl₂ and MeOH (10 mL, 1:1, v/v) and stirred with K₂CO₃ (0.50 g, 3.62 mmol) at rt for 2h. The reaction mixture was then diluted with CH₂Cl₂ (10 mL) and successively washed with brine (20 mL) and water (20 mL). The organic phase was dried over Na₂SO₄ and evaporated to dryness. The resulting crude was purified by silica gel column chromatography (0-50% EtOAc in petroleum ether, v/v) to afford **90** (0.47 g, 58%) as a white solid material. *R*_f = 0.3 (25% EtOAc in petroleum ether, v/v); ESI-HRMS *m/z* 279.0783 ([M+Na]⁺, C₁₉H₁₂O·Na⁺, calc 279.0780); ¹H NMR (DMSO-*d*₆) δ 8.59 (d, 1H, *J* = 10.0 Hz, Py), 8.35-8.29 (m, 4H, Py), 8.26 (d, 1H, *J* = 10.0 Hz, Py), 8.20-8.16 (m, 2H, Py),

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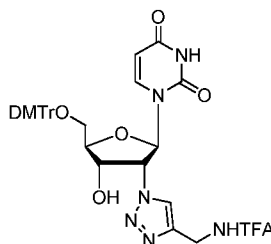
8.09 (t, 1H, $J = 7.5$ Hz, Py), 6.41-6.39 (d, 1H, ex, $J = 5.0$ Hz, OH), 6.34-6.31 (dd, 1H, $J = 5.0$ Hz, 2.5 Hz, HC(OH)), 3.60 (d, 1H, $J = 2.5$ Hz, HC $\equiv$ C); ^{13}C NMR (DMSO- d_6) δ 135.0, 130.7, 130.5, 130.1, 127.34, 127.31 (Py), 127.28 (Py), 127.25 (Py), 126.2 (Py), 125.3 (Py), 125.2 (Py), 124.65 (Py), 124.59 (Py), 124.1, 123.8, 123.7 (Py), 85.5, 76.6 (HC $\equiv$ C), 60.8 (HC(OH)).

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1-(Pyren-1-yl)-prop-2-yn-1-one (92): The Jones reagent (2.67 M CrO₃ in 3M H₂SO₄, 1.0 mL, 2.67 mmol) was added to a solution of alcohol **90** (180 mg, 0.67 mmol) in acetone (10 mL), and the reaction mixture was stirred under an ambient atmosphere at rt for 2h, whereupon it was diluted with EtOAc (20 mL), neutralized by drop-wise addition of 6M NaOH (1.0 mL) under stirring, and sequentially washed with water (30 mL) and sat. aq. NaHCO₃ (30 mL). The organic phase was dried over Na₂SO₄, evaporated to dryness, and the resulting crude purified by silica gel column chromatography (0-20% EtOAc in petroleum ether, v/v) to furnish **92** (130 mg, 75%) as a brightly yellow solid material. $R_f = 0.6$ (50% EtOAc in petroleum ether, v/v); ESI-HRMS m/z 277.0626 ([M+Na]⁺, C₁₉H₁₀O·Na⁺, calc 277.0624); ^1H NMR (CDCl₃) δ 9.48 (d, 1H, $J = 10.0$ Hz), 8.94 (d, 1H, $J = 8.0$ Hz), 8.28-8.23 (m, 3H), 8.19-8.14 (m, 2H), 8.07-8.02 (m, 2H), 3.53 (s, 1H); ^{13}C NMR (CDCl₃) δ 179.3, 135.8, 132.2 (Py), 131.31, 131.25 (Py), 131.14, 131.05 (Py), 130.6, 128.4, 127.35 (Py), 127.29 (Py), 127.1 (Py), 126.8 (Py), 124.97 (Py), 124.96, 124.95, 124.2 (Py), 124.1, 82.6, 80.1 (HC $\equiv$ C).

4-(Pyren-1-yl)-but-1-yne (94): An oven-dried flask was charged with pyrene-1-carboxaldehyde (230 mg, 1.00 mmol) and activated zinc (100 mg, 1.50 mmol) and placed under an argon atmosphere. Anhydrous THF (5 mL) and propargyl bromide (0.20 mL, 1.79 mmol) were added and the reaction mixture was stirred at 45 °C for 4h. Sat. aq. NH₄Cl (1 mL) was added and the mixture was extracted with EtOAc (2 x 20 mL). The organic phase was washed with brine (20 mL) and evaporated to dryness. The resulting crude was purified by silica gel column chromatography (0-30% EtOAc in petroleum ether, v/v) to afford a crude white solid material (145 mg), which ^1H NMR suggested to be a ~9:1 mixture of the desired 1-(pyren-1-yl)-but-3-yn-1-ol and the corresponding allene isomer. Et₃SiH (0.20 mL, 1.25 mmol) and boron trifluoride etherate (0.20 mL, 1.62) were added to a solution of the crude mixture in CH₂Cl₂ (5 mL), which then was stirred at rt for 1h. The reaction mixture was diluted with CH₂Cl₂ (20 mL) and sat. aq. NaHCO₃ (2 mL), and successively washed with brine (20 mL) and water (20 mL). The organic phase was dried over Na₂SO₄, evaporated to dryness under reduced pressure, and the resulting crude purified by silica gel column chromatography (0-3% EtOAc in petroleum ether, v/v) to afford **94** (80 mg, 31%) as a white solid material. $R_f = 0.5$ (5% EtOAc in petroleum ether, v/v); ESI-HRMS m/z 277.0973 ([M+Na]⁺, C₂₀H₁₄·Na⁺, calc 279.0988); ^1H NMR (CDCl₃) δ 8.27-8.25 (d, 1H, $J = 9.5$ Hz, Py), 8.17-8.14 (m, 2H, Py), 8.12-8.10 (m, 2H, Py), 8.01 (ap s, 2H), 8.00-7.96 (t, 1H, $J = 8.0$ Hz, Py), 7.92-7.90 (d, 1H, $J = 7.5$ Hz, Py), 3.59 (t, 2H, $J = 7.7$ Hz, CH₂CH₂C $\equiv$ CH), 2.72 (dt, 2H, $J = 7.7$ Hz, 2.5 Hz, CH₂C $\equiv$ CH), 2.03 (t, 1H, $J = 2.5$ Hz, HC $\equiv$ C); ^{13}C

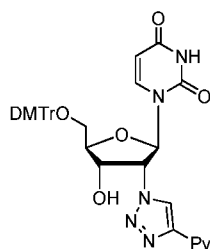
NMR (CDCl₃) δ 134.7, 131.6, 131.1, 130.5, 128.9, 127.8 (Py), 127.7 (Py), 127.5 (Py), 127.1 (Py), 126.1 (Py), 125.31, 125.27 (Py), 125.2, 125.1 (Py), 125.0 (Py), 123.2 (Py), 84.0, 69.6 (HC $\equiv$ C), 32.8 (CH₂CH₂C $\equiv$ CH), 21.0 (CH₂C $\equiv$ CH).

**104V**

5'-O-(4,4'-Dimethoxytrityl)-2'-C-[4-(2,2,2-trifluoroacetamidomethyl)-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (104V): Nucleoside **102** (0.40 g, 0.70 mmol), 2,2,2-trifluoro-*N*-(prop-2-

ynyl)acetamide **Av** (105 mg, 0.70 mmol), sodium ascorbate (70 mg, 0.35 mmol), CuSO₄·5H₂O (5 mg, 0.02 mmol) and THF/*t*-BuOH/H₂O (5 mL) were mixed, reacted (14h at rt), worked up and purified (50-100% EtOAc in petroleum ether, v/v) as described above except that the organic phase was successively washed with brine and water. Nucleoside **104V** (0.42 g, 83%) was obtained as a yellow solid material. *R*_f = 0.3 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 745.2225

([M+Na]⁺, C₃₅H₃₄F₃N₆O₈·Na⁺, calc 745.2204); ¹H NMR (DMSO-*d*₆) δ 11.40 (d, 1H, ex, *J* = 2.0 Hz, H3), 10.02 (t, 1H, *J* = 6.0 Hz, NHCOCF₃), 8.01 (s, 1H, Tz), 7.81 (d, 1H, *J* = 8.0 Hz, H6), 7.43-7.22 (m, 9H, DMTr), 6.93-6.88 (m, 4H, DMTr), 6.42 (d, 1H, *J* = 4.5 Hz, H1'), 5.79 (d, 1H, ex, *J* = 6.0 Hz, 3'-OH), 5.50 (dd, 1H, *J* = 7.0 Hz, 4.5 Hz, H2'), 5.45 (dd, 1H, *J* = 8.0 Hz, 2.0 Hz, H5), 4.52 (m, 1H, H3'), 4.47 (d, 2H, *J* = 5.5 Hz, CH₂NHCO), 4.24-4.20 (m, 1H, H4'), 3.75 (s, 6H, CH₃O), 3.38-3.30 (m, 2H, H5' – partial overlap with H₂O); ¹³C NMR (DMSO-*d*₆) δ 162.8, 158.09, 158.08, 156.2 (q, ¹*J*_{CF} = 36 Hz, COCF₃), 150.1, 144.6, 142.4, 140.5 (C6), 135.3, 135.1, 129.7 (DMTr), 127.8 (DMTr), 127.7 (DMTr), 126.7 (DMTr), 124.5 (Tz), 115.8 (q, *J*_{CF} = 288 Hz, CF₃), 113.2 (DMTr), 101.9 (C5), 87.1 (C1'), 85.8, 83.2 (C4'), 68.8 (C3'), 64.5 (C2'), 62.8 (C5'), 55.0 (CH₃O), 34.5 (CH₂NHCO); ¹⁹F-NMR (DMSO-*d*₆) δ -74.2.

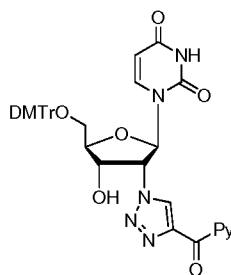
**104W**

5'-O-(4,4'-Dimethoxytrityl)-2'-C-[4-(pyrene-1-yl)-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine

(104W): Nucleoside **102** (0.28 g, 0.49 mmol), 1-ethynylpyrene **98** (130 mg, 0.58 mmol), sodium

ascorbate (200 mg, 1.00 mmol), CuSO₄·5H₂O (25 mg, 0.10 mmol) and THF/*t*-BuOH/H₂O (10 mL) were mixed, reacted (7h at 75 °C), worked up and purified (40-70% EtOAc in petroleum ether, v/v) as described above to provide nucleoside **104W** (140 mg, 35%) as an off-white solid material. *R*_f =

- 5 0.5 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 820.277 ([M+Na]⁺, C₄₈H₃₉N₅O₇·Na⁺, calc 820.274); ¹H NMR (DMSO-*d*₆) δ 11.46 (d, 1H, ex, *J* = 1.5 Hz, NH), 8.87 (d, 1H, *J* = 9.0 Hz, Py), 8.80 (s, 1H, Tz), 8.41-8.33 (m, 4H, Py), 8.27 (d, 1H, *J* = 9.2 Hz, Py), 8.26-8.22 (m, 2H, Py); 8.12 (t, 1H, *J* = 7.5 Hz, Py), 7.91 (d, 1H, *J* = 8.0 Hz, H6), 7.48-7.20 (m, 9H, DMTr), 6.96-6.90 (m, 4H, DMTr), 6.65 (d, 1H, *J* = 5.0 Hz, H1'), 5.95 (d, 1H, ex, *J* = 6.0 Hz, 3'-OH), 5.69 (dd, 1H, *J* = 7.0 Hz, 5.0 Hz, H2'), 5.54 (dd, 1H, *J* = 8.0 Hz, 1.5 Hz, H5), 4.69-4.64 (m, 1H, H3'), 4.40-4.36 (m, 1H, H4'),
10 3.76 (s, 6H, CH₃O), 3.46-3.36 (m, 2H, H5'); ¹³C NMR (DMSO-*d*₆) δ 162.9, 158.2, 150.3, 145.7, 144.7, 140.8 (C6), 135.4, 135.2, 130.9, 130.6, 130.3, 129.78 (DMTr), 129.76 (DMTr), 128.0 (Py), 127.9 (DMTr), 127.73 (DMTr), 127.67 (Py), 127.5, 127.3 (Py), 127.0 (Py), 126.8 (DMTr), 126.4 (Py), 125.7 (Tz), 125.5 (Py), 125.16, 125.15 (Py), 125.09 (Py), 124.8 (Py), 124.3, 123.9, 113.3 (DMTr), 102.1 (C5), 87.4 (C1'), 85.9, 83.4 (C4'), 69.1 (C3'), 64.9 (C2'), 63.1 (C5'), 55.0 (CH₃O).



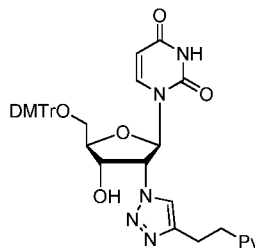
104X

5'-O-(4,4'-Dimethoxytrityl)-2'-C-[4-(pyrene-1-ylcarbonyl)-1H-1,2,3-triazol-1-yl]-2'-

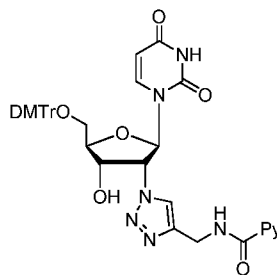
deoxyuridine (104X): Nucleoside **102** (0.28 g, 0.49 mmol), 1-(pyren-1-yl)-prop-2-yn-1-one **92** (140

- mg, 0.55 mmol), sodium ascorbate (200 mg, 1.00 mmol), CuSO₄·5H₂O (25 mg, 0.10 mmol) and
20 THF/*t*-BuOH/H₂O (10 mL) were mixed, reacted (5h at rt), worked up and purified (40-90% EtOAc in petroleum ether, v/v) as described above to provide nucleoside **104X** (0.25 g, 60%) as yellow solid material. *R*_f = 0.4 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 848.267 ([M+Na]⁺, C₄₉H₃₉N₅O₈·Na⁺, calc 848.270); ¹H NMR (DMSO-*d*₆) δ 11.46 (br s, 1H, ex, NH), 8.96 (s, 1H, Tz), 8.51-8.28 (m, 8H, Py), 8.17 (t, 1H, *J* = 7.5 Hz, Py), 7.83 (d, 1H, *J* = 8.0 Hz, H6), 7.44-7.21 (m, 9H, DMTr), 6.93-6.88 (m, 4H, DMTr), 6.55 (d, 1H, *J* = 5.0 Hz, H1'), 5.90 (d, 1H, ex, *J* = 5.0 Hz, 3'-OH),
25 5.68 (dd, 1H, *J* = 7.0 Hz, 5.0 Hz, H2'), 5.53 (dd, 1H, *J* = 8.0 Hz, 2.0 Hz, H5), 4.64-4.58 (m, 1H, H3'), 4.31-4.26 (m, 1H, H4'), 3.74 (s, 6H, CH₃O), 3.40-3.30 (m, 2H, H5'); ¹³C NMR (DMSO-*d*₆) δ 188.3, 162.9, 158.1, 150.2, 147.2, 144.6, 140.8 (C6), 135.4, 135.2, 133.0, 131.8, 131.5 (Tz), 130.6, 130.0, 129.74 (DMTr), 129.72 (DMTr), 129.4 (Py), 129.1 (Py), 128.9, 127.9, 127.84 (DMTr), 127.80 (Py),
30 127.7 (DMTr), 127.2 (Py), 126.8 (Py), 126.7 (DMTr), 126.5 (Py), 126.1 (Py), 124.01 (Py), 123.98

(Py), 123.8, 123.5, 113.2 (DMTr), 102.0 (C5), 87.4 (C1'), 85.8, 83.3 (C4'), 69.0 (C3'), 65.0 (C2'), 63.0 (C5'), 55.0 (CH₃O).

**104Y**

- 5 **5'-O-(4,4'-Dimethoxytrityl)-2'-C-[4-{2-(pyrene-1-yl)ethyl}-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (104Y):** Nucleoside **102** (0.34 g, 0.60 mmol), 4-(pyren-1-yl)-but-1-yne **94** (160 mg, 0.63 mmol), sodium ascorbate (0.25 g, 1.25 mmol), CuSO₄·5H₂O (31 mg, 0.12 mmol) and THF/*t*-BuOH/H₂O (10 mL) were mixed, reacted (2h at rt), worked up and purified (50-100% EtOAc in petroleum ether, v/v) as described above to provide nucleoside **104Y** (0.33 g, 67%) as a white solid material. *R*_f = 0.3 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 848.3046 ([M+Na]⁺, C₅₀H₄₃N₅O₇·Na⁺, calc 848.3055); ¹H NMR (DMSO-*d*₆) δ 11.44 (s, 1H, ex, NH), 8.40 (d, 1H, *J* = 9.0 Hz, Py), 8.30-8.19 (m, 4H, Py), 8.13 (ap s, 2H, Py), 8.06 (t, 1H, *J* = 8.0 Hz, Py), 8.01 (s, 1H, Tz), 7.95 (d, 1H, *J* = 8.0 Hz, Py), 7.82 (d, 1H, *J* = 8.0 Hz, H6), 7.44-7.41 (m, 2H, DMTr), 7.36-7.23 (m, 7H, DMTr), 6.94-6.90 (m, 4H, DMTr), 6.44 (d, 1H, *J* = 5.0 Hz, H1'), 5.79 (d, 1H, ex, *J* = 6.0 Hz, 3'-OH), 5.49-5.45 (m, 2H, H5, H2'), 4.54-4.49 (m, 1H, H3'), 4.27-4.22 (m, 1H, H4'), 3.75 (s, 6H, CH₃O), 3.72-3.66 (m, 2H, CH₂CH₂), 3.40-3.30 (m, 2H, H5'), 3.19-3.14 (m, 2H, CH₂CH₂); ¹³C NMR (DMSO-*d*₆) δ 162.8, 158.12, 158.11, 150.2, 145.8, 144.6, 140.5 (C6), 135.6, 135.4, 135.1, 130.8, 130.3, 129.7 (DMTr), 129.4, 128.0, 127.8 (DMTr), 127.7 (DMTr), 127.5 (Py), 127.4 (Py), 127.3 (Py), 126.7 (DMTr), 126.5 (Py), 126.1 (Py), 124.93 (Py), 124.88 (Py), 124.8 (Py), 124.2, 124.1, 123.4 (Tz), 123.2 (Py), 113.2 (DMTr), 102.0 (C5), 87.1 (C1'), 85.9, 83.3 (C4'), 68.9 (C3'), 64.3 (C2'), 62.9 (C5'), 55.0 (CH₃O), 32.6 (CH₂CH₂), 27.3 (CH₂CH₂).

**104Z**

- 25 **5'-O-(4,4'-Dimethoxytrityl)-2'-C-[4-(pyrene-1-yl)carboxamidomethyl-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (104Z):** Nucleoside **102** (0.40 g, 0.70 mmol), *N*-(prop-2-ynyl)pyrene-1-carboxamide **100** (200 mg, 0.71 mmol), sodium ascorbate (50 mg, 0.25 mmol), CuSO₄·5H₂O (5 mg, 0.02 mmol) and THF/*t*-BuOH/H₂O (5 mL) were mixed, reacted (8h at rt), worked up and purified (50-100%

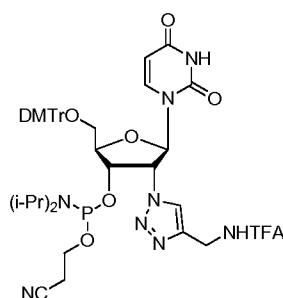
EtOAc in petroleum ether, v/v) as described above except that the organic phase was successively washed with brine and water. Nucleoside **104Z** (0.49 g, 83%) was obtained a yellow solid material. $R_f = 0.2$ (EtOAc); MALDI-HRMS m/z 877.2979 ($[M+Na]^+$, $C_{50}H_{42}N_6O_8 \cdot Na^+$, calc 877.2956); 1H NMR (DMSO- d_6) δ 11.43 (s, 1H, ex, H3), 9.26 (t, 1H, ex, $J = 6.0$ Hz, NHCO), 8.53-8.52 (d, 1H, $J = 9.5$ Hz, Ar), 8.36-8.34 (m, 3H, Ar), 8.27-8.22 (m, 3H, Ar), 8.17-8.11 (m, 3H, Ar, Tz), 7.85 (d, 1H, $J = 8.5$ Hz, H6), 7.44-7.43 (m, 2H, DMTr), 7.35-7.24 (m, 7H, DMTr), 6.93-6.89 (m, 4H, DMTr), 6.50 (d, 1H, $J = 4.7$ Hz, H1'), 5.87 (d, 1H, ex, $J = 5.5$ Hz, 3'-OH), 5.56 (dd, 1H, $J = 7.0$ Hz, 4.7 Hz, H2'), 5.47 (d, 1H, $J = 8.0$ Hz, H5), 4.71 (d, 2H, $J = 6.0$ Hz, CH_2NHCO), 4.58-4.54 (m, 1H, H3'), 4.30-4.25 (m, 1H, H4'), 3.75 (s, 6H, CH_3O), 3.41-3.32 (m, 2H, H5'); ^{13}C NMR (DMSO- d_6) δ 168.8, 162.9, 158.13, 158.12, 150.2, 144.7, 144.6, 140.5 (C6), 135.4, 135.2, 131.6, 131.5, 130.7, 130.2, 129.8 (DMTr), 128.3 (Ar), 128.1 (Ar), 127.9 (DMTr), 127.8, 127.7 (DMTr), 127.1 (Ar), 126.8 (DMTr), 126.5 (Ar), 125.7 (Ar), 125.5 (Ar), 125.2 (Ar), 124.7 (Ar), 124.3 (Ar), 124.2 (Tz), 123.7, 123.6, 113.2 (DMTr), 102.0 (C5), 87.1 (C1'), 85.9, 83.3 (C4'), 69.0 (C3'), 64.5 (C2'), 62.9 (C5'), 55.0 (CH_3O), 35.0 (CH_2NHCO).

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General phosphitylation protocol for preparation of 106V-106Z (description for ~3 mmol scale):

The appropriate nucleoside **104** was co-evaporated with anhydrous CH_2Cl_2 (5 mL) and redissolved in anhydrous CH_2Cl_2 (reagent quantities and solvent volumes are specified below). To this was added *N,N*-diisopropylethylamine (DIPEA), 0.45 M tetrazole in CH_3CN and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (PN2-reagent). The reaction mixture was stirred at rt until analytical TLC indicated complete conversion (reaction time specified below) whereupon cold abs. EtOH (0.5 mL) was added. The reaction mixture was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (eluent specified below). The crude material was triturated from cold petroleum ether to afford phosphoramidite **106** (yields specified below).

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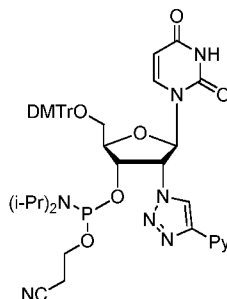
106V

3'-O-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphiny)-5'-O-(4,4'-dimethoxytrityl)-2'-C-[4-(2,2,2-trifluoroacetamidomethyl)-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (106V):

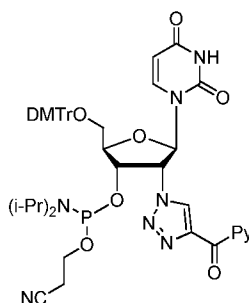
Nucleoside **104V** (0.29 g, 0.40 mmol), DIPEA (0.10 mL, 0.57 mmol), tetrazole in CH_3CN (0.45 M, 1.0 mL, 0.45 mmol), PN2-reagent (0.15 mL, 0.46 mmol) and anhydrous CH_2Cl_2 (1 mL) were mixed, reacted (3h),

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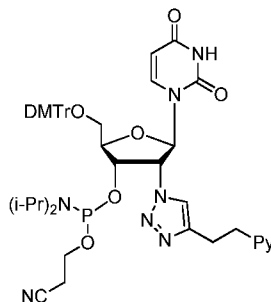
- worked up and purified (50-90% EtOAc in petroleum ether, v/v) as described above except that: a) the reaction mixture was extracted with EtOAc (5 mL) after addition of EtOH, followed by drying of the organic phase over anhydrous Na₂SO₄ and evaporation to dryness under reduced pressure and b) trituration was not performed. Phosphoramidite **106V** (0.24 g, 67%) was obtained as a white solid material. $R_f = 0.3$ (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 945.3322 ([M+Na]⁺, C₄₄H₅₀F₃N₈O₉·Na⁺, calc 945.3283); ³¹P NMR (CDCl₃) δ 152.0, 149.8; ¹⁹F NMR (CDCl₃) δ -75.7.

**106W**

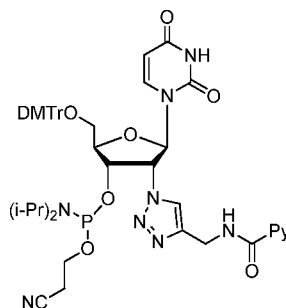
- 3'-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2'-C-[4-(pyrene-1-yl)-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (106W):** Nucleoside **104W** (230 mg, 0.29 mmol), DIPEA (0.10 mL, 0.57 mmol), tetrazole in CH₃CN (0.45 M, 1.0 mL, 0.45 mmol), PN2-reagent (0.20 mL, 0.62 mmol) and anhydrous CH₂Cl₂ (2 mL) were mixed, reacted (4h), worked up and purified (0-4% MeOH/CH₂Cl₂, v/v) as described above to afford **106W** (180 mg, 62%) as a white powder. $R_f = 0.35$ (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 1020.3855 ([M+Na]⁺, C₅₇H₅₆N₇O₈P·Na⁺, calc 1020.3826); ³¹P NMR (CDCl₃) δ 152.0, 150.5.

**106X**

- 3'-O-(N,N-Diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)-2'-C-[4-(pyrene-1-ylcarbonyl)-1H-1,2,3-triazol-1-yl]-2'-deoxyuridine (106X):** Nucleoside **104X** (150 mg, 0.18 mmol), DIPEA (0.10 mL, 0.57 mmol), tetrazole in CH₃CN (0.45 M, 0.6 mL, 0.27 mmol), PN2-reagent (0.12 mL, 0.37 mmol) and anhydrous CH₂Cl₂ (2 mL) were mixed, reacted (3.5h), worked up and purified (0-4% MeOH/CH₂Cl₂, v/v) as described above to afford **106X** (110 mg, 59%) as a yellow solid material. $R_f = 0.4$ (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 1048.3779 ([M+Na]⁺, C₅₈H₅₆N₇O₉P·Na⁺, calc 1048.3775); ³¹P NMR (CDCl₃) δ 152.4, 150.9.

**106Y**

3'-O-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphiny)-5'-O-(4,4'-dimethoxytrityl)-2'-C-[4-{2-(pyrene-1-yl)ethyl}-1*H*-1,2,3-triazol-1-yl]-2'-deoxyuridine (**106Y**): Nucleoside **104Y** (0.33 g, 0.40 mmol), DIPEA (0.10 mL, 0.57 mmol), tetrazole in CH₃CN (0.45 M, 1.5 mL), PN2-reagent (0.25 mL, 0.78 mmol) and anhydrous CH₂Cl₂ (2 mL) were mixed, reacted (3.5h), worked up and purified (0-4% MeOH/CH₂Cl₂, v/v) as described above to afford **106Y** (210 mg, 51%) as a white powder. *R*_f = 0.45 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1048.4147 ([M+Na]⁺, C₅₉H₆₀N₇O₈P·Na⁺, calc 1048.4139); ³¹P NMR (CDCl₃) δ 151.6, 150.6.

**106Z**

3'-O-(*N,N*-Diisopropylamino-2-cyanoethoxyphosphiny)-5'-O-(4,4'-dimethoxytrityl)-2'-C-[4-(pyrene-1-yl)carboxamidomethyl]-1*H*-1,2,3-triazol-1-yl]-2'-deoxyuridine (**106Z**): Nucleoside **104Z** (0.36 g, 0.42 mmol), DIPEA (0.10 mL, 0.57 mmol), tetrazole in CH₃CN (0.45 M, 1.0 mL, 0.45 mmol), PN2-reagent (0.15 mL, 0.46 mmol) and anhydrous CH₂Cl₂ (1 mL) were mixed, reacted (3h), worked up and purified (50-90% EtOAc in petroleum ether, v/v) as described above except that: a) the reaction mixture was extracted with EtOAc (5 mL) after addition of EtOH, followed by drying of the organic phase over anhydrous Na₂SO₄ and evaporation to dryness under reduced pressure and b) trituration was not performed. Phosphoramidite **106Z** (0.28 g, 67 %) was obtained as a white solid material. *R*_f = 0.3 (5% MeOH in CH₂Cl₂, v/v); MALDI-HRMS *m/z* 1077.3984 ([M+Na]⁺, C₅₉H₅₉N₈O₉P·Na⁺, calc 1077.4035); ³¹P NMR (CDCl₃) δ 151.9, 150.1.

Synthesis and purification of single-stranded probes modified with monomers **122V/W/X/Y/Z**:

Synthesis of modified oligodeoxyribonucleotides was performed on an Expedite 8909 DNA

Synthesizer using 0.2 μmol scale succinyl linked LCAA-CPG (long chain alkyl amine controlled pore

glass) columns with a pore size of 500Å. Standard protocols for incorporation of DNA phosphoramidites were used. A ~50-fold molar excess of modified phosphoramidites in anhydrous acetonitrile (at 0.05 M) was used during hand-couplings using the conditions specified in the main manuscript. Moreover, extended oxidation (45s) was employed during hand-couplings. Cleavage from solid support and removal of protecting groups was accomplished upon treatment with 32% aq. ammonia (55 °C, 20 h). Purification of all modified oligonucleotides was performed by ion-pair reverse phase HPLC as described below followed by detritylation (80% aq. AcOH) and precipitation from acetone (-18 °C for 12-16h).

Purification of crude oligonucleotides was performed on a Varian Prostar HPLC system equipped with an XTerra MS C18 pre-column (10 µm, 7.8 x 10 mm) and an XTerra MS C18 column (10 µm, 7.8 x 150 mm) using a 0.05 mM TEAA (triethylammonium acetate) buffer - 25% water/acetonitrile (v/v) gradient. The identity of synthesized oligonucleotides was established through MALDI-MS/MS analysis recorded in positive ions mode on a Quadrupole Time-Of-Flight Tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K) using anthranilic acid as a matrix (Table 61, while purity (>80%) was verified by RP-HPLC running in analytical mode.

Table 61
MALDI-Tof MS-Analysis of Single-Stranded Probes Modified with 122W/X/Y/Z

Sequence	Calc.(M+H) ⁺	Expt. (M+H) ⁺
5'-CGCAA A 122W A AACGC	4188.8	4188.9
5'-CGCAA C 122W C AACGC	4140.8	4140.9
5'-CGCAA G 122W G AACGC	4220.8	4220.8
5'-CGCAA T 122W T AACGC	4170.8	4170.8
5'-GCGTT 122W AT TTGCG	4223.8	4223.8
5'-GCGTT TA 122W TTGCG	4223.8	4223.9
5'-GCGTT 122W A 122W TTGCG	4476.8	4477.0
5'-CGCAA A 122X A AACGC	4216.8	4216.9
5'-CGCAA C 122X C AACGC	4168.8	4169.0
5'-CGCAA G 122X G AACGC	4248.8	4248.8
5'-CGCAA T 122X T AACGC	4198.8	4198.9
5'-GCGTT 122X AT TTGCG	4251.8	4251.9
5'-GCGTT TA 122X TTGCG	4251.8	4251.9
5'-GCGTT 122X A 122X TTGCG	4532.8	4532.9
5'-CGCAA A 122Y A AACGC	4216.8	4216.9
5'-CGCAA C 122Y C AACGC	4168.8	4169.0
5'-CGCAA G 122Y G AACGC	4248.8	4248.9
5'-CGCAA T 122Y T AACGC	4198.8	4198.9
5'-GCGTT 122Y AT TTGCG	4251.8	4251.9
5'-GCGTT TA 122Y TTGCG	4251.8	4251.9
5'-GCGTT 122Y A 122Y TTGCG	4532.9	4533.1
5'-CGCAA A 122Z A AACGC	4245.8	4245.9
5'-CGCAA C 122Z C AACGC	4197.8	4197.9
5'-CGCAA G 122Z G AACGC	4277.8	4278.1
5'-CGCAA T 122Z T AACGC	4227.8	4227.9

5'-GCGTT <u>122Z</u> AT TTGCG	4280.8	4280.9
5'-GCGTT TA <u>122Z</u> TTGCG	4280.8	4280.8
5'-GCGTT <u>122ZA122Z</u> TTGCG	4590.9	4590.9
5'-GTG ATA <u>122V</u> GC	2835.5	2835.9
5'-GTG A <u>122V</u> A TGC	2835.5	2835.9
5'-G <u>122V</u> G ATA TGC	2835.5	2835.8
5'-GTG ATA <u>122Z</u> GC	3063.6	3063.9
5'-GTG A <u>122Z</u> A TGC	3063.6	3063.9
5'-G <u>122Z</u> G ATA TGC	3063.6	3063.8

Thermal Denaturation Studies involving probes modified with monomers 122V/W/X/Y/Z:

Concentrations of oligonucleotides were estimated using the following extinction coefficients

(OD/ μ mol): dG (12.01), dA (15.20), dT (8.40), dC (7.05); rG (13.70), rA (15.40), rU (10.00), rC

- 5 (9.00); **122V** (19.96), **122W** (31.08), **122X** (35.60), **122Y** (27.62) and **122Z** (30.95) [values for monomers **122V-122Z** were estimated through A_{260} measurements of the corresponding phosphoramidites in 1% aq. DMSO solutions]. Each strand was thoroughly mixed and denatured by heating to 80-85 °C followed by cooling to the starting temperature of the experiment. Quartz optical cells with a path length of 10 mm were used. Thermal denaturation temperatures (T_m values [°C]) of duplexes (1.0 μ M 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 20 °C below T_m to 20 °C above T_m . A temperature ramp of 0.5 °C/min was used in all experiments. Reported T_m -values are averages of two experiments within ± 1.0 °C.

Certain embodiments pertain probes modified with monomers **122W/X/Y/Z**. Thermal denaturation studies for duplexes between such probes and matched or mismatched DNA targets are disclosed in Tables 62 and 63. The examples provided therein, demonstrate that probes modified with monomers **122W/X/Y/Z**, display universal thermal hybridization properties, i.e., they display significantly similar thermal affinity toward matched and mismatched nucleic acid targets, more commonly single-stranded nucleic acid targets, even more commonly single-stranded DNA and/or single-stranded RNA.

Table 62

T_m -Values of Duplexes Between Probes Modified with Monomers 122W/X/Y/Z and Complementary or Centrally Mismatched DNA Targets^[a]

Probe	T_m (ΔT_m) [°C]		Mismatch ΔT_m [°C]		
	B	A	C	G	T
5'-CGCAA ATA AACGC	48.5		-10.0	-5.0	-9.0
5'-CGCAA CTC AACGC	55.5		-13.5	-9.5	-9.0

5'-CGCAA GTG AACGC	55.5	-13.0	-9.5	-
				10.0
5'-CGCAA TTT AACGC	48.5	-11.0	-9.0	-
				11.0
5'-CGCAA A(122W)A AACGC	48.0 (-0.5)	+1.0	+1.5	5
5'-CGCAA C(122W)C AACGC	53.5 (-2.0)	+0.5	+2.5	0
5'-CGCAA G(122W)G AACGC	51.5 (-4.0)	+1.0	-4.5	0.0
5'-CGCAA T(122W)T AACGC	47.0 (-1.5)	+2.5	-0.5	+2.0
5'-CGCAA A(122X)A AACGC	46.5 (-2.0)	+1.0	+0.5	+1.0
				5
5'-CGCAA C(122X)C AACGC	52.0 (-3.5)	-1.5	0.0	-0.5
5'-CGCAA G(122X)G AACGC	52.5 (-3.0)	+0.5	-7.0	-0.5
5'-CGCAA T(122X)T AACGC	44.5 (-4.0)	+1.0	-1.0	0.0
5'-CGCAA A(122Y)A AACGC	49.5(+1.0)	+1.5	0.0	+1.0
				)
5'-CGCAA C(122Y)C AACGC	50.5 (-5.0)	-5.0	-1.0	-2.5
5'-CGCAA G(122Y)G AACGC	53.0 (-2.5)	+1.5	-3.5	+0.5
5'-CGCAA T(122Y)T AACGC	44.5 (-4.0)	-2.0	-2.0	-1.5
5'-CGCAA A(122Z)A AACGC	47.0 (-1.5)	-5.5	-2.5	-4.0
5'-CGCAA C(122Z)C AACGC	51.5 (-4.0)	-6.5	-1.0	-4.0
5'-CGCAA G(122Z)G AACGC	52.0 (-3.5)	-2.0	-6.0	-4.0
5'-CGCAA T(122Z)T AACGC	45.5 (-3.0)	-4.5	-5.0	-4.0

- ^[a] T_m 's determined as maximum of the first derivative of denaturation curves (A_{260} vs T) recorded in T_m -buffer ($[Na^+] = 110$ mM, $[Cl^-] = 100$ mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)) using 1.0 μ M of each strand. T_m 's are averages of at least two measurements within 1.0 °C. " ΔT_m " = change in T_m relative to unmodified reference duplex. "Mismatch ΔT_m " = change in T_m relative to fully matched duplex (B = A). "Avg Mismatch ΔT_m seq" = average of all three "Mismatch ΔT_m "-values for a given probe. "Avg Mismatch ΔT_m series" = average of all twelve "Mismatch ΔT_m "-values of all four studied sequences within a monomer series. " $\pm$ " denotes standard deviation. DNA targets: 3'-GCGTT TBT TTGCG, 3'-GCGTT GBG TTGCG, 3'-GCGTT CBC TTGCG and 3'-GCGTT ABA TTGCG.

10

15

Table 63

Additional T_m -Values of Duplexes Between Probes Modified with Monomers 122W/X/Y/Z and Complementary or Centrally Mismatched DNA Targets.^[a]

Probe	B=	DNA: 5'-CGCAA ABA AACGC			
		T_m (ΔT_m) [°C]	Mismatch ΔT_m [°C]		
		T	A	C	G
3'-GCGTT TAT TTGCG		48.5	-10.0	-10.0	-5.5
3'-GCGTT TA 122W TTGCG		46.5 (-2.0)	-17.0	-9.0	-12.0
3'-GCGTT 122W AT TTGCG		41.0 (-7.5)	-11.5	-10.0	-5.5
3'-GCGTT 122WA122W TTGCG		35.0 (-13.5)	nt	-3.5	Nt
3'-GCGTT TA 122X TTGCG		44.0 (-4.5)	-9.0	-7.5	-5.0
3'-GCGTT 122X AT TTGCG		42.0 (-6.5)	-10.5	-11.5	-6.0
3'-GCGTT 122XA122X TTGCG		36.5 (-12.0)	-2.5	-4.5	+0.5
3'-GCGTT TA 122Y TTGCG		40.0 (-8.5)	-7.0	-7.0	-5.0
3'-GCGTT 122Y AT TTGCG		42.5 (-6.0)	-6.0	-2.5	-7.0
3'-GCGTT 122YA122Y TTGCG		34.5 (-14.0)	-4.5	-3.5	-4.5
3'-GCGTT TA 122Z TTGCG		42.5 (-6.0)	-7.0	-8.5	-7.5
3'-GCGTT 122Z AT TTGCG		44.0 (-4.5)	-11.5	-12.0	-9.0
3'-GCGTT 122ZA122Z TTGCG		39.5 (-9.0)	-2.5	-0.5	-3.5

^[a] Conditions and definitions as described in footnote of Table above. "nt" = no transition.

5

Steady-state fluorescence emission spectra involving probes modified with monomers

122V/W/X/Y/Z: Spectra of oligonucleotides modified with pyrene-functionalized monomers

122W/122X/122Y/122Z and the corresponding duplexes with complementary or mismatched

DNA/RNA targets were recorded in non-deoxygenated thermal denaturation buffer (each strand 1.0

10 μ M) using an excitation wavelength of $\lambda_{ex} = 350$ nm for **122W/122Y/122Z** or $\lambda_{ex} = 400$ nm for **122X**, excitation slit 5.0 nm, emission slit 5.0 nm and a scan speed of 600 nm/min. Experiments were performed at ambient temperature (~20 °C).

Determination of quantum yields involving probes modified with monomers 122V/W/X/Y/Z:

15 Relative fluorescence emission quantum yields (Φ_F) of modified nucleic acids (SSP or duplex) were determined using the following equation: Φ_F (NA) = [Φ_F (std)/ α (std)] $\times$ [IFI (NA) / A_{ex} (NA)] $\times$ [n (NA)/ n (std)]² where Φ_F (std) is the fluorescence emission quantum yield of standard; α (std) is the slope of the integrated fluorescence intensity vs. optical intensity plot made for the standard; IFI (NA) is the integrated fluorescence intensity ($\lambda_{em} = 360$ -510 nm for monomer **122W/122Y/122Z**; λ_{em} = 425-625 nm for monomer **122X**; $\lambda_{em} = 360$ -600 nm for standards); A_{ex} (NA) is the optical density of the sample at the utilized excitation wavelength ($\lambda_{ex} = 350$ nm for monomer **122W/122Y/122Z**; $\lambda_{ex} = 400$ nm for monomer **122X**; $\lambda_{ex} = 350$ nm for standards; optical densities of all solutions at the excitation wavelengths were between 0.01 and 0.10); n (NA) and n (std) are refractive indexes of solvents used for sample and standard respectively ($n_{water} = 1.33$, $n_{ethanol} = 1.36$, and $n_{cyclohexane} = 1.43$).

The validity of this method under our experimental set-up was ascertained by determining the quantum yield of anthracene in ethanol with respect to 9,10-diphenylanthracene in cyclohexane ($\Phi_F = 0.86$). The measured value of $\Phi_F = 0.28$ is in excellent agreement with the reported value of ($\Phi_F = 0.27$). Subsequently, the literature value for anthracene in ethanol was used as the standard for
 5 determination of quantum yields of SSPs and duplexes.

Optical spectroscopy studies involving probes modified with monomers 122V/W/X/Y/Z. UV-Vis absorption spectra of oligonucleotides modified with monomers **122W-122Z** were recorded in absence or presence of complementary or centrally mismatched DNA targets, in order to gain
 10 additional insights into the mechanism that governs the observed universal hybridization characteristics (FIG. 27); hybridization-induced intercalation of pyrene moieties is known to induce subtle bathochromic shifts.

Single-stranded probes modified with monomer **122W** display a single unstructured maximum in the pyrene region ($\lambda_{\max} \sim 351$ nm, FIG. 27), while duplexes with complementary,
 15 mismatched or abasic DNA targets display two resolved maxima at ~ 351 nm and ~ 365 nm. The lack of defined peaks for the single stranded probes (SSPs) precludes analysis of bathochromic shifts. Single-stranded probes modified with monomer **122X** display two broad and virtually equally intense peaks which renders exact determination of absorption maxima unfeasible ($\lambda_{\max} \sim 385$ nm and ~ 415 nm, FIG. 27). Hybridization with complementary DNA results in subtle bathochromic shifts, while
 20 more pronounced shifts are observed upon hybridization with mismatched or abasic DNA. The pyrene maxima are red-shifted relative to those of unconjugated pyrenes chromophores, which suggests electronic coupling between the pyrene and triazole moieties. Single-stranded probes modified with monomer **122Y** or monomer **122Z**, on the other hand, have structured absorption spectra with two maxima in the 'normal' region (i.e., $\lambda_{\max} \sim 333/348$ nm and $\sim 332/346$ nm,
 25 respectively, FIG. 27). Hybridization of these probes with complementary, mismatched or abasic DNA target strands results in subtle bathochromic shifts ($\Delta\lambda_{\max}$ between +1 and +3 nm, FIG. 27, Table 64. Thus, the absorption data are consistent with the hypothesis that the pyrene moieties of monomers **122W-122Z** intercalate into the duplex core upon hybridization with DNA targets.

30

Table 64

Pyrene Absorption Maxima of Oligonucleotides Modified With 122Y or 122Z in the Absence (SSP) or Presence of Matched (M) or Centrally Mismatched (MM) DNA Targets.^[a]

Sequence	$\lambda_{\max}/\text{nm} (\lambda_{\max})$				
	SSP	+M (A)	+MM (C)	+MM (G)	+MM (T)
5'-CGCAA A(122Y)A AACGC	348	349 (+1)	349 (+1)	349 (+1)	349 (+1)
5'-CGCAA C(122Y) C AACGC	347	349 (+2)	349 (+2)	350 (+3)	350 (+3)
5'-CGCAA G(122Y) G AACGC	348	349 (+1)	349 (+1)	349 (+1)	349 (+1)
5'-CGCAA T(122Y) T AACGC	346	349 (+3)	349 (+3)	349 (+3)	349 (+3)
5'-CGCAA A(122Z)A AACGC	346	348 (+2)	349 (+3)	349 (+3)	349 (+3)

5'-CGCAA C(122Z)C AACGC	346	349 (+3)	349 (+3)	349 (+3)	347 (+1)
5'-CGCAA G(122Z)G AACGC	347	348 (+1)	350 (+3)	349 (+2)	350 (+3)
5'-CGCAA T(122Z)T AACGC	346	348 (+2)	348 (+2)	349 (+3)	349 (+3)

^[a] Recorded in T_m -buffer at $T = 20\text{ }^{\circ}\text{C}$ using $1.0\text{ }\mu\text{M}$ of each strand. SSP = single-stranded probe. Nucleotide opposite of modification is mentioned in parenthesis. "M" = matched, "MM" = mismatched.

5 Next, steady-state fluorescence emission spectra and fluorescence emission quantum yields were determined for oligonucleotides modified with **122W/X/Y/Z** in absence or presence of complementary or centrally mismatched DNA targets (FIG. 28 and Table 65).

Single-stranded probes modified with monomer **122W** display two structured emission peaks at $\lambda_{em} \sim 390\text{ nm}$ and 405 nm (FIG. 28). The single-stranded probe with a central A**122W**A-
10 context has higher fluorescence quantum yield than SSPs in other contexts ($\Phi_F = 0.27$ vs $0.07/0.05/0.05$, Table 65). The spectra of the corresponding duplexes with complementary DNA have a similar shape and sequence dependency, affirming that the pyrene moiety is in close contact with the neighboring nucleobases (FIG. 28, Table 65). The extensive decreases in fluorescence quantum yield (Table 65) upon hybridization with matched or mismatched DNA targets further
15 corroborate this hypothesis. The probe with T**122W**T-context exhibits considerably smaller changes, presumably since the fluorophore interacts with the neighboring and only weakly quenching adenine moieties upon target binding (FIG. 28).

Fluorescence emission spectra of single-stranded probes modified with monomer **122X** and the corresponding duplexes with complementary or mismatched DNA targets display broad and
20 unstructured emission peaks with maxima at $\lambda_{em} \sim 490\text{ nm}$ (FIG. 28). SSPs are strongly quenched with the probe having the G(**122X**)G-context displaying the lowest intensity ($\Phi_F < 0.04$, Table 65). Quantum yields are markedly increased upon hybridization of the probes with A(**122X**)A- and T(**122X**)T-sequence contexts with complementary/mismatched DNA targets (Table 65). In contrast, probes with C(**122X**)C- and G(**122X**)G-sequence contexts display hybridization-induced decreases in
25 fluorescence intensity (Table 65). One interpretation of these observations is that the conjugated pyrene moiety of monomer **122X** intercalates into the base stack where it is quenched by neighboring cytosine and guanine moieties (but not quenched by adenine and thymine moieties).

The fluorescence emission spectra of single-stranded probes modified with monomer **122Y** and the corresponding duplexes with matched or mismatched DNA targets, display two well-resolved
30 pyrene peaks at $\lambda_{em} \sim 380\text{ nm}$ and 400 nm , with an additional shoulder at $\lambda_{em} \sim 420\text{ nm}$ (FIG. 28). Very low quantum yields are observed ($\Phi_F < 0.03$, Table 65), except for the single-stranded probe with A(**122Y**)A sequence context. Hybridization of single-stranded probes modified with monomer **122Y** with complementary or mismatched DNA targets generally results in decreased fluorescence intensity, which is consistent with an intercalating binding mode for the pyrene moiety.

35 The fluorescence emission spectra of single-stranded probes modified with monomer **122Z** the corresponding duplexes with matched or mismatched DNA targets display an unstructured peak

at $\lambda_{em} \sim 410$ nm with a weaker shoulder at $\lambda_{em} \sim 390$ nm (FIG. 28). The quantum yields of SSPs range from moderate to high and closely align with the previously discussed quenching trends of nucleobases ($\Phi_F = 0.05$ -0.58, Table 65). Hybridization with matched or mismatched DNA targets generally results in decreases or minor increases in quantum yields and intensity (Table 65).

5

Table 65
Relative Fluorescence Emission Quantum Yield (Φ_F) of Single-Stranded Probes Modified with Monomer 122W/X/Y/Z in the Absence (SSP) or Presence of Matched (M) or Centrally Mismatched (MM) DNA Targets.^[a]

10

Sequence	Φ_F				
	SSP	+M (A)	+MM (C)	+MM (G)	+MM (T)
5'-CGCAA A(122W)A AACGC	0.27	0.08	0.09	0.06	0.08
5'-CGCAA C(122W)C AACGC	0.07	0.02	0.02	0.02	0.01
5'-CGCAA G(122W)G AACGC	0.05	0.03	0.01	0.02	0.01
5'-CGCAA T(122W)T AACGC	0.05	0.07	0.06	0.06	0.04
5'-CGCAA A(122X)A AACGC	0.02	0.25	0.33	0.10	0.25
5'-CGCAA C(122X)C AACGC	0.02	<0.01	<0.01	<0.01	<0.01
5'-CGCAA G(122X)G AACGC	<0.01	<0.01	<0.01	<0.01	<0.01
5'-CGCAA T(122X)T AACGC	0.04	0.16	0.35	0.04	0.32
5'-CGCAA A(122Y)A AACGC	0.09	0.02	0.02	0.02	0.03
5'-CGCAA C(122Y)C AACGC	0.01	0.02	<0.01	<0.01	<0.01
5'-CGCAA G(122Y)G AACGC	0.03	0.01	0.01	0.01	<0.01
5'-CGCAA T(122Y)T AACGC	0.01	<0.01	<0.01	<0.01	<0.01
5'-CGCAA A(122Z)A AACGC	0.58	0.52	0.78	0.29	0.79
5'-CGCAA C(122Z)C AACGC	0.24	0.15	0.17	0.15	0.19
5'-CGCAA G(122Z)G AACGC	0.05	0.04	0.02	0.02	0.03
5'-CGCAA T(122Z)T AACGC	0.27	0.57	0.58	0.31	0.52

^[a] Relative to quantum yield of anthracene in ethanol (0.27). Recorded in T_m -buffer at $T = 20$ °C using 1.0 μ M concentration of each strand and $\lambda_{ex} = 350$ nm and $\lambda_{em} = 360$ -510 nm (monomers **122W**, **122Y** and **122Z**) or $\lambda_{ex} = 400$ nm and $\lambda_{em} = 425$ -625 nm (monomer **122X**). Nucleotide opposite of modification is mentioned in parenthesis.

15

Very similar quantum yields are observed for the four duplexes between a particular probe and matched/mismatched DNA targets. Collectively, these observations indicate a) that the fluorophore is in a similar electronic environment within the duplex core regardless of the nucleotide opposite of the monomer, and b) that the opposing nucleotide is not strongly involved in base pairing and possibly even pushed into an extrahelical position (FIG. 29). Along the lines, it is interesting to note that placement of pyrene-functionalized C-glycosides in DNA duplexes opposite of abasic sites, which are generated via enzyme-mediated extrahelical flipping of the opposing nucleotide, is known to be stabilizing.

20

25 Universal hybridization – RNA targets: A representative subset of modified oligonucleotides (TBT/CBT-contexts) was studied with respect to thermal denaturation, absorption and fluorescence

properties with complementary/mismatched RNA targets. Briefly described: a) incorporation of monomer **122W** or **122X** into oligonucleotides results in similar decreases in thermal affinity toward complementary RNA as toward DNA, while oligonucleotides modified with monomers **122Y** or **122Z** are more destabilizing (Table 66); b) oligonucleotides modified with monomers **122W** or **122X** display robust universal hybridization characteristics (compare 'mismatch ΔT_m '-values for **ON6/ON8/ON12** and **ON2/ON4**, Table 66), while oligonucleotides modified with monomers **122Y** or **122Z** do not; c) pyrene absorption spectra of duplexes between modified oligonucleotides and complementary or centrally mismatched RNA targets are very similar to those of the corresponding DNA duplexes;; and d) hybridization of modified oligonucleotides to RNA targets results in very similar changes in fluorescence intensity as with DNA targets.

Thus, the results indicate that the universal RNA hybridization characteristics of oligonucleotides modified with monomer **122W/122X** also are be governed by a similar mechanism as universal DNA hybridization (FIG. 29).

Table 66
 T_m -Values of Duplexes Between Centrally Modified Oligonucleotides and Complementary or Centrally Mismatched RNA Targets.^[a]

ON	Sequence	B=	T_m (ΔT_m) [°C]	Mismatch ΔT_m [°C]		
			A	C	G	U
2	5'-CGCAA CTC AACGC		51.5	-15.5	-3.0	-13.5
4	5'-CGCAA TTT AACGC		40.5	-19.0	-3.5	-17.0
6	5'-CGCAA C 122W C AACGC		47.0 (-4.5)	+1.0	+0.0	+0.5
8	5'-CGCAA T 122W T AACGC		42.0 (+1.5)	+3.0	+0.5	+1.5
12	5'-CGCAA T 122X T AACGC		38.0 (-2.5)	+1.0	+0.0	+1.0
14	5'-CGCAA C 122Y C AACGC		43.5 (-8.0)	-2.0	-5.0	-4.0
16	5'-CGCAA T 122Y T AACGC		36.0 (-4.5)	+1.5	-1.0	-1.0
18	5'-CGCAA C 122Z C AACGC		44.5 (-7.0)	-3.0	-8.0	-7.0
20	5'-CGCAA T 122Z T AACGC		36.5 (-4.0)	-12.0	-7.5	-13.0

^[a] RNA targets: 3'-GCGUU **GBG** UUGCG and 3'-GCGUU **ABA** UUGCG.

Synthesis of 260. Co-evaporated **82Q** (3.40g, 4.39 mmol) with anhydrous 1,2-dichloroethane (2 x 30 mL), redissolved in anhydrous pyridine (55 mL) and cooled to 0°C. Dimethylaminopyridine (DMAP, 55 mg, 0.44 mmol) was added followed by dropwise addition of acetic anhydride (1.25 mL, 13.18 mmol). After stirring at rt for 12 h, the reaction mixture was diluted with EtOAc (150 mL) and washed with H₂O (80 mL) and sat. aq. NaHCO₃ (80 mL). The product was co-evaporated with EtOH:toluene (2:1, 3 x 30 mL) and suspended in AcOH:H₂O (4:1, 55 mL) and stirred overnight. The reaction mixture was then evaporated to dryness and purified via silica gel column chromatography (0-5% MeOH/CH₂Cl₂, v/v). The appropriate fractions were pooled and evaporated to dryness. The product was then coevaporated in anhydrous pyridine:CH₂Cl₂ (1:1, 2 x 30 mL), redissolved in pyridine:CH₂Cl₂ (1:1, 44 mL) and cooled to -20°C. Methanesulfonyl chloride (MsCl, 0.75 mL, 9.62

mmol) was added dropwise over 30 min and allowed to stir at -20°C for 2h whereupon the crude was diluted with CH₂Cl₂ (80 mL) and washed with sat. aq. NaHCO₃ (50 mL). The aqueous layer was back-extracted with CH₂Cl₂ (3 x 15 mL) and the organic layers were evaporated to dryness via co-evaporation with EtOH:toluene (2:1, 3 x 30 mL). The crude was purified by silica gel column chromatography (0-3% MeOH/CH₂Cl₂, v/v) to afford nucleoside **260** (1.43 g, 55%) as a white foam. R_f: 0.4 (5%, MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 614.1600 ([M+Na]⁺, C₃₀H₂₉N₃O₈S⁺Na⁺, Calc. 614.1568); ¹H NMR (DMSO-*d*₆) δ 11.48 (d, 1H, ex, J = 2.20 Hz, NH), 8.03-8.36 (m, 8H, py), 7.93 (d, 1H, J = 7.96 Hz, py), 7.73 (d, 1H, J = 8.23 Hz, H6), 6.39 (d, 1H, J = 7.41, H1'), 5.67 (dd, 1H, J = 2.20 Hz, 8.23 Hz, H5), 5.39 (dd, 1H, J = 3.57 Hz, 6.56 Hz, H3'), 4.33-4.38 (m, 5H, CH₂Py, H4', H5'), 3.83 (dd, 1H, J = 6.59 Hz, 7.41 Hz, H2'), 3.20 (s, 3H, CH₃ (Ms)), 2.33 (s, 3H, NCH₃), 2.13 (s, 3H, CH₃ (Ac)); ¹³C NMR (DMSO-*d*₆) δ 169.7, 162.7, 150.5, 140.6 (C6), 131.9, 130.7, 130.3, 130.2, 129.1, 127.7 (py), 127.3 (py), 127.0 (py), 126.8 (py), 126.1 (py), 125.1 (py), 124.4 (py), 124.2, 123.8, 123.7 (py), 102.6 (C5), 83.7 (C5), 79.9 (C4'), 71.7 (C3'), 69.1 (C5'), 64.9 (C2'), 57.5 (CH₂Py), 37.7 (NCH₃), 36.8 (CH₃, Ms), 20.9 (CH₃, Ac).

15

Synthesis of 262. Nucleoside **260** (770 mg, 1.30 mmol) was coevaporated in anhydrous 1,2-dichloroethane (3 x 6 mL) and suspended in absolute EtOH (25 mL) whereupon NaHCO₃ (275 mg, 3.25 mmol) was added and the suspension was heated to reflux under Argon atmosphere for 4 days. The reaction mixture was then diluted with CH₂Cl₂ and the salts were filtered and washed with CH₂Cl₂. The CH₂Cl₂ layer was evaporated to dryness and purified via silica gel column chromatography (0-7% MeOH/CH₂Cl₂, v/v) to afford nucleoside **262** (338 mg, 52%) as a white foam. R_f: 0.3 (10%, MeOH in CH₂Cl₂, v/v); MALDI-HRMS m/z 522.1985 ([M+Na]⁺, C₂₉H₂₉N₃O₅Na⁺, Calc. 522.1999); ¹H NMR (DMSO-*d*₆) δ 8.40 (d, 1H, J = 9.33 Hz, Py), 8.24-8.29 (m, 2H, Py), 8.21 (d, 1H, J = 7.96 Hz, Py), 8.13-8.16 (m, 2H, Py), 8.05-8.11 (m, 2H, Py), 7.97 (d, 1H, J = 7.96 Hz, Py), 7.92 (d, 1H, J = 7.68 Hz, H6), 6.34 (d, 1H, J = 8.51 Hz, H1'), 5.82 (d, 1H, J = 7.68 Hz, H5), 5.53 (d, 1H, ex, J = 4.94 Hz, 3'-OH), 5.11 (t, 1H, ex, J = 5.21 Hz, 5'-OH), 4.42-4.52 (m, 3H, CH₂Py, H3'), 4.11-4.28 (m, 2H, CH₂CH₃), 3.97-4.02 (m, 1H, H4'), 3.57-3.63 (m, 2H, H5'), 3.47 (dd, 1H, J = 5.21 Hz, 8.51 Hz, H2'), 2.36 (s, 3H, NCH₃), 1.07 (t, 3H, J = 7.14 Hz, CH₂CH₃); ¹³C NMR (DMSO-*d*₆) δ 169.3, 155.0, 138.1 (C6), 132.7, 130.7, 130.3, 130.1, 129.0, 127.5 (Py), 127.3 (Py), 126.9 (Py), 126.8 (Py), 126.1 (Py), 125.02 (Py), 125.00 (Py), 124.4 (Py), 124.1, 123.8, 123.6 (Py), 108.4 (C5), 87.6 (C4'), 85.0 (C1'), 71.2 (C3'), 68.8 (C2'), 64.2 (CH₂, OEt), 61.7 (C5'), 57.1 (CH₂Py), 39.0 (NCH₃), 13.6 (CH₃, OEt).

Synthesis of 264. Compound **262** (315 mg, 0.63 mmol) was coevaporated with anhydrous 1,2-dichloroethane (2 x 5 mL) and redissolved in anhydrous pyridine (6 mL). Added DMTr-Cl (260 mg, 0.76 mmol) and DMAP (8 mg, 0.06 mmol) and let stir at ambient temperature for 14 h whereupon the crude mixture was diluted with CHCl₃ (80 mL) and washed with sat. aq. NaHCO₃ (30 mL) and H₂O

(30 mL). The aqueous layer was back-extracted with CHCl_3 (3 x 15 mL) and the combined organic layers dried over Na_2SO_4 and evaporated to dryness. The crude was purified by silica gel column chromatography (0-2.5 % $\text{MeOH}/\text{CH}_2\text{Cl}_2$, v/v) to afford nucleoside **264** (445 mg, 88%) as a pale orange foam. R_f : 0.7 (10%, MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 824.3319 ($[\text{M}+\text{Na}]^+$, $\text{C}_{50}\text{H}_{47}\text{N}_3\text{O}_7\text{Na}^+$, Calc. 824.3306); ^1H NMR ($\text{DMSO}-d_6$) δ 8.40 (d, 1H, $J = 9.33$ Hz, Py), 8.24-8.30 (m, 2H, Py), 8.18 (d, 1H, $J = 7.96$ Hz, Py), 8.14 (s, 2H, Py) 8.03-8.11 (m, 2H, Py), 7.99 (d, $J = 7.68$ Hz, Py), 7.68 (d, 1H, $J = 7.68$ Hz, H6), 7.19-7.39 (m, 9H, DMTr), 6.83-6.90 (m, 4H, DMTr), 6.32 (d, 1H, $J = 7.96$ Hz, H1'), 5.59-5.64 (m, 2H, 3'-OH, H5), 4.51 (m, 2H, CH_2Py), 4.42-4.48 (m, 1H, H3'), 4.08-4.24 (m, 3H, H4', CH_2CH_3), 3.71 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.51-3.58 (m, 1H, H2'), 3.31-3.35 (m, 1H, H5'A), 3.14-3.20 (m, 1H, H5'B), 2.42 (s, 3H, NCH_3), 1.05 (t, 3H, CH_2CH_3); ^{13}C NMR ($\text{DMSO}-d_6$) δ 169.2, 158.09, 158.08, 154.9, 144.5, 138.1 (C6), 135.3, 135.0, 132.6, 130.7, 130.2, 130.1, 129.7 (Ar), 129.6 (Ar), 129.0, 127.8 (Ar), 127.6 (Ar), 127.5 (Ar), 127.3 (Ar), 126.9 (Ar), 126.8 (Ar), 126.7 (Ar), 126.1 (Ar), 125.0 (Ar), 124.4 (Ar), 124.1, 123.9, 123.6 (Ar), 113.2 (Ar), 113.1 (Ar), 108.1 (C5), 85.9, 85.7 (C4'), 85.3 (C1'), 71.0 (C3'), 68.1 (C2'), 64.2 (CH_2CH_3), 63.9 (C5'), 57.1 (CH_2Py), 55.0 (OCH_3), 38.8 (NCH_3), 13.6 (CH_2CH_3).

Synthesis of 266. Nucleoside **264** (435 mg, 0.54 mmol) was flushed with argon and cooled to 0°C over a freezing bath. A solution of 1,1,3,3-tetramethylguanidine (TMG, 0.68 mL, 5.42 mmol) in anhydrous pyridine (10 mL) was flushed with argon and cooled to 0°C over a freezing bath. After cooling, the pyridine solution was saturated with hydrogen sulfide for 1 h while maintaining the temperature of the bath below 0°C . The solution was then transferred to the pre-cooled flask containing nucleoside **264** and allowed to reach room temperature while stirring for 72 h whereupon the mixture was diluted with EtOAc (100 mL) and washed with conc. aq. NaHCO_3 (50 mL) and H_2O (50 mL). The aqueous layer was then back-extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic layers were evaporated to dryness and co-evaporated with EtOH:toluene (2:1, 3 x 15 mL). The crude was then purified via silica gel column chromatography (0-70% EtOAc/ petroleum ether, v/v) to afford nucleoside **268** (353 mg, 82%) as a white foam. R_f : 0.8 (80%, EtOAc in petroleum ether, v/v); MALDI-HRMS m/z 812.2765 ($[\text{M}+\text{Na}]^+$, $\text{C}_{48}\text{H}_{43}\text{N}_3\text{O}_6\text{SNa}^+$, Calc. 812.2797); ^1H NMR ($\text{DMSO}-d_6$) δ 12.76 (br d, 1H, ex, $J = 1.65$ Hz, NH), 8.50 (d, 1H, $J = 9.33$ Hz, Py), 8.01-8.30 (m, 8H, Py), 7.74 (d, 1H, $J = 8.23$ Hz, H6), 7.18-7.41 (m, 10H, DMTr, H1'), 6.81-6.91 (m, 4H, DMTr), 5.61 (dd, 1H, $J = 1.65$ Hz, 8.23 Hz, H5), 5.54 (d, 1H, ex, $J = 4.94$, 3'-OH), 4.42-4.58 (m, 3H, CH_2Py , H3'), 4.06-4.11 (m, 1H, H4'), 3.71 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.45-3.53 (m, 1H, H2'), 3.30-3.37 (m, 1H, H5'A), 3.15-3.24 (m, 1H, H5'B), 2.43 (s, 3H, NCH_3); ^{13}C NMR ($\text{DMSO}-d_6$) δ 176.6, 159.0, 158.1, 144.5, 140.8 (C6), 135.3, 135.0, 132.6, 130.7, 130.3, 130.2, 129.7 (Ar), 129.6 (Ar), 129.2, 128.0 (Ar), 127.9 (Ar), 127.6 (Ar), 127.3 (Ar), 126.9 (Ar), 126.8 (Ar), 126.7 (Ar), 126.1 (Ar), 125.0 (Ar), 124.9, 124.4 (Ar), 124.1, 123.9 (Ar), 123.8, 113.24 (Ar), 113.21 (Ar), 106.9 (C5), 88.0 (C1'), 86.0, 85.3 (C4'), 70.9 (C3'), 68.6 (C2'), 63.9 (C5'), 57.6 (CH_2Py), 55.0 (OCH_3), 39.2 (NCH_3).

Synthesis of 268. Nucleoside **266** (150 mg, 0.19 mmol) was added to a flame dried 10 mL round-bottom flask and dissolved in an. CH_2Cl_2 (2 mL) whereupon anhydrous diisopropylethylamine (DIPEA, 165 μL , 0.95 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (PCI-reagent, 85 μL , 0.38 mmol) were added. The reaction mixture was stirred at ambient temperature under an argon atmosphere for 3.5 h whereupon it was quenched with 1 mL cold EtOH, evaporated to dryness, and purified by silica gel column chromatography (0-55% EtOAc/petroleum ether (v/v) followed by precipitation in cold petroleum ether to afford nucleoside **268** (153 mg, 81%) was a white foam. R_f : 0.6 (50%, EtOAc in Pet Ether, v/v); MALDI-HRMS m/z 1012.3843 ($[\text{M}+\text{Na}]^+$, $\text{C}_{57}\text{H}_{60}\text{N}_5\text{O}_7\text{PS}\cdot\text{Na}^+$, Calc. 1012.3887); ^{31}P NMR (CDCl_3) δ 150.9, 149.6.

Synthesis of single-stranded probes modified with monomer 270. Briefly, the monomer was introduced into probes using compound **268**, MeCN as solvent and DCI as an activator (15 min hand-coupling, 90-99% yield). 10% tert-butylhydroperoxide (tBuOOH) in an. MeCN was used as an oxidizer (2 x 5 min oxidation, 10:87:3, tBuOOH, MeCN, H_2O) whereas 2,6-aminopurine 2'-deoxyriboside monomers were coupled using MeCN and 4,5-dicyanoimidazole activator, 15 min, 95-99%. Strands were cleaved from solid support 15-17h, rt, NH_4OH . Purified via RP-HPLC, with DMT cleavage and precipitation overnight in freezer.

Probes modified with non-pairing (or bulged) monomers 402-4, 402-N or 402-9. Particular embodiments pertain to probes that are modified with monomers **402-4**, **402-N** or **402-9**. These monomers were incorporated into probes (e.g., oligonucleotides) using **400-4**, **400-N** or **400-9** as suggested by commercial vendors. The composition of the probes was verified by MALDI-MS analysis (Table 67) whereas the purity (>80%, unless stated otherwise) was verified by ion-exchange HPLC using a LaChrom L-7000 system (VWR International) equipped with a Gen-Pak Fax column (100 mm $\times$ 4.6 mm). A representative protocol involves the use of an isocratic hold of 95% A-buffer for 5 min, followed by a linear gradient to 70% B-buffer over 41 min at a flow rate of 0.75 mL/min (A-buffer: 25 mM Tris-Cl, 1 mM EDTA, pH 8.0; B-buffer: 1 M NaCl).

Table 67
MS-Data of Representative Probes Modified with Intercalator-Functionalized Monomers 120Y, 120'W, 140'X and/or 140'Y and Non-Pairing (or Bulged) Monomers 402-4, 402-N Or 402-9.

Probe	Calc. Mass	Exp. Mass
5'- TC(140'X) AGA TAG TTG AC(140'X) ACC	5901.1	5901.7
3'- AGG (120Y)CT ATC AAC TGG (120Y)GG	5984.1	5984.2
5'- TC(140'X) AGA TAG (402-9) TTG AC(140'X) ACC	6206.2	6206.3
3'- AGG (120Y)CT ATC (402-9) AAC TGG (120Y)GG	6123.2	6123.5
5'- TC(140'X) AGA TAG (402-9) ₃ TTG AC(140'X) ACC	6650.4	6650.6
3'- AGG (120Y)CT ATC (402-9) ₃ AAC TGG (120Y)GG	6567.4	6567.7
5'- TC(140'X) AGA TAG (402-4) TTG AC(140'X) ACC	6136.1	6136.4
3'- AGG (120Y)CT ATC (402-4) AAC TGG (120Y)GG	6053.1	6053.3
5'- TC(140'X) AGA TAG (402-4) ₃ TTG AC(140'X) ACC	6440.1	6440.4
3'- AGG (120Y)CT ATC (402-4) ₃ AAC TGG (120Y)GG	6357.2	6358.5
5'- TC(140'X) AGA TAG (402-N) TTG AC(140'X) ACC	6137.1	6137.4
3'- AGG (120Y)CT ATC (402-N) AAC TGG (120Y)GG	6054.1	6054.3
5'- TC(140'X) AGA TAG (402-N) ₃ TTG AC(140'X) ACC	6360.1	6360.2
3'- AGG (120Y)CT ATC (402-N) ₃ AAC TGG (120Y)GG	6443.1	6443.8

5

Table 68
MS-Data of Additional Single-Stranded Probes Modified with Intercalator-Functionalized Monomers 120Y, 120'W and 140'X as well as Non-Pairing (or Bulged) Monomers 402-4, 402-N Or 402-9 as cell as a Cy3 Fluorophore

10

Probe	Mass Calc.	Mass Obs.
5'- <u>Cy3</u> (120'W)GC CC(120Y) GTG <u>402-9</u> CC(140'X) TG	5619.6	5619.3
3'- T(140'X)G GGA (140'X)AC <u>402-9</u> GGG (120'W)C <u>Cy3</u>	5731.7	5731.2
5'- <u>Cy3</u> (120'W)GC CC(120Y) GTG <u>402-4</u> CC(140'X) TG	5549.5	5549.2
3'- T(140'X)G GGA (140'X)AC <u>402-4</u> GGG (120'W)C <u>Cy3</u>	5661.6	5661.3
5'- <u>Cy3</u> (120'W)GC CC(120Y) GTG <u>402-N</u> CC(140'X) TG	5550.5	5550.0
3'- T(140'X)G GGA (140'X)AC <u>402-N</u> GGG (120'W)C <u>Cy3</u>	5662.6	5662.3

Experimental protocol for electrophoretic mobility shift assays used to study targeting of nucleic acid targets, more commonly, dsDNA, as illustrated by FIGS. 24-26 and FIGS. 30-39:

100 pmol of hairpin oligonucleotide was labeled with digoxigenine-ddUTP following recommended
 5 protocol by Roche Applied Bioscience. Equal volumes of 100 nM solution of digoxigenine labeled
 target hairpin duplex and varying concentration (e.g., 0.5 μ M, 1 μ M, 5 μ M, 10 μ M, 50 μ M) of stock
 probe duplex solution in 1X HEPES buffer (50 mM HEPES, 100mM NaCl, 5 mM MgCl₂, pH 7.2,
 10% sucrose, 1mg/mL spermine tetrahydrochloride) were mixed and incubated for three hours before
 loading on 15% non-denaturing polyacrilamide gel. After 2-3 hrs of electrophoresis at 100V and 0 °C
 10 the oligonucleotide complexes were transferred to a positively charged nylon membrane by
 electroblotting, which was then processed according to recommended protocol for
 chemiluminescence detection by Roche Applied Bioscience. The chemiluminescence was captured
 on X-ray film and bands were quantified using Quantity One software.

15 **Additional working examples of dsDNA-targeting via the method disclosed in FIG 1 using modified double-stranded probes.** A central embodiment entails the use of probes, more
 commonly double-stranded probes, with certain interstrand zipper monomer arrangements, more
 commonly one or more +1 interstrand zipper arrangements, for targeting of nucleic acid targets, more
 commonly dsDNA, via the method shown in FIG 1. FIG. 34 and Table 69 show the results from
 20 additional working examples hereof. Thus, various double-stranded probes with one or more +1
 interstrand zipper arrangements of monomer **120Y** were incubated at 200-fold excess (6.88 μ M) with
 a structured dig-labeled model dsDNA target, i.e., a hairpin-loop (HP) with a double-stranded target
 stem region that is is connected via a loop of ten thymidines [**HP1**: 5'-GGTATATATAGGC-(T10)-
 GCCTATATATACC] at room temperature for 15h. Hepes buffer (50 mM Hepes, 100 mM NaCl,
 25 10 mM MgCl₂, 20% sucrose, 2.9 mM spermine, pH 7.2) was used. FIG. 34 illustrates recognition
 as monitored by the formation of the probe:**HP1** complex, which has retarded mobility on non-
 denaturing PAGE compared to **HP1** (for related concept see FIG 30). The degree of invasion (=
 recognition) is calculated from the ratio of the signal [100 (probe:**HP1**)/**HP1**] in FIG. 34 and
 tabulated in Table 69. The data demonstrate that probes with one or more +1 interstrand
 30 arrangements of the disclosed monomers recognize the double-stranded stem of the structured
 dsDNA target. Without being limited to a theory of operation, probes with two or more +1
 interstrand arrangements of the disclosed monomers recognize dsDNA targets with similar or
 significantly greater efficiency (e.g., see entries 4-8) than probes with a single +1 interstrand
 arrangements of the disclosed monomers (e.g., see entries 1-3).

35

**Recognition Efficiency of Double-Stranded Probes Modified with one or More
+1 Interstrand Zipper Arrangements of Monomer 120Y**

Name	Probe	Invasion (%)
120Y-P1	5'- GG(120Y) ATA TAT AGG C 3'- CCA (120Y)AT ATA TCC G	48
120Y-P2	5'- GGT A(120Y)A TAT AGG C 3'- CCA TA(120Y) ATA TCC G	46
120Y-P3	5'- GGT ATA TA(120Y) AGG C 3'- CCA TAT ATA (120Y)CC G	24
120Y-P4	5'- GG(120Y) A(120Y)A TAT AGG C 3'- CCA (120Y)A(120Y) ATA TCC G	86
120Y-P5	5'- GG(120Y) ATA (120Y)AT AGG C 3'- CCA (120Y)AT A(120Y)A TCC G	45
120Y-P6	5'- GG(120Y) ATA TA(120Y) AGG C 3'- CCA (120Y)AT ATA (120Y)CC G	35
120Y-P7	5'- GGT A(120Y)A (120Y)AT AGG C 3'- CCA TA(120Y) A(120Y)A TCC G	82
120Y-P8	5'- GG(120Y) A(120Y)A (120Y)A(120Y) AGG C 3'- CCA (120Y)A(120Y) A(120Y)A (120Y)CC G	100

5 FIG. 35 illustrates targeting of structured dsDNA **HP1** using various concentrations of
120Y-P4. Addition of probes with two +1 interstrand arrangements of the disclosed monomers
results in a dose-dependent recognition of the structured dsDNA target. At low probe excess (e.g., 5-
10 fold relative to target **HP1**) little recognition is observed (as seen by the pale upper band). At
higher probe excess (e.g., 50-500 fold relative to target **HP1**) significant (or complete) recognition is
10 observed. Without being limited to a single mode of operation, and excess of 50-fold or greater fold
probe excess (relative to target) may be needed to facilitate significant recognition.

FIG 36 provides the results of a representative control experiment demonstrating the
recognition specificity of the disclosed probes. Probe **120Y-P4** is incubated at 200-fold excess with
structured dsDNAs (**HP1-HP7**; for sequences, see Table 70 below) that have an isosequential (**HP1**)

or non-isosequential double-stranded DNA target region (**HP2-HP7**). Only when probe **120Y-P4** is incubated with its isosequential (correct) target, significant recognition is observed (Lane 1). Lanes 2-7 show no or scant levels of recognition. This demonstrates that targeting of dsDNA using the disclosed probes is highly specific.

5

Table 70
Sequence of Isosequential and Non-Isosequential dsDNA Targets used in FIGS. 34-36

Target	Sequence	
HP1	5'- GGT ATA TAT AGG C (T) ₁₀ G CCT ATA TAT ACC	10
HP2	5'- GGT ATT TAT AGG C (T) ₁₀ G CCT ATA AAT ACC	
HP3	5'- GGT ATG TAT AGG C (T) ₁₀ G CCT ATA CAT ACC	
HP4	5'- GGT ATC TAT AGG C (T) ₁₀ G CCT ATA GAT ACC	15
HP5	5'- GGT ATA TAA AGG C (T) ₁₀ G CCT TTA TAT ACC	
HP6	5'- GGT ATA TAG AGG C (T) ₁₀ G CCT CTA TAT ACC	20
HP7	5'- GGT ATA TAC AGG C (T) ₁₀ G CCT GTA TAT ACC	

FIG. 37 demonstrates that the observed 'low mobility band' (upper band) truly is a recognition complex that is comprised of both probes strands and both target strands (see FIG. 1). In other words, FIG. 37 demonstrates that the mode of operation is as illustrated in FIG. 1. With reference to FIG. 37, lane 1: DIG-labeled **HP1** only; lane 2: DIG-labeled 'upper' probe strand only (i.e., 5'- GG(**120Y**)A(**120Y**)ATATAGGC-DIG); lane 3: DIG-labeled 'lower' probe strand only (3'- DIG-CCA(**120Y**)A(**120Y**)ATATCCG); lane 4: probe with DIG-labeled upper strand (5'- GG(**120Y**)A(**120Y**)ATATAGGC-DIG + 3'-CCA(**120Y**)A(**120Y**)ATATCCG) incubated with unlabeled **HP1**; lane 5: probe with DIG-labeled lower strand (5'-GG(**120Y**)A(**120Y**)ATATAGGC + 3'-DIG-CCA(**120Y**)A(**120Y**)ATATCCG) incubated with unlabeled **HP1**; lane 6: unlabeled probe **120Y-P4** incubated with labeled structured target **HP1**. With continued reference to FIG. 37, lane 1: shows where unreacted target is located; Lane 2+3: shows where unreacted probes are located; Lanes 4-6 concern real recognition experiments and show the same 'low mobility band' (upper band) regardless if whether only the target or only one of the probe strands was labelled with digoxigenin (dig).

35

FIG. 38 concerns a control experiment demonstrating that both probe strands are needed to facilitate recognition of double-stranded DNA. Incubation of structured dsDNA target **HP1** with only the upper (lane 2; 5'-GG(**120Y**)A(**120Y**)ATATAGGC) or lower (lane 3; 3'-CCA(**120Y**)A(**120Y**)ATATCCG) strand of **120Y-P4**, does not result in formation of a recognition complex (note absence of upper spot). This demonstrates that both strands of the disclosed double-stranded probes are needed to facilitate recognition of dsDNA (lane 4). In lane 1, only dsDNA target **HP1** is present. All probe strands are added at 200-fold excess.

FIG. 39 illustrates the results of a control experiment demonstrating that an unmodified probe (i.e., without any incorporations of disclosed monomers) does not result in recognition of dsDNA.

Table 71 provides data from dose-response and time-course studies where the listed probes were incubated with structured dsDNA target **HP1** at a) different concentrations but fixed time (dose-response, used to determine Kd 50% = probe concentration that results in 50% binding) or b) fixed concentration + varying time (time-course; used to determine TC 50% = time to accomplish 50% recognition). With reference to Table 71, probes with a greater number of +1 zipper monomer arrangements display more effective recognition (lower Kd-values, i.e., **120Q**-based probes 1.72 vs 0.76 vs 0.30 uM; **120Y**-based probes 10.30, 2.95, 0.34 uM). Without being limited to a single mode of theory, probes with +1 zipper arrangements of monomer **120Q** are more efficient than monomer **120Y** (lower Kd-values). Recognition is faster for probes with +1 zipper arrangements of monomer **120Q** than monomer **120Y** in some but not all cases

Table 71
Representative Results from Dose-Response and Time-Course
Studies using Certain Disclosed Probes

Sequence	KD 50% (μ M)	TC 50% (min)
5' - GG(120Q) ATA TAT AGG C 3' - CCA (120Q)AT ATA TCC G	1.72	560
5' - GG(120Q) A(120Q)A TAT AGG C 3' - CCA (120Q)A(120Q) ATA TCC G	0.76	205
5' - GG(120Q) A(120Q)A (120Q)A(120Q) AGG C 3' - CCA (120Q)A(120Q) A(120Q)A (120Q)CC G	0.3	29
5'- GG(120Y) ATA TAT AGG C 3'- CCA (120Y)AT ATA TCC G	10.3	N/A
5'- GG(120Y) A(120Y)A TAT AGG C 3'- CCA (120Y)A(120Y) ATA TCC G	2.95	62

5'- GG(120Y) A(120Y)A (120Y)A(120Y) AGG C 3'- CCA (120Y)A(120Y) A(120Y)A (120Y)CC G	0.34	6
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FIGS. 40-42 illustrate the results of recognition experiments where probes modified with intercalator-functionalized monomers **120Y**, **120'W**, **140'X** and/or **140'Y** and non-pairing (or bulged) monomers **402-4**, **402-N** or **402-9** (for probes, see Table 67 above) are incubated with an isosequential structured dsDNA target (i.e., 5'-GGT GGT CAA CTA TCT GGA-(T10)-TCC AGA TAG TTG ACC ACC at room temperature or 37 °C. These figures illustrate quantification results from a gel mobility shift assay (similar to FIG 30), where a 200-fold excess of the listed double-stranded probes containing one (either in up or down strand) or two (= symmetric) 'bulges' (= positioned opposite of each other) were used.

The data show that some of probes with one or two bulges display dsDNA-recognition efficiencies that differ from the 'regular' probe (P; i.e., not containing such a bulged monomer) at both evaluated temperatures. More specifically, at 37 °C: probes with two +1 interstrand arrangements of **120Y** and **140'X** that are additionally modified with (**402-4**), (**402-N**), (**402-4**)₃ and (**402-N**)₃ in both strands (=symmetric bulges) display similar or more efficient dsDNA-targeting than the regular probe. At room temperature, all probes that are modified with **402-4**, **402-9**, **402-N**, (**402-4**)₃, (**402-9**)₃ or (**402-N**)₃ in both strands display similar or more efficient dsDNA-targeting than the regular probe. A probe containing one bulge, in some cases, displays greater recognition efficiency than a regular probe (P) without the bulges. Without being limited to one a theory of operation, introduction of one or more bulge monomers may be beneficial for the dsDNA-recognition events disclosed herein.

Table 72 shows the degree of recognition (based on gel electrophoresis assay; probes used at 200-fold excess) upon incubation of structured isosequential dsDNA target **HP1** [= 5'-GGTATATATAGGC-(T10)-GCCTATATATACC] with probes that are modified with two +1 interstrand zipper arrangements of monomer **120Y** and one or more non-pairing (bulged) monomers **402-9**.

Table 72
Dsdna-Recognition Efficiency of Double-Stranded Probes that are Modified
with +1 Interstrand Zipper Arrangements of Monomer 120Y and Non-Pairing
(Bulged) Monomer 402-9

5

Probe	Degree of recognition (%)	
	8 °C	20 °C
5'- GGT A(120Y)A (120Y)AT AGG C 3'- CCA TA(120Y) A(120Y)A TCC G	41	90
5'- GG(402-9)T A(120Y)A (120Y)ATAGG C 3'- CC(402-9)ATA(120Y)A(120Y)ATCC G	57	96
5'- GGTA(120Y)A(120Y)AT AG(402-9)GC 3'- CCATA(120Y)A(120Y)A TC(402-9)CG	71	91
5'- GG(402-9)TA(120Y)A(120Y)ATAG(402-9)GC 3'- CC(402-9)ATA(120Y)A(120Y)ATC(402-9)CG	2	2
5'- GG(402-9)TA(120Y)A(120Y)ATAGGC 3'- CCATA(120Y)A(120Y)ATC(402-9)CG	4	20
5'- GGT A(120Y)A(120Y)AT AG(402-9)GC 3'- CC(402-9)ATA(120Y)A(120Y)A TCCG	13	43
5'- GG(402-9)TA(120Y)A (120Y)AT AGG C 3'- CCATA(120Y) A(120Y)A TCC G	49	99
5'- GGTA(120Y)A (120Y)AT AG(402-9)GC 3'- CCATA(120Y)A(120Y)ATCCG	49	99
5'- GGTA(120Y)A(120Y)ATAGGC 3'- CC(402-9)ATA(120Y)A(120Y)ATCCG	37	100
5'- GGTA(120Y)A (120Y)AT AGG C 3'- CCATA(120Y)A(120Y)ATC(402-9)CG	46	102
5'- GG(402-9)TA(120Y)A(120Y)ATAG(402-9)GC 3'- CCATA(120Y)A(120Y)ATCCG	75	93
5'- GGT A(120Y)A (120Y)AT AGG C 3'- CC(402-9)ATA(120Y)A(120Y)ATC(402-9)CG	66	92
5'- GG(402-9)TA(120Y)A (120Y)ATAG(402-9)GC 3'- CC(402-9)ATA(120Y)A(120Y)ATCC G	19	3
5'- GG(402-9)TA(120Y)A (120Y)AT AG(402-9)GC 3'- CCATA(120Y)A(120Y)ATC(402-9)CG	6	1

Particular embodiments involve the use of double-stranded probes with certain interstrand zipper monomer arrangements, more commonly one or more +1 interstrand zipper arrangements, that are additionally modified with fluorophore-quencher or fluorophore-fluorophore quencher pairs, and which enable targeting of nucleic acid targets, more commonly dsDNA, via the method shown in FIG 43, under the simultaneous generation of an optical signal, more commonly a fluorescent signal. Table 73 below, provides working examples of this embodiment where Cy3 and BHQ2 (= Black Hole Quencher 2) are used as a representative fluorophore-quencher pair. With reference to Table 73, probe-target duplexes (products of recognition reaction) display significantly higher thermostability than the probe, which enables detection via the method illustrated by FIG. 43.

Table 73

MS Of Representative Examples of Double-Stranded Probes with +1 Interstrand Zipper Monomer Arrangements, which are Additionally Modified With Fluorophore-Quencher Pairs (BHQ2 = Black Hole Quencher 2)

Sequence	Mass Calc.	Mass Obs.
5'- <u>Cy3</u> (120'W)GC CC(120Y) GTG CC(140'X) TG	5397.5	5396.0
3'- <u>BHQ2</u> T(140'X)G GGA (140'X)AC GGG (120'W)C	5558.4	5558.9
5'-(120'W)GC CC(120Y) GTG CC(140'X) TG <u>BHQ2</u>	5446.4	5445.9
3'-T(140'X)G GGA (140'X)AC GGG (120'W)C <u>Cy3</u>	5509.6	5508.6

Particular embodiments involve the use of double-stranded probes with certain interstrand zipper monomer arrangements, more commonly one or more +1 interstrand zipper arrangements, where the oligonucleotide backbone is chemically modified. Table 74 below, provides a working example hereof, where the backbone of double-stranded probe is fully modified with the phosphorothioate-DNA backbone modification.

Table 74

MS of Representative Examples of Double-Stranded Probes with +1 Interstrand Zipper Monomer Arrangements, where the Backbone of Double-Stranded Probe is Fully Modified with the Phosphorothioate-DNA [Ps-DNA] Backbone Modification.

Probe	Mass Calc.	Mass Obs.
5'-[ps-DNA]- <u>Cy3</u> (120'W)GC CC(120Y) GTG CC(140'X) TG	5621.5	5622.0
3'-[ps-DNA]-T(140'X)G GGA (140'X)AC GGG (120'W)C-Cy3-5'	5733.6	5734.0

Additional information concerning subject matter similar to that disclosed herein is provided by International Patent Application No. PCT/US2010/048520, which is incorporated herein by reference.

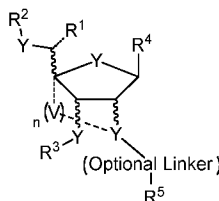
In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes
5 within the scope and spirit of these claims.

10

I claim:

1. A probe, comprising:

a pair of monomers comprising a first monomer having a formula

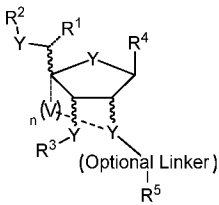


5

where each Y independently is selected from carbon, oxygen, sulfur, a triazole, oxazole, tetrazole, isoxazole, and NR^b , wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl; V is selected from carbon, oxygen, sulfur, and NR^b ; n ranges from 0 to 4; R^1 and R^2 are selected from hydrogen, aliphatic, aryl, aryl aliphatic, and a heteroatom-containing moiety; R^3 is a heteroatom-containing functional group; R^4 is selected from any natural or non-natural nucleobase; R^5 is selected from an intercalator suitable for intercalating within a nucleic acid; “optional linker” is selected from linkers comprising alkyl linkers, amide linkers, carbamate linkers, carbonate linkers, urea linkers, and combinations thereof;

10

a second monomer having a formula



15

wherein Y, V, R^1 , R^2 , R^3 , R^4 , R^5 and “optional linker” are as stated for the first monomer; and

at least one nucleotide selected from a natural nucleotide, a non-natural nucleotide, and combinations thereof.

20

2. The probe according to claim 1 wherein the heteroatom-containing functional group is selected from ether (R^aOR^b), hydroxyl (R^aOH), silyl ether ($\text{R}^a\text{R}^b\text{R}^c\text{SiOR}^d$), phosphine ($\text{PR}^a\text{R}^b\text{R}^c$), thiol (R^aSH), thioether/sulfide (R^aSR^b), disulfide (R^aSSR^b), isothiocyanate (R^aNCS), isocyanate (R^aNCO), amine (NH_2 , NHR^a , NR^aR^b), amide ($\text{R}^a\text{NR}^b\text{C}(\text{O})\text{R}^c$), ester ($\text{R}^a\text{OC}(\text{O})\text{R}^b$), halogen (I, Br, Cl, F), carbonate ($\text{R}^a\text{OC}(\text{O})\text{OR}^b$), carboxyl ($\text{R}^a\text{C}(\text{O})\text{OH}$), carboxylate (R^aCOO^-), ester ($\text{R}^a\text{C}(\text{O})\text{OR}^b$), ketone ($\text{R}^a\text{C}(\text{O})\text{R}^b$), phosphate ($\text{R}^a\text{OP}(\text{O})(\text{OH})_2$), phosphoryl ($\text{R}^a\text{P}(\text{O})(\text{OH})_2$), sulfinyl ($\text{R}^a\text{S}(\text{O})\text{R}^b$), sulfonyl ($\text{R}^a\text{SO}_2\text{R}^b$), carbonothioyl ($\text{R}^a\text{C}(\text{S})\text{R}^b$ or $\text{R}^a\text{C}(\text{S})\text{H}$), sulfinio ($\text{R}^a\text{S}(\text{O})\text{OH}$), sulfo ($\text{R}^a\text{SO}_3\text{H}$), amide ($\text{R}^a\text{C}(\text{O})\text{NR}^b\text{R}^c$), azide (N_3), nitrile (R^aCN), isonitrile ($\text{R}^a\text{N}^+\text{C}^-$), and nitro (R^aNO_2); R^a represents the remaining monomer structure, which is attached to the abovementioned functional

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groups at the position indicated for R¹; and R^b, R^c, and R^d independently are hydrogen, aliphatic, aryl, heteroaliphatic, heteroaryl, and any combination thereof.

3. The probe according to claim 1 wherein R² and R³ independently are selected from a heteroatom functional group comprising phosphorous, sulfur, nitrogen, oxygen, selenium, and/or a metal.

4. The probe according to claim 1 wherein R² and R³ independently are selected from a phosphate group of a natural nucleotide, non-natural nucleotide, or combinations thereof.

5. The probe according to claim 1 wherein R³ has a formula



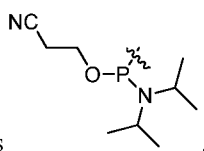
where Y is selected from oxygen, sulfur, NR^b where R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, heteroaryl, and W is selected from phosphorus, SH, or SeH.

6. The probe according to claim 1 wherein R³ is $\begin{array}{c} \text{O} \\ \parallel \\ \text{S}^{\ominus}\text{---P---} \\ | \\ \text{O} \end{array}$, or $\begin{array}{c} \text{O} \\ \parallel \\ \text{O}^{\ominus}\text{---P---} \\ | \\ \text{O} \end{array}$.

7. The probe according to claim 1 where R³ has a formula



where W is phosphorus, and each Z independently is selected from ether, thioether, hydroxyl, and NR^b.

8. The probe according to claim 1 wherein R³ is 

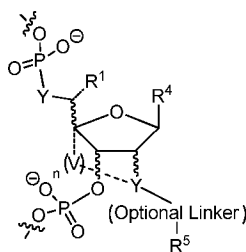
9. The probe according to claim 1 wherein R⁴ is selected from adenine, guanine, cytosine, uracil, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one, or any derivative thereof.

10. The probe according to claim 1 wherein the intercalator is a hydrocarbon or an aromatic heterocycle.

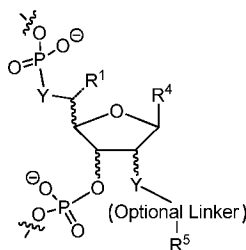
11. The probe according to claim 1 wherein the intercalator is a hydrocarbon selected from pyrene, coronene, perylene, anthracene, naphthalene and functionalized derivatives thereof.

12. The probe according to claim 1 wherein the intercalator is an aromatic heterocycle selected from a porphyrin, nucleobase, metal chelator, azapyrene, thiazole orange, ethidium, an indole, a pyrrole, a benzimidazole, and modified analogs thereof.

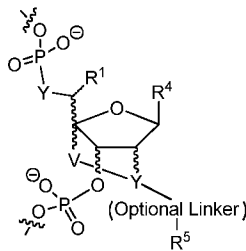
13. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula



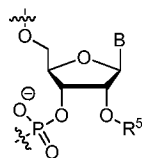
14. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula



15. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula

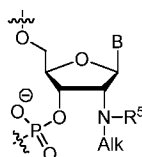


16. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula



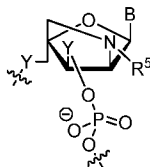
wherein B is selected from uracil, guanine, cytosine, adenine, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one, or any derivative thereof.

- 5 17. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula



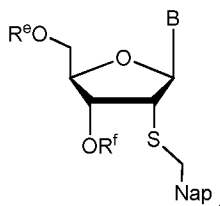
wherein B is selected from uracil, guanine, cytosine, adenine, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one, or any derivative thereof, and alk is
10 C1-C10 alkyl.

18. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula

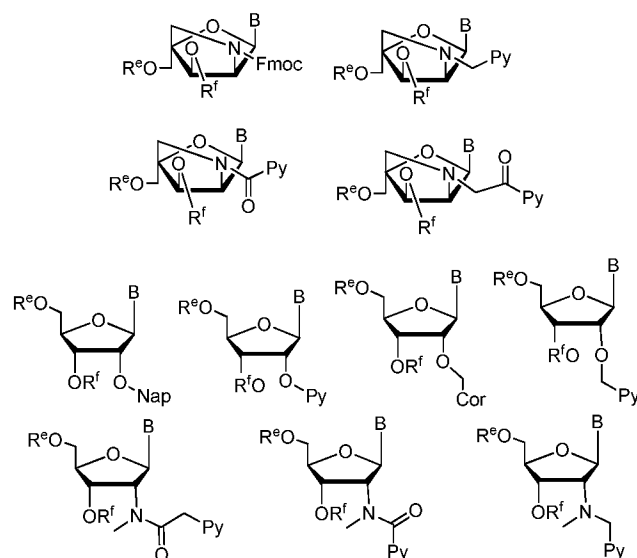


- 15 wherein B is selected from uracil, guanine, cytosine, adenine, or thymine.

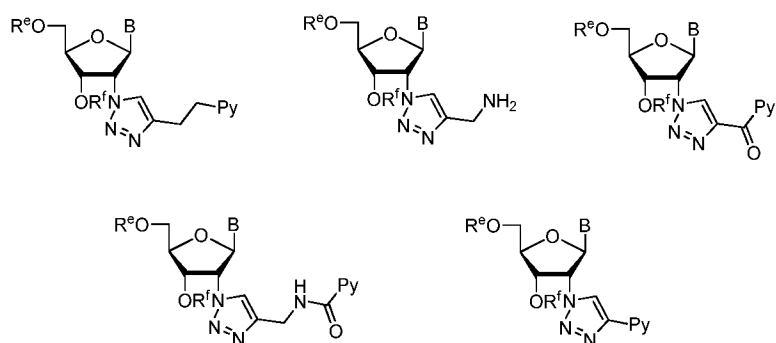
19. The probe according to claim 1 wherein either the first monomer or the second monomer has a formula



- 20 20. The probe according to claim 1 wherein either the first monomer or the second monomer has any one of the following formulas:



5



wherein B is selected from uracil, guanine, cytosine, adenine, thymine, 2-thiouracil, 2,6-diaminopurine, inosine, 3-pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one, or any derivative thereof; R^e is H, DMTr, or phosphate; and R^f is H, (N(i-Pr)₂)P(OCH₂CH₂CN), or phosphate.

21. The probe according to claim 1 wherein the at least one natural nucleotide is selected from adenine, guanine, cytosine, uracil, and thymine.

22. The probe according to claim 1 wherein the at least one non-natural nucleobase is selected from C-5 functionalized pyrimidines, C6-functionalized pyrimidines, C7-functionalized 7-deazapurines, C8-functionalized purines, 2,6-diaminopurine, 2-thiouracil, 4-thiouracil, deoxyinosine and 3-(2'-deoxy-β-D-ribofuranosyl)pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one.

23. The probe according to claim 1 wherein the at least one natural nucleotide, unnatural nucleotide, and combinations thereof is selected to substantially match at least one nucleotide of a corresponding nucleic acid sequence.

5 24. The probe according to claim 1 having a formula

$$5'(B_1B_2...B_m)(\underline{XA})(B_1B_2...B_n)(\underline{XA})_f(B_1B_2...B_o)(\underline{XA})_g(B_1B_2...B_p)(\underline{XA})_h(B_1B_2...B_q)(\underline{XA})_i(B_1B_2...B_r)(\underline{XA})_j(B_1B_2...B_s)$$

$$3'(C_1C_2...C_m)(\underline{DP})(C_1C_2...C_n)(\underline{DP})_f(C_1C_2...C_o)(\underline{DP})_g(C_1C_2...C_p)(\underline{DP})_h(C_1C_2...C_q)(\underline{DP})_i(C_1C_2...C_r)(\underline{DP})_j(C_1C_2...C_s)$$

10 wherein B_1 , B_2 and B_{m-s} may be any natural or non-natural nucleotide, wherein $m-r$ ranges from zero to about 28; f , g , h , i and j range from zero to 10; X is the first monomer; A is the complement Watson-Crick base pairing nucleotide of P; C is any natural or non-natural nucleotide capable of Watson-Crick base pairing with any one of B_1 , B_2 and B_{m-s} ; P is the second monomer, and D is the complement Watson-Crick base pairing nucleotide of X.

15 25. The probe according to claim 1 wherein the probe is selected to recognize a predetermined sequence of a nucleic acid target.

20 26. The probe according to claim 19 wherein the nucleic acid target is single-stranded or double-stranded.

 27. The probe according to claim 1 further comprising one or more bulge monomers that do not participate in base pairing.

25 28. The probe according to claim 1 wherein the first monomer and the second monomer are arranged in a manner that substantially weakens the duplex's thermal stability.

 29. The probe according to claim 22 wherein the duplex has a thermal melting temperature which is substantially similar to that of a nucleic acid duplex.

30 30. The probe according to claim 23 wherein the unmodified nucleic acid duplex does not comprise a monomer having the formula of claim 1.

35 31. The probe according to claim 1 wherein the first monomer and the second monomer are arranged in a +n or -n zipper orientation, wherein n ranges from 0 to about 10.

 32. The probe according to claim 25 wherein the first monomer and the second monomer are arranged in a +n orientation, wherein n is 1.

33. The probe according to claim 1 further comprising one or more additional pairs of monomer pairs.

5 34. The probe according to claim 1 further comprising a signal generating moiety capable of being detected.

35. The probe according to claim 28 wherein the signal generating moiety is selected from a fluorophore, a member of a specific binding pair, a nanoparticle, and combinations thereof.

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36. The probe according to claim 29 wherein the member of a specific binding pair is biotin.

37. The probe according to claim 30 further comprising a secondary entity that facilitates cell-uptake, is a quencher, is crosslinking reagent capable of forming bonds between the probe and nucleic acids, proteins, sugars, lipids or other biomolecules; a nucleic acid cargo, single-stranded DNA, single-stranded RNA, double-stranded DNA, double-stranded RNA, plasmid, or gene.

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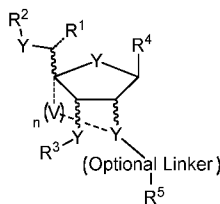
38. The probe according to claim 1 wherein the probe is used in solution, on a solid surface, or in combination with a colloidal material.

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39. A probe selected from any of SEQ ID Nos. 1-253.

40. A method for associating a probe with a target, comprising:
selecting a probe comprising a monomer having a formula

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where each Y independently is selected from carbon, oxygen, sulfur, a triazole, an oxazole, a tetrazole, an isoxazole, or is NR^b , wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl; V is selected from carbon, oxygen, sulfur, and NR^b ; n ranges from 0 to 4; R^1 and R^2 are selected from hydrogen, aliphatic, aryl, aryl aliphatic, and a heteroatom-containing moiety; R^3 is a heteroatom-containing functional group; R^4 is selected from any natural or

30

non-natural nucleobase; R⁵ is selected from any functional group suitable for coupling to or associating with a nucleic acid;

at least one natural nucleotide, unnatural nucleotide, and combinations thereof;

exposing a nucleic acid target to the probe; and

5 detecting the probe.

41. The method according to claim 40 wherein the probe is selected to substantially match a target nucleic acid sequence.

10 42. The method according to claim 40 wherein the nucleic acid target comprises one or more polypurine units.

43. The method according to claim 40 wherein the nucleic acid target does not comprise a polypurine unit.

15 44. The method according to claim 40 wherein the nucleic acid target is a mixed-sequence, structured nucleic acid.

20 45. The method according to claim 44 wherein the nucleic acid target is a mixed-sequence, hairpin DNA target.

46. The method according to claim 40 wherein the nucleic acid target is isosequential with the probe.

25 47. The method according to claim 40 wherein exposing comprises incubating the nucleic acid target with the probe.

48. The method according to claim 40 wherein the nucleic acid target is incubated with about a 5-fold excess of the probe to a 5,000,000-fold excess of the probe.

30 49. The method according to claim 40 wherein the nucleic acid target is incubated with about a 5-fold excess of the probe to about a 500-fold excess of the probe.

35 50. The method according to claim 40 wherein the probe is detected by fluorescence spectroscopy, electrophoresis, absorption spectroscopy, flow cytometry, and combinations thereof.

51. The method according to claim 40 wherein the probe is selected from SEQ. ID Nos. 1-253.

52. The method according to claim 40 wherein the nucleic acid target is single-stranded or double-stranded.

53. The method according to claim 40 wherein the nucleic acid target is double-stranded and the probe is double stranded.

54. The method according to claim 40 wherein the probe further comprises one or more bulge monomers that do not participate in base pairing.

55. The method according to claim 40 wherein the monomer is positioned in the probe in a manner that substantially weakens a duplex's thermal stability.

56. The method according to claim 55 wherein the duplex has a thermal melting temperature which is substantially similar to that of an unmodified nucleic acid duplex.

57. The method according to claim 41 wherein a first monomer and a second monomer are arranged in a +n or -n zipper orientation, wherein n ranges from 0 to about 10.

58. The method according to claim 57 wherein the first monomer and the second monomer are arranged in a +n orientation, wherein n is 1.

59. The method according to claim 40 wherein the target is a DNA target selected from second insulin, PPAR gamma, and CEBP promoters.

60. The method according to claim 40 wherein the probe is used for gender determination in mammals.

61. The method according to claim 60 wherein the mammals are ungulates.

62. The method according to claim 60 wherein the mammals are ruminants.

63. The method according to claim 60 wherein the mammals are bovines, equines or porcines.

64. The method according to claim 40 wherein the target is isosequential (relative to a probe) double stranded DNA target regions, including stems of molecular beacons, target regions embedded within PCR amplicons, target regions embedded within circular or linearized plasmids, target regions embedded within genomic DNA, and target regions embedded within microorganisms.

5

65. The method according to claim 40 wherein the target is selected from a nucleic acid sequence associated with B cell and T cell leukemias, lymphomas, breast cancer, colon cancer, and neurological cancers.

10

66. The method according to claim 40 wherein the target is selected from the EGFR gene (7p12; *e.g.*, GENBANK™ Accession No. NC_000007, nucleotides 55054219-55242525), the C-MYC gene (8q24.21; *e.g.*, GENBANK™ Accession No. NC_000008, nucleotides 128817498-128822856), D5S271 (5p15.2), lipoprotein lipase (LPL) gene (8p22; *e.g.*, GENBANK™ Accession No. NC_000008, nucleotides 19841058-19869049), RB1 (13q14; *e.g.*, GENBANK™ Accession No. NC_000013, nucleotides 47775912-47954023), p53 (17p13.1; *e.g.*, GENBANK™ Accession No. NC_000017, complement, nucleotides 7512464-7531642), N-MYC (2p24; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 151835231-151854620), CHOP (12q13; *e.g.*, GENBANK™ Accession No. NC_000012, complement, nucleotides 56196638-56200567), FUS (16p11.2; *e.g.*, GENBANK™ Accession No. NC_000016, nucleotides 31098954-31110601), FKHR (13p14; *e.g.*, GENBANK™ Accession No. NC_000013, complement, nucleotides 40027817-40138734), as well as, for example: ALK (2p23; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 29269144-29997936), Ig heavy chain, CCND1 (11q13; *e.g.*, GENBANK™ Accession No. NC_000011, nucleotides 69165054..69178423), BCL2 (18q21.3; *e.g.*, GENBANK™ Accession No. NC_000018, complement, nucleotides 58941559-59137593), BCL6 (3q27; *e.g.*, GENBANK™ Accession No. NC_000003, complement, nucleotides 188921859-188946169), MALF1, AP1 (1p32-p31; *e.g.*, GENBANK™ Accession No. NC_000001, complement, nucleotides 59019051-59022373), TOP2A (17q21-q22; *e.g.*, GENBANK™ Accession No. NC_000017, complement, nucleotides 35798321-35827695), Tmprss (21q22.3; *e.g.*, GENBANK™ Accession No. NC_000021, complement, nucleotides 41758351-41801948), ERG (21q22.3; *e.g.*, GENBANK™ Accession No. NC_000021, complement, nucleotides 38675671-38955488); ETV1 (7p21.3; *e.g.*, GENBANK™ Accession No. NC_000007, complement, nucleotides 13897379-13995289), EWS (22q12.2; *e.g.*, GENBANK™ Accession No. NC_000022, nucleotides 27994271-28026505); FLI1 (11q24.1-q24.3; *e.g.*, GENBANK™ Accession No. NC_000011, nucleotides 128069199-128187521), PAX3 (2q35-q37; *e.g.*, GENBANK™ Accession No. NC_000002, complement, nucleotides 222772851-222871944), PAX7 (1p36.2-p36.12; *e.g.*, GENBANK™ Accession No. NC_000001, nucleotides 18830087-18935219), PTEN (10q23.3; *e.g.*, GENBANK™ Accession No. NC_000010, nucleotides 89613175-89716382), AKT2

35

(19q13.1-q13.2; *e.g.*, GENBANK™ Accession No. NC_000019, complement, nucleotides 45431556-45483036), MYCL1 (1p34.2; *e.g.*, GENBANK™ Accession No. NC_000001, complement, nucleotides 40133685-40140274), REL (2p13-p12; *e.g.*, GENBANK™ Accession No. NC_000002, nucleotides 60962256-61003682) and CSF1R (5q33-q35; *e.g.*, GENBANK™ Accession No. NC_000005, complement, nucleotides 149413051-149473128).

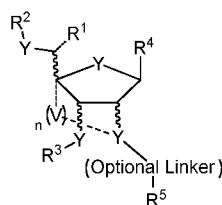
67. The method according to claim 40 wherein the target is selected from a virus or other microorganism and the probe is used to detect and/or identify the microorganism.

68. The method according to claim 40 wherein the target is selected from the genome of an oncogenic or pathogenic virus, a bacterium or an intracellular parasite selected from *Plasmodium* species, *Leishmania* (sp.), *Cryptosporidium parvum*, *Entamoeba histolytica*, *Giardia lamblia*, *Toxoplasma*, *Eimeria*, *Theileria*, and *Babesia*.

69. The method according to claim 40 wherein the target is selected from human adenovirus A (NC_001460), human adenovirus B (NC_004001), human adenovirus C (NC_001405), human adenovirus D (NC_002067), human adenovirus E (NC_003266), human adenovirus F (NC_001454), human astrovirus (NC_001943), human BK polyomavirus (V01109; GI:60851) human bocavirus (NC_007455), human coronavirus 229E (NC_002645), human coronavirus HKU1 (NC_006577), human coronavirus NL63 (NC_005831), human coronavirus OC43 (NC_005147), human enterovirus A (NC_001612), human enterovirus B (NC_001472), human enterovirus C (NC_001428), human enterovirus D (NC_001430), human erythrovirus V9 (NC_004295), human foamy virus (NC_001736), human herpesvirus 1 (Herpes simplex virus type 1) (NC_001806), human herpesvirus 2 (Herpes simplex virus type 2) (NC_001798), human herpesvirus 3 (Varicella zoster virus) (NC_001348), human herpesvirus 4 type 1 (Epstein-Barr virus type 1) (NC_007605), human herpesvirus 4 type 2 (Epstein-Barr virus type 2) (NC_009334), human herpesvirus 5 strain AD169 (NC_001347), human herpesvirus 5 strain Merlin Strain (NC_006273), human herpesvirus 6A (NC_001664), human herpesvirus 6B (NC_000898), human herpesvirus 7 (NC_001716), human herpesvirus 8 type M (NC_003409), human herpesvirus 8 type P (NC_009333), human immunodeficiency virus 1 (NC_001802), human immunodeficiency virus 2 (NC_001722), human metapneumovirus (NC_004148), human papillomavirus-1 (NC_001356), human papillomavirus-18 (NC_001357), human papillomavirus-2 (NC_001352), human papillomavirus-54 (NC_001676), human papillomavirus-61 (NC_001694), human papillomavirus-cand90 (NC_004104), human papillomavirus RTRX7 (NC_004761), human papillomavirus type 10 (NC_001576), human papillomavirus type 101 (NC_008189), human papillomavirus type 103 (NC_008188), human papillomavirus type 107 (NC_009239), human papillomavirus type 16 (NC_001526), human papillomavirus type 24 (NC_001683), human papillomavirus type 26 (NC_001583), human

papillomavirus type 32 (NC_001586), human papillomavirus type 34 (NC_001587), human papillomavirus type 4 (NC_001457), human papillomavirus type 41 (NC_001354), human papillomavirus type 48 (NC_001690), human papillomavirus type 49 (NC_001591), human papillomavirus type 5 (NC_001531), human papillomavirus type 50 (NC_001691), human papillomavirus type 53 (NC_001593), human papillomavirus type 60 (NC_001693), human papillomavirus type 63 (NC_001458), human papillomavirus type 6b (NC_001355), human papillomavirus type 7 (NC_001595), human papillomavirus type 71 (NC_002644), human papillomavirus type 9 (NC_001596), human papillomavirus type 92 (NC_004500), human papillomavirus type 96 (NC_005134), human parainfluenza virus 1 (NC_003461), human parainfluenza virus 2 (NC_003443), human parainfluenza virus 3 (NC_001796), human parechovirus (NC_001897), human parvovirus 4 (NC_007018), human parvovirus B19 (NC_000883), human respiratory syncytial virus (NC_001781), human rhinovirus A (NC_001617), human rhinovirus B (NC_001490), human spumaretrovirus (NC_001795), human T-lymphotropic virus 1 (NC_001436), human T-lymphotropic virus 2 (NC_001488), Epstein-Barr Virus (EBV) or a Human Papilloma Virus (HPV, *e.g.*, HPV16, HPV18), Respiratory Syncytial Virus, a Hepatitis Virus (*e.g.*, Hepatitis C Virus), a Coronavirus (*e.g.*, SARS virus), an Adenovirus, a Polyomavirus, a Cytomegalovirus (CMV), Herpes Simplex Virus (HSV), Her1, Her2, Her3, Her4, EGFR1, EGFR2, EGFR3, EGFR4, ErbB-1, ErbB-2, ErbB-3 and ErbB-4.

20 70. A kit, comprising a nucleic acid probe comprising at least one monomer having a formula



where each Y independently is selected from carbon, oxygen, sulfur, a triazole, oxazole, tetrazole, isoxazole, and NR^b, wherein R^b is selected from hydrogen, aliphatic, aryl, heteroaliphatic, and heteroaryl; V is selected from carbon, oxygen, sulfur, and NR^b; n ranges from 0 to 4; R¹ and R² are selected from hydrogen, aliphatic, aryl, aryl aliphatic, and a heteroatom-containing moiety; R³ is a heteroatom-containing functional group; R⁴ is selected from any natural or non-natural nucleobase; R⁵ is selected from an intercalator suitable for intercalating within a nucleic acid; “optional linker” is selected from linkers comprising alkyl linkers, amide linkers, carbamate linkers, carbonate linkers, urea linkers, and combinations thereof;

71. The kit according to claim 70 where the probe comprises at least one bulge monomer.

72. The kit according to claim 70 comprising a dsDNA probe.
73. The kit according to claim 70 comprising a sequence selected from SEQ ID NOs.
5 1-254.
74. The kit according to claim 70 for gender determination in mammals.
75. The kit according to claim 70 for sexing mammalian embryos.
10
76. The kit according to claim 74 where the mammal is an ungulate.
77. The kit according to claim 75 where the mammal is a bovine, equine or porcine.

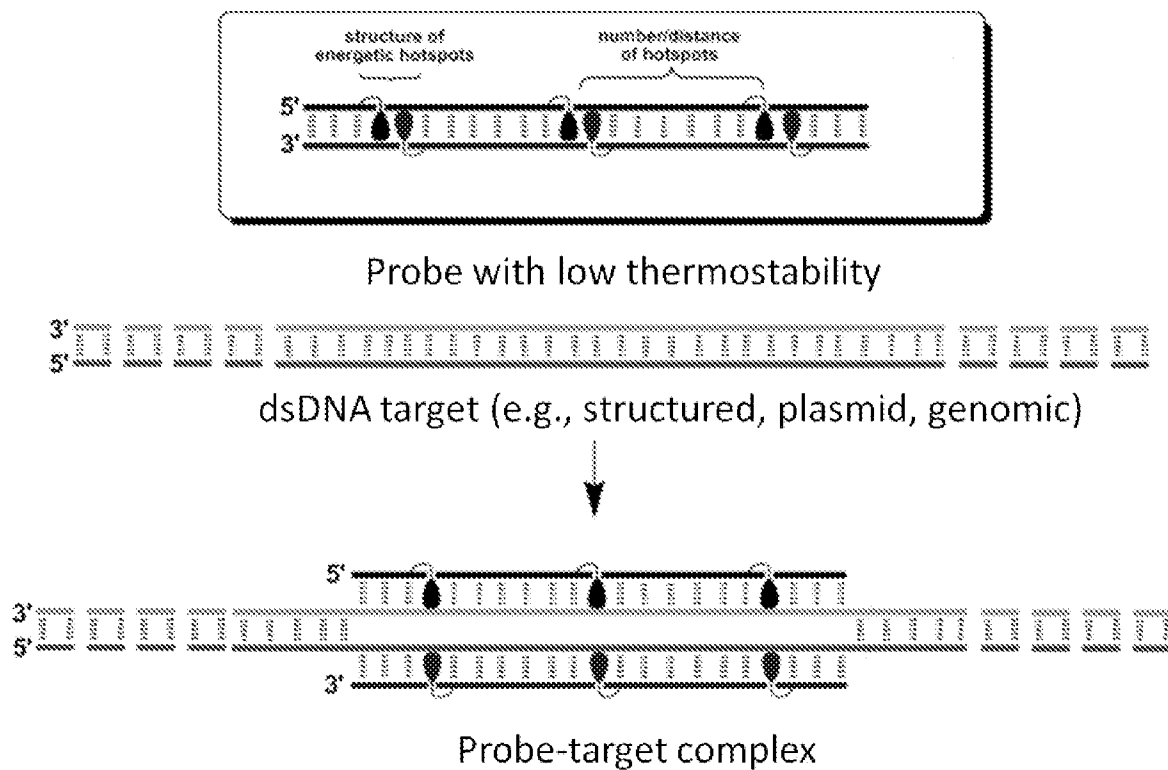


FIG. 1

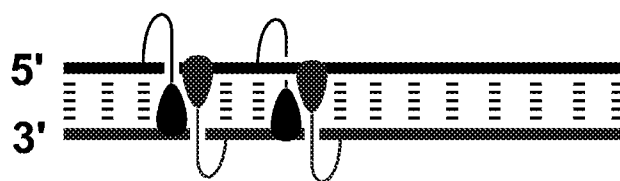


FIG. 2

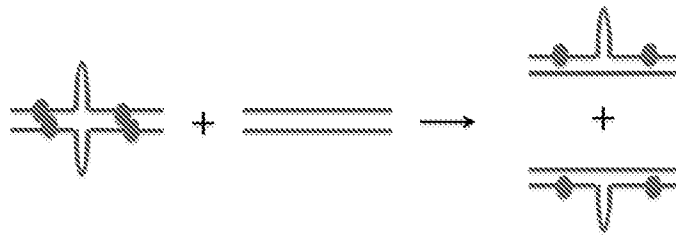


FIG. 3A

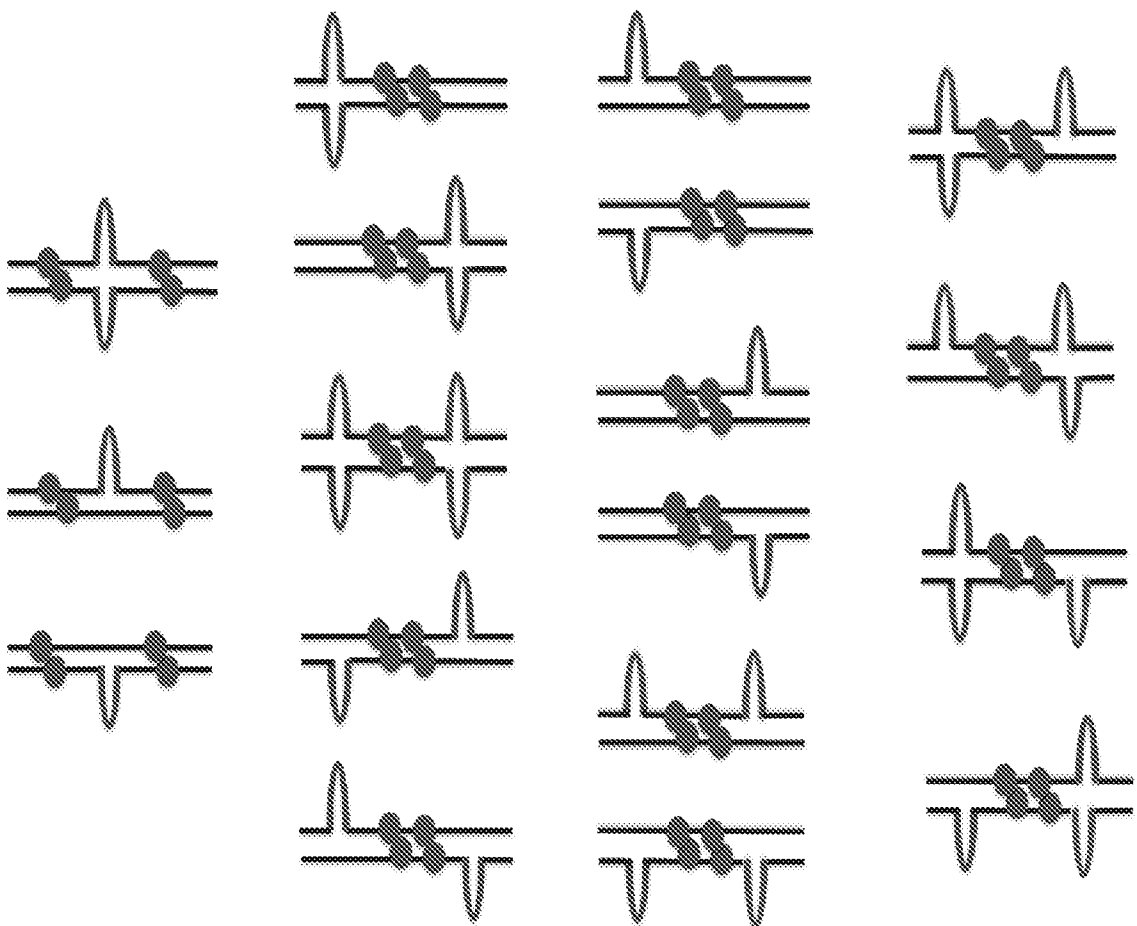


FIG. 3B

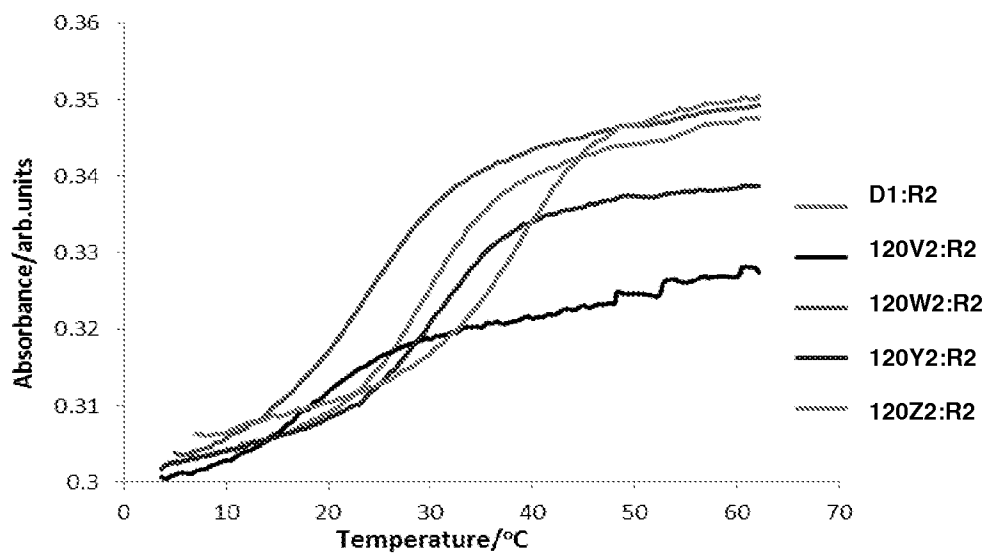


FIG. 4

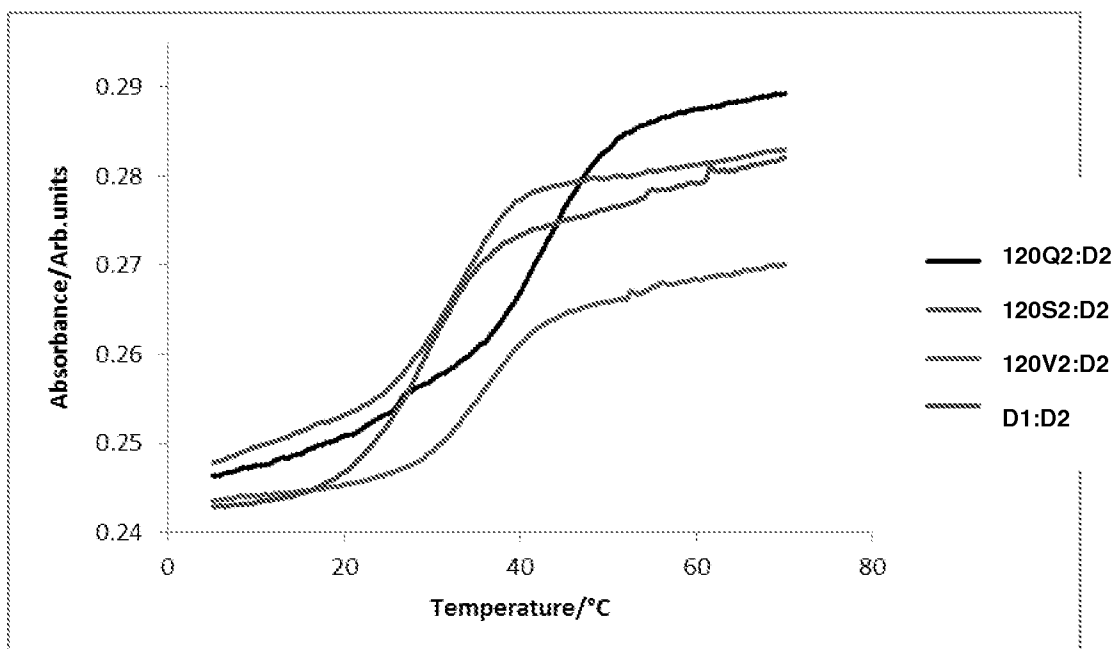


FIG. 5

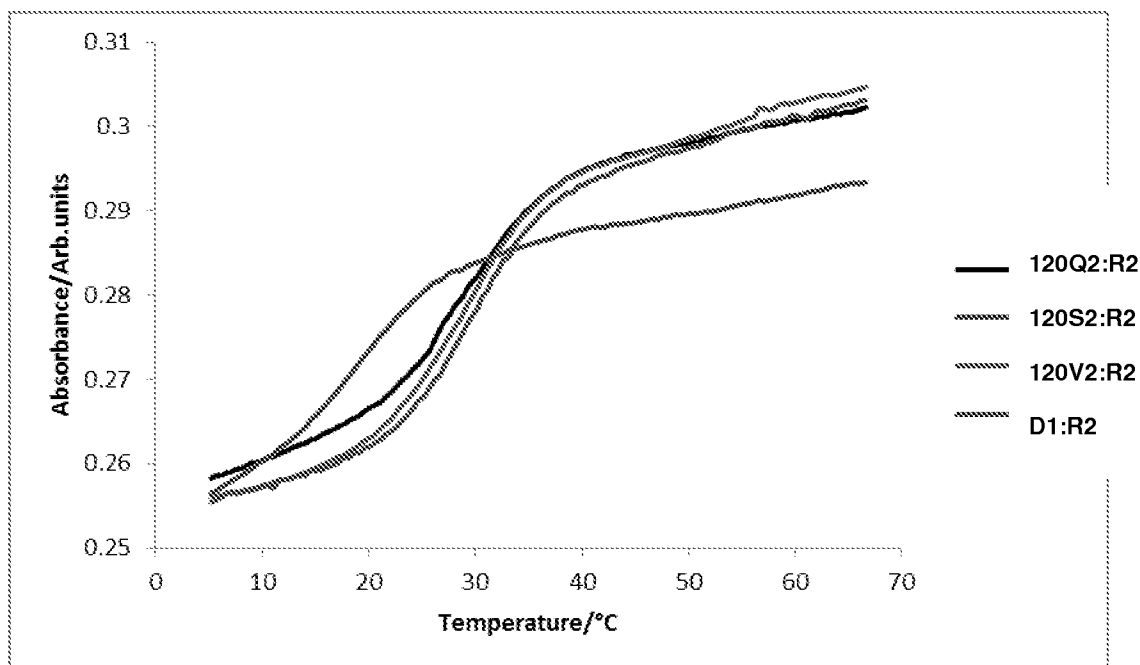


FIG. 6

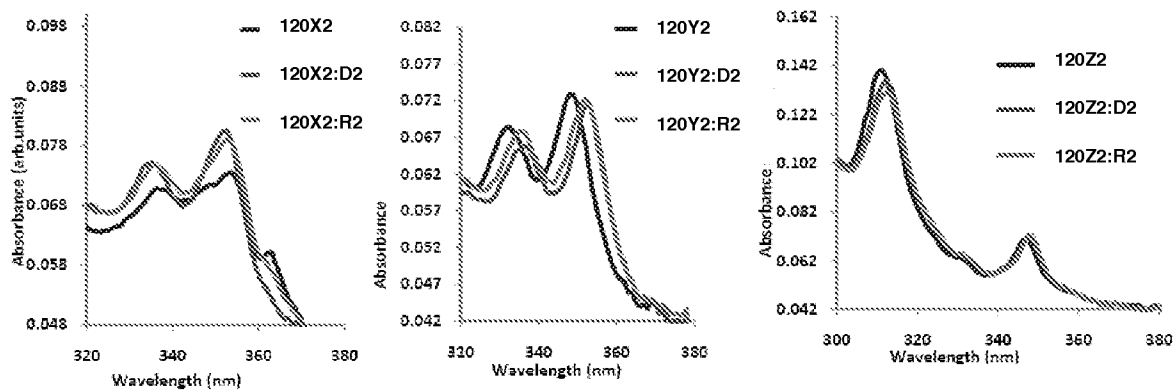


FIG. 7

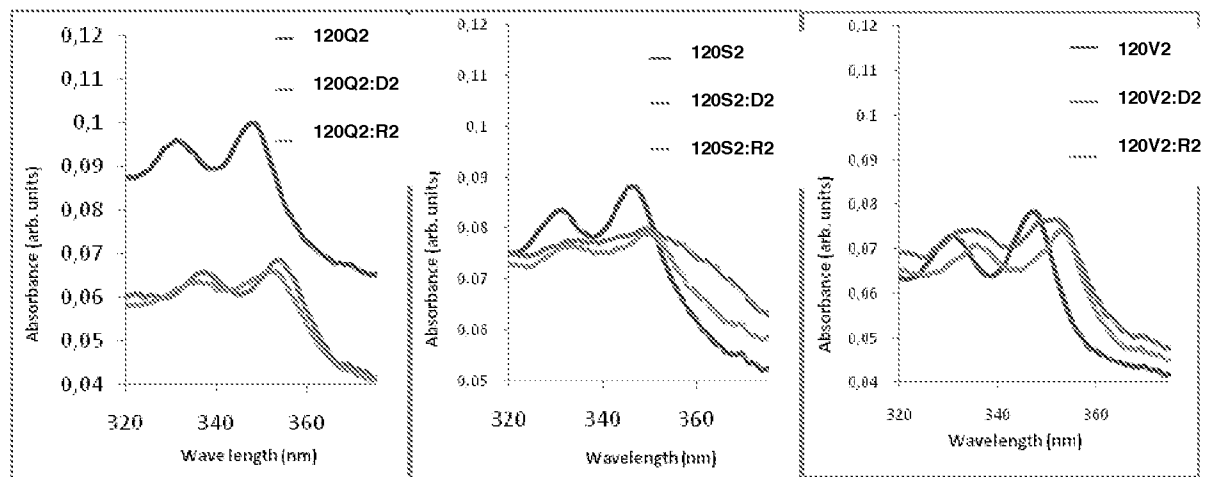


FIG. 8

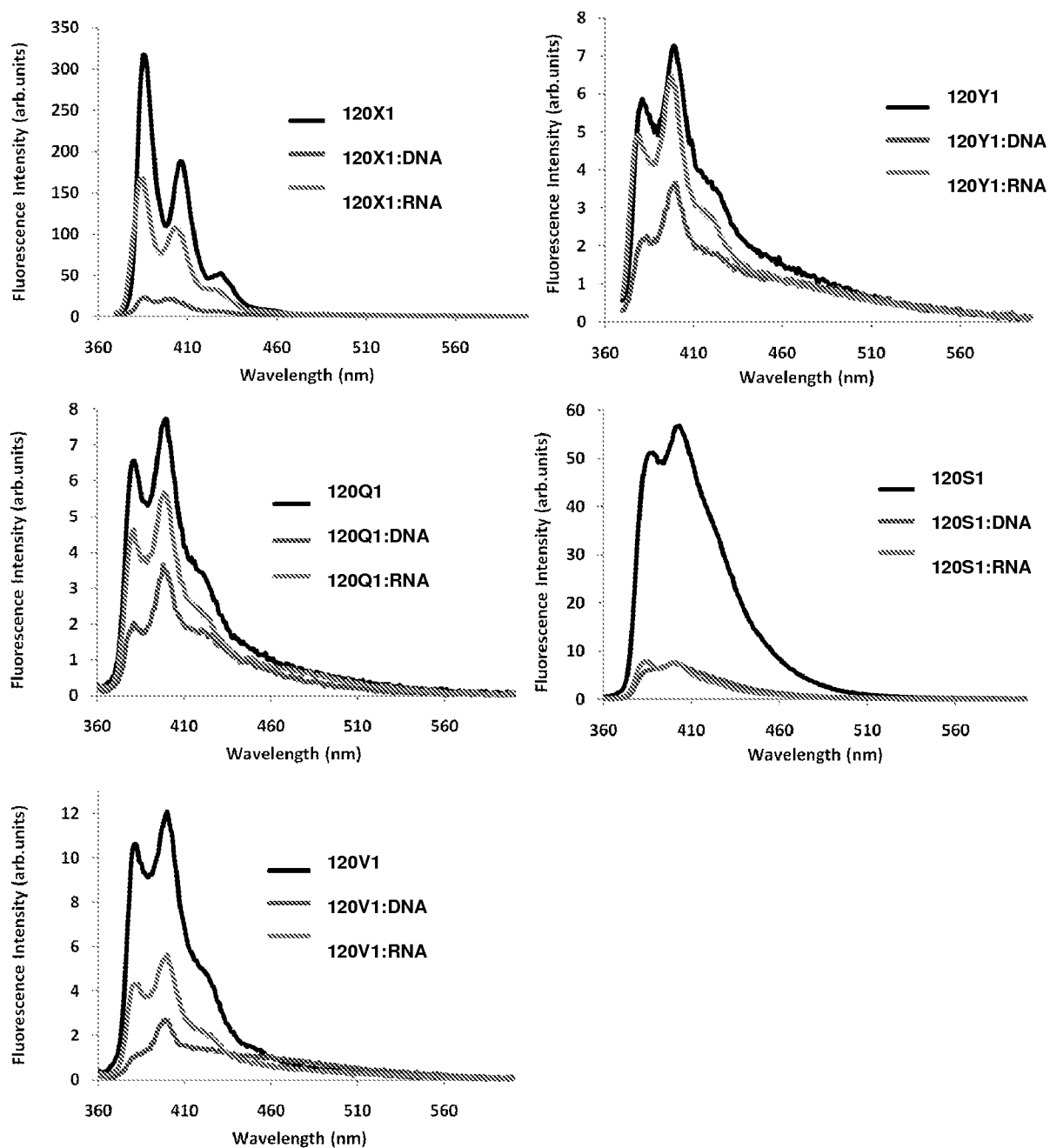


FIG. 9

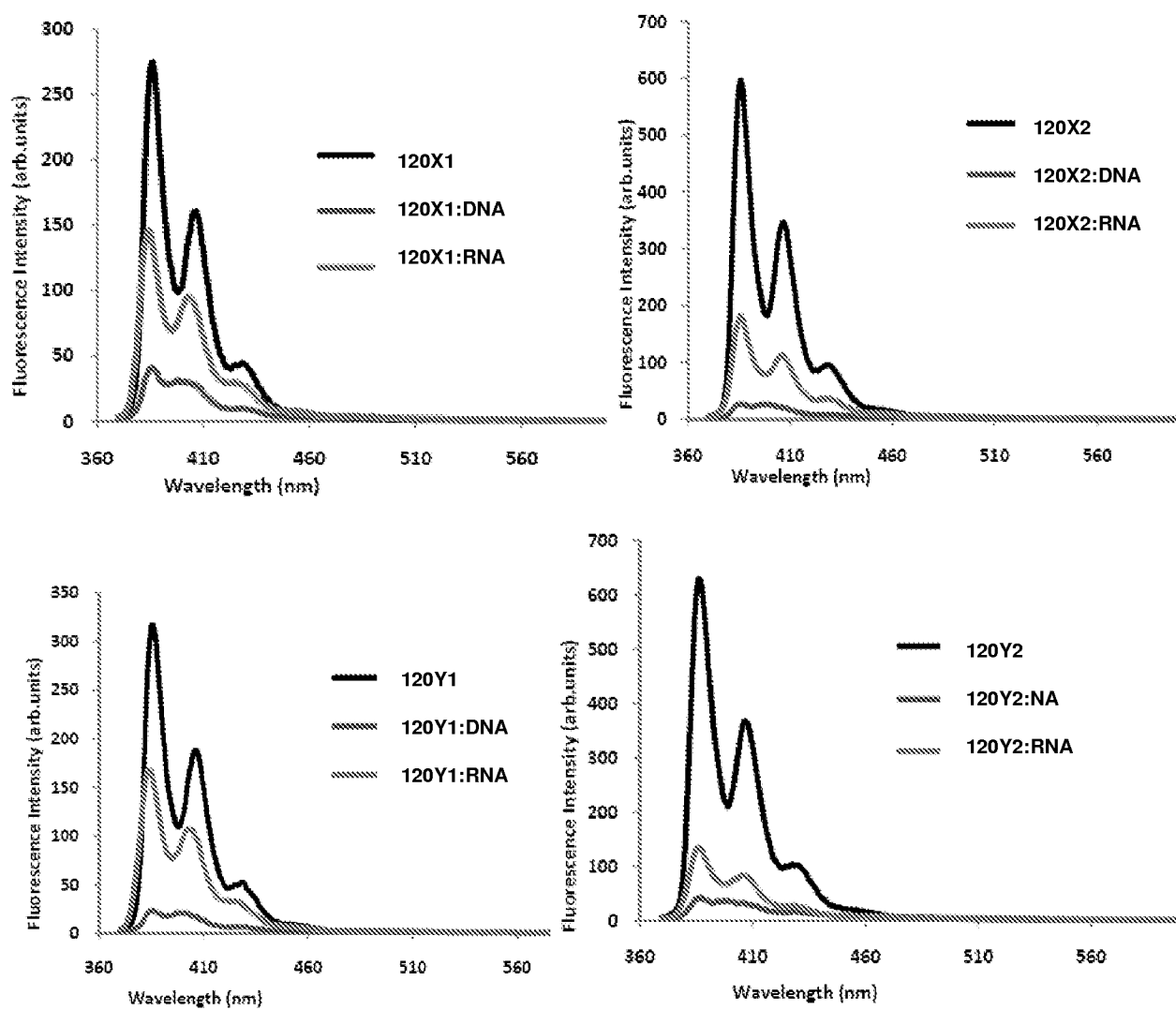


FIG. 10

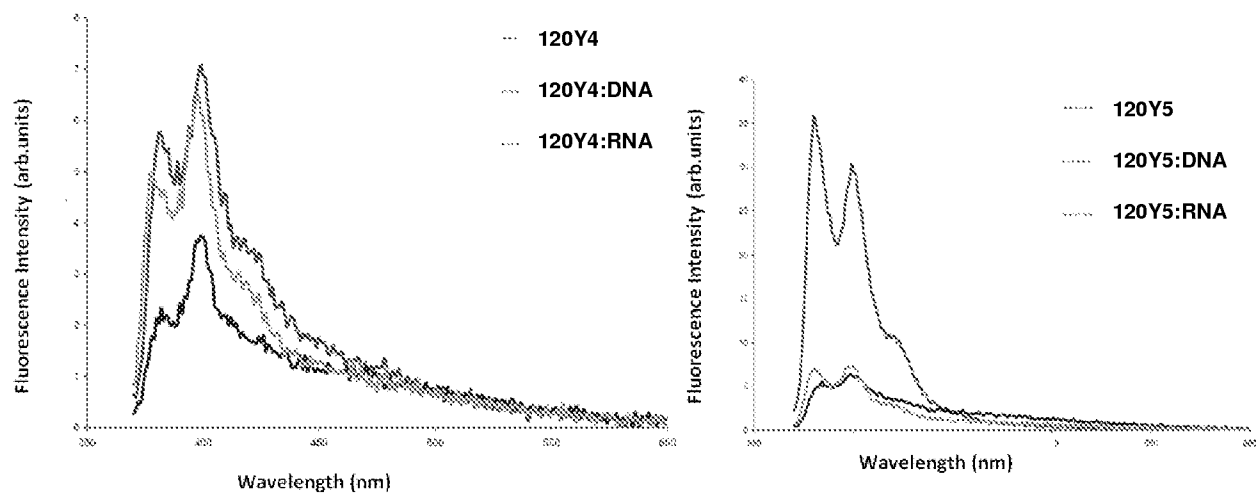


FIG. 11

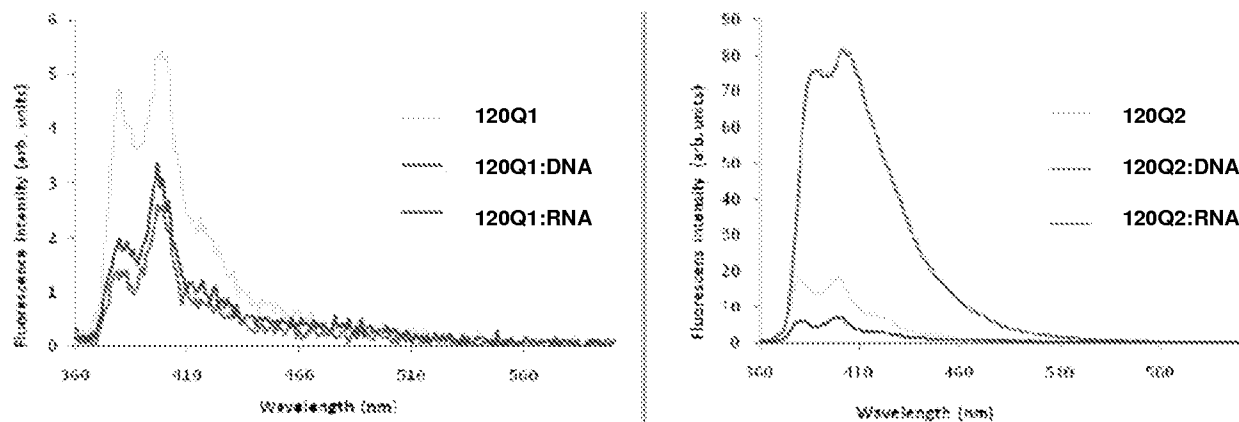


FIG. 12

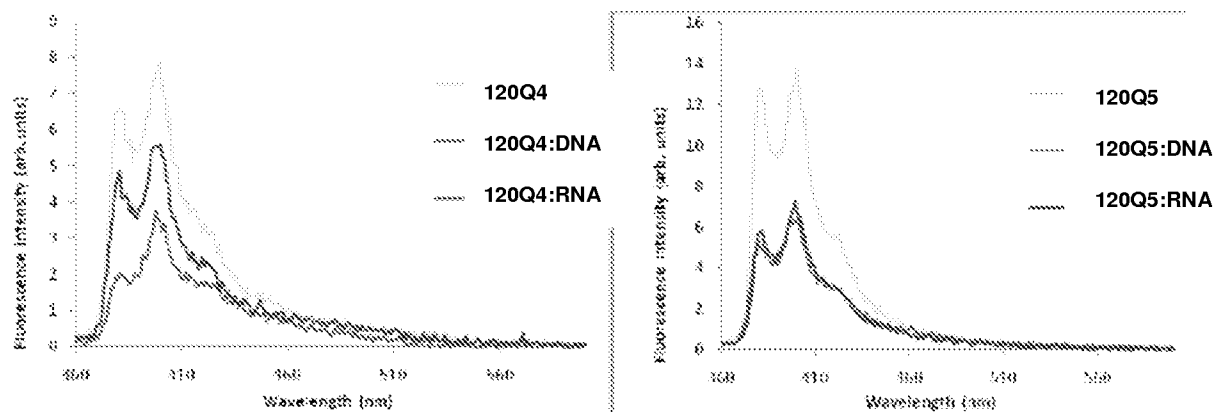


FIG. 13

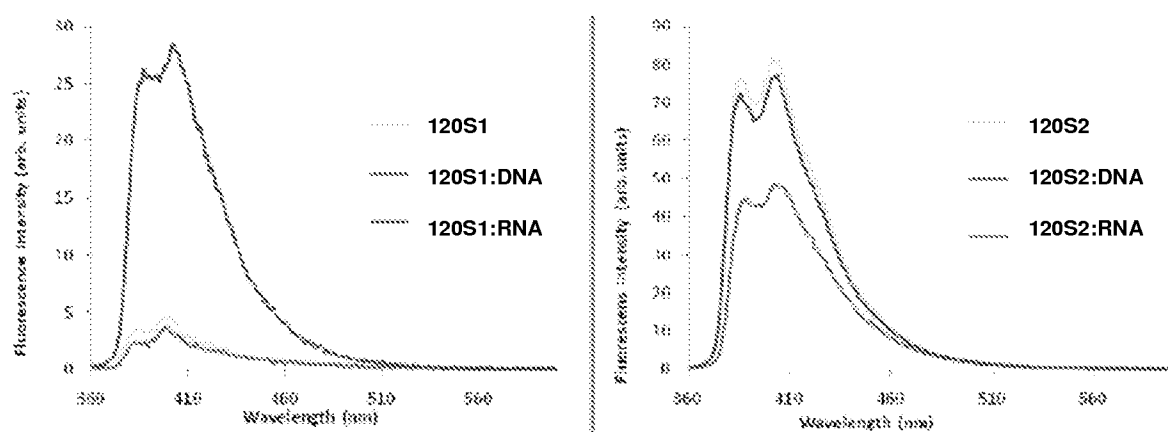


FIG. 14

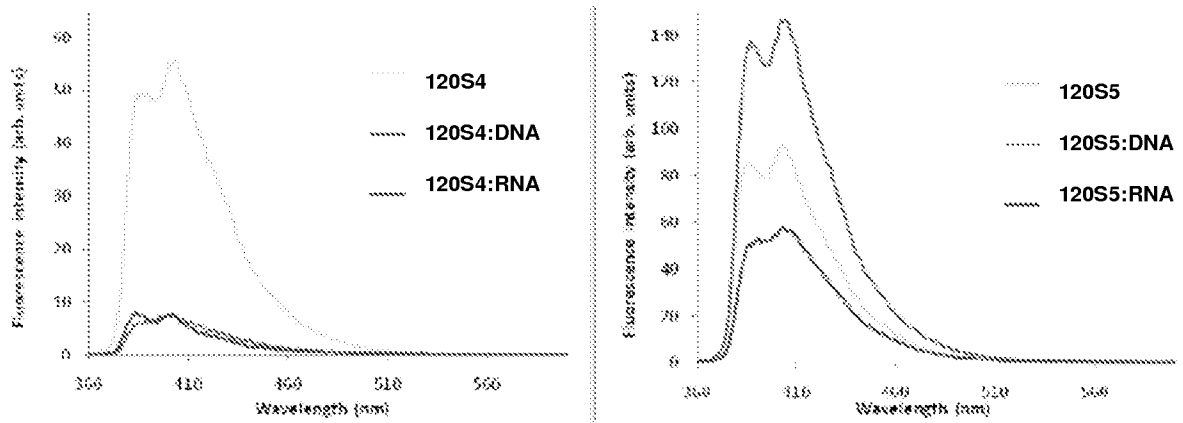


FIG. 15

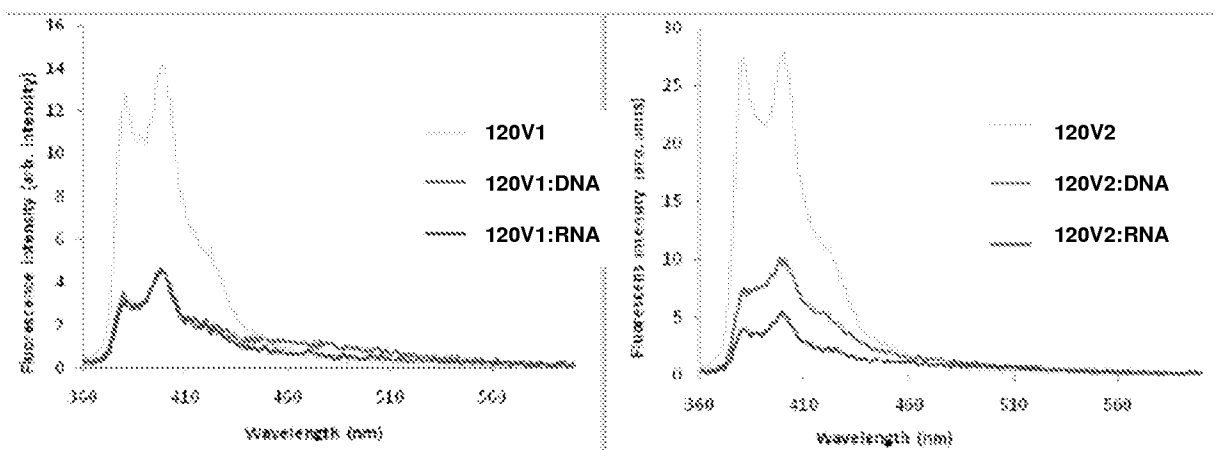


FIG. 16

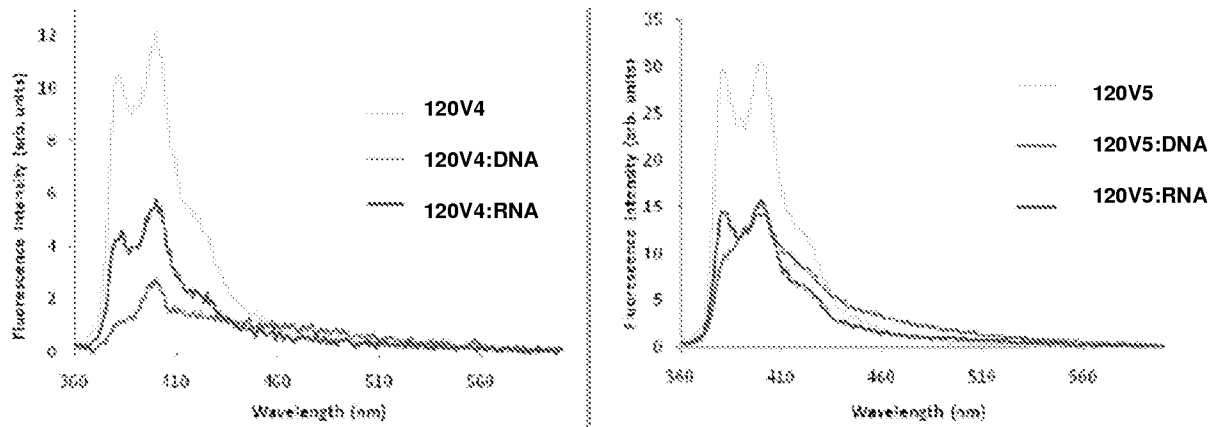


FIG. 17

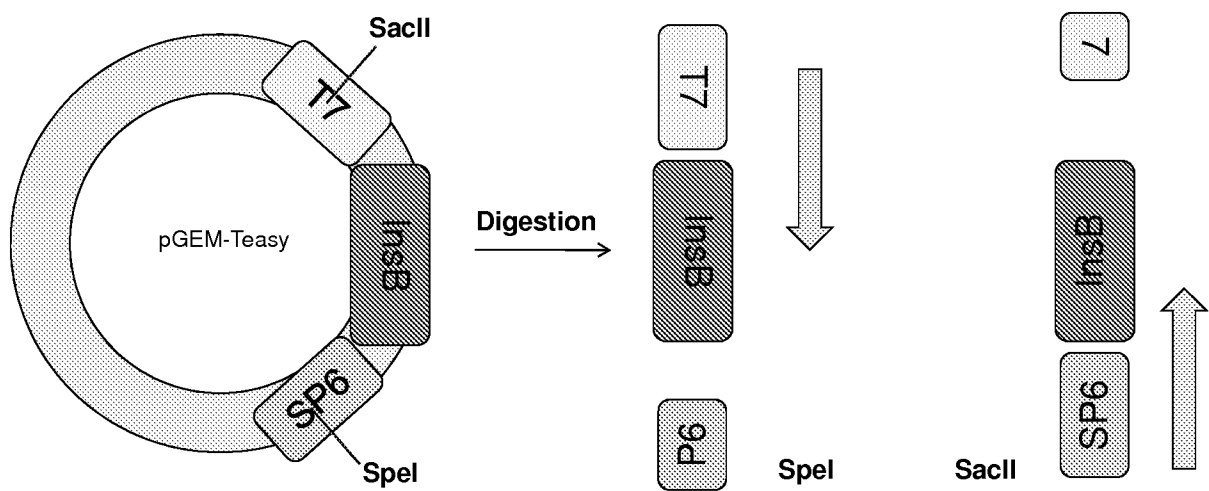


FIG. 18

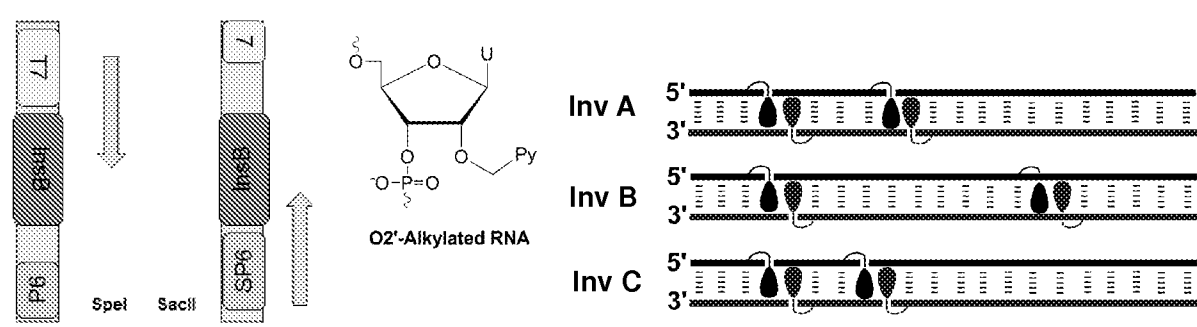


FIG. 19

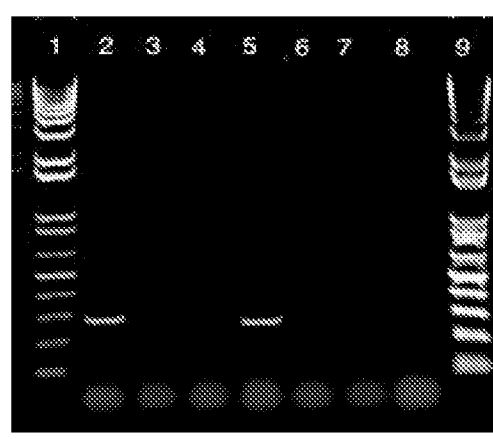


FIG. 20

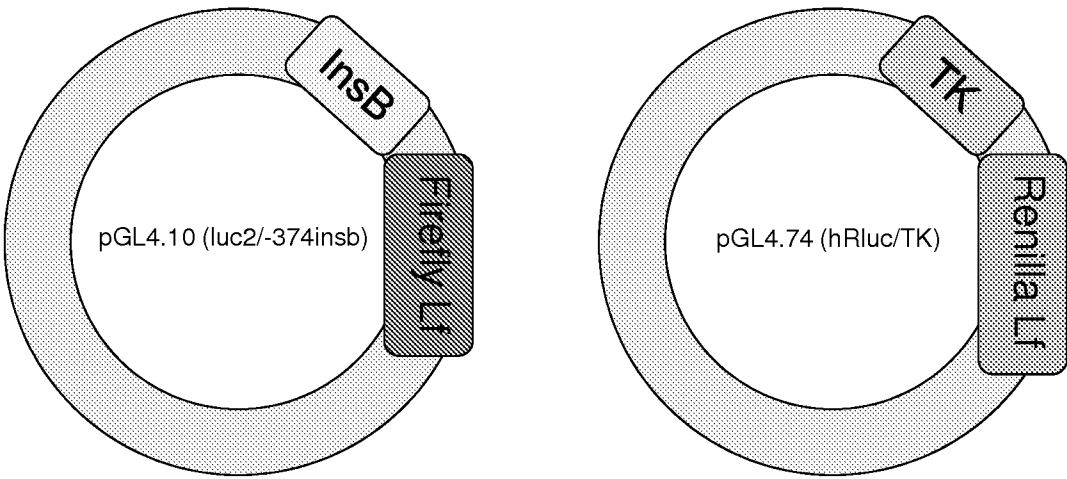


FIG. 21

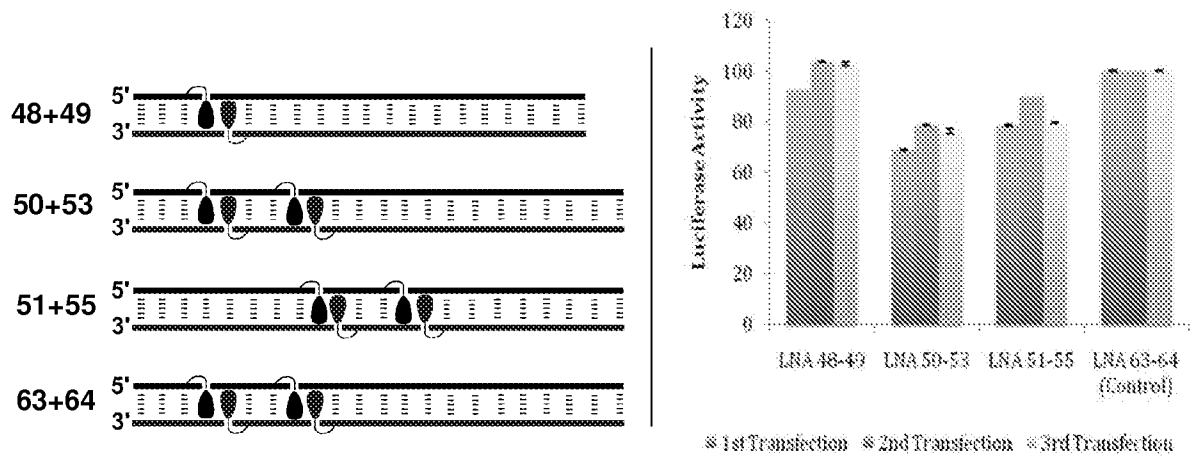


FIG. 22

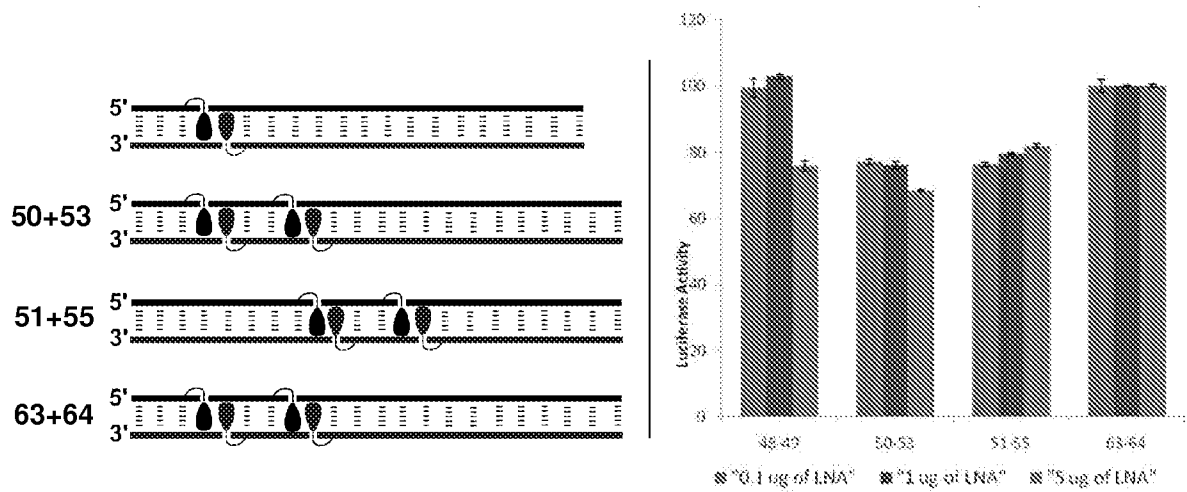


FIG. 23

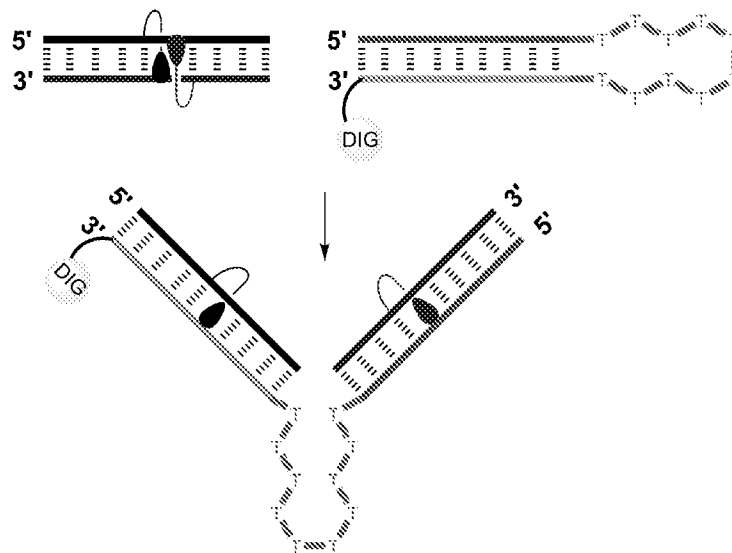


FIG. 24

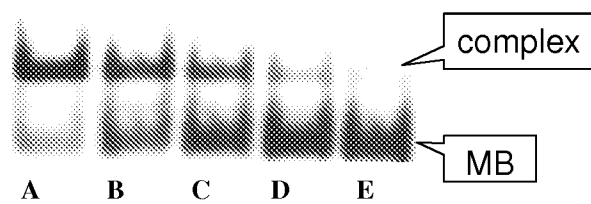


FIG. 25

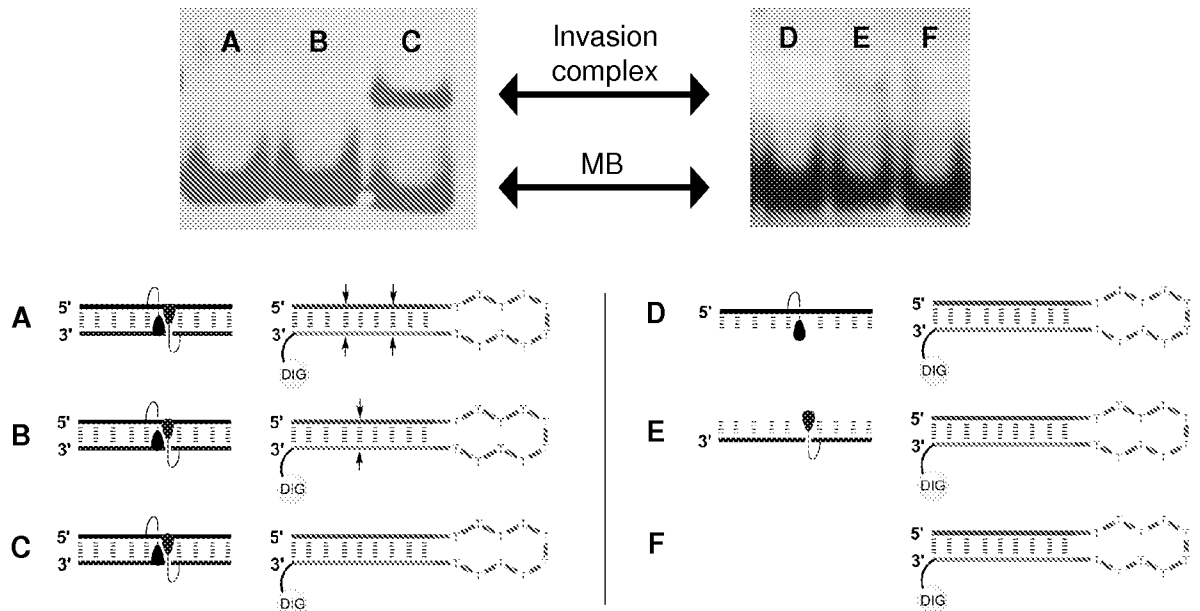


FIG. 26

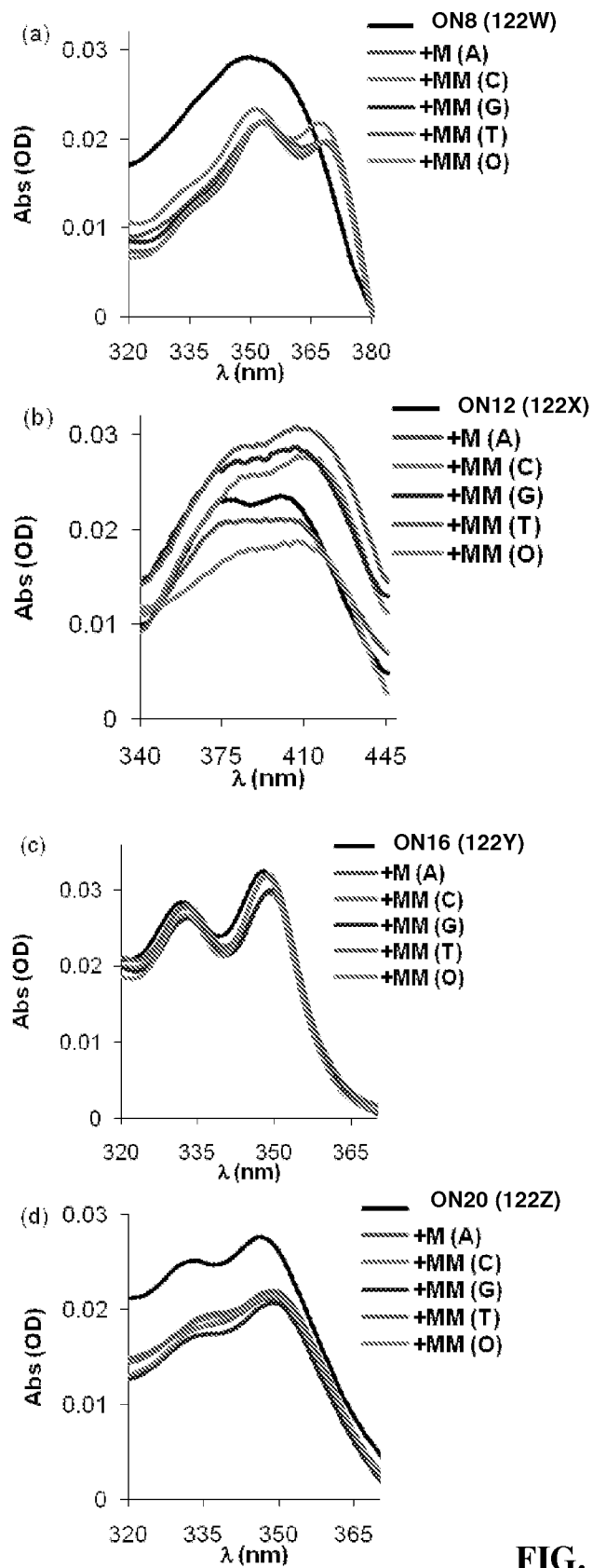


FIG. 27

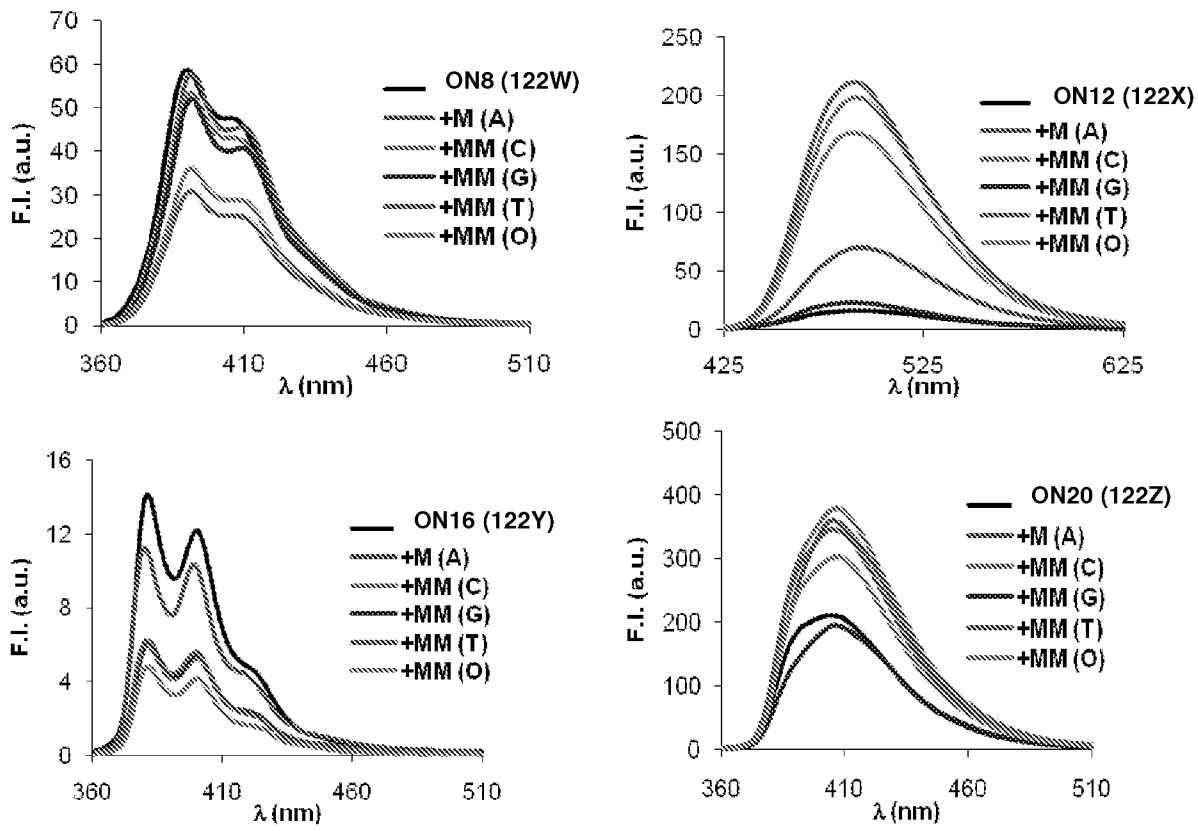
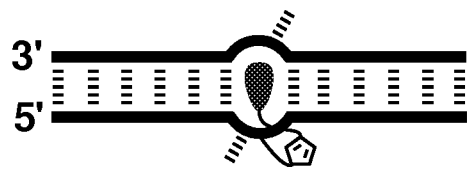


FIG. 28



universal hybridization
via base-flipping

FIG. 29

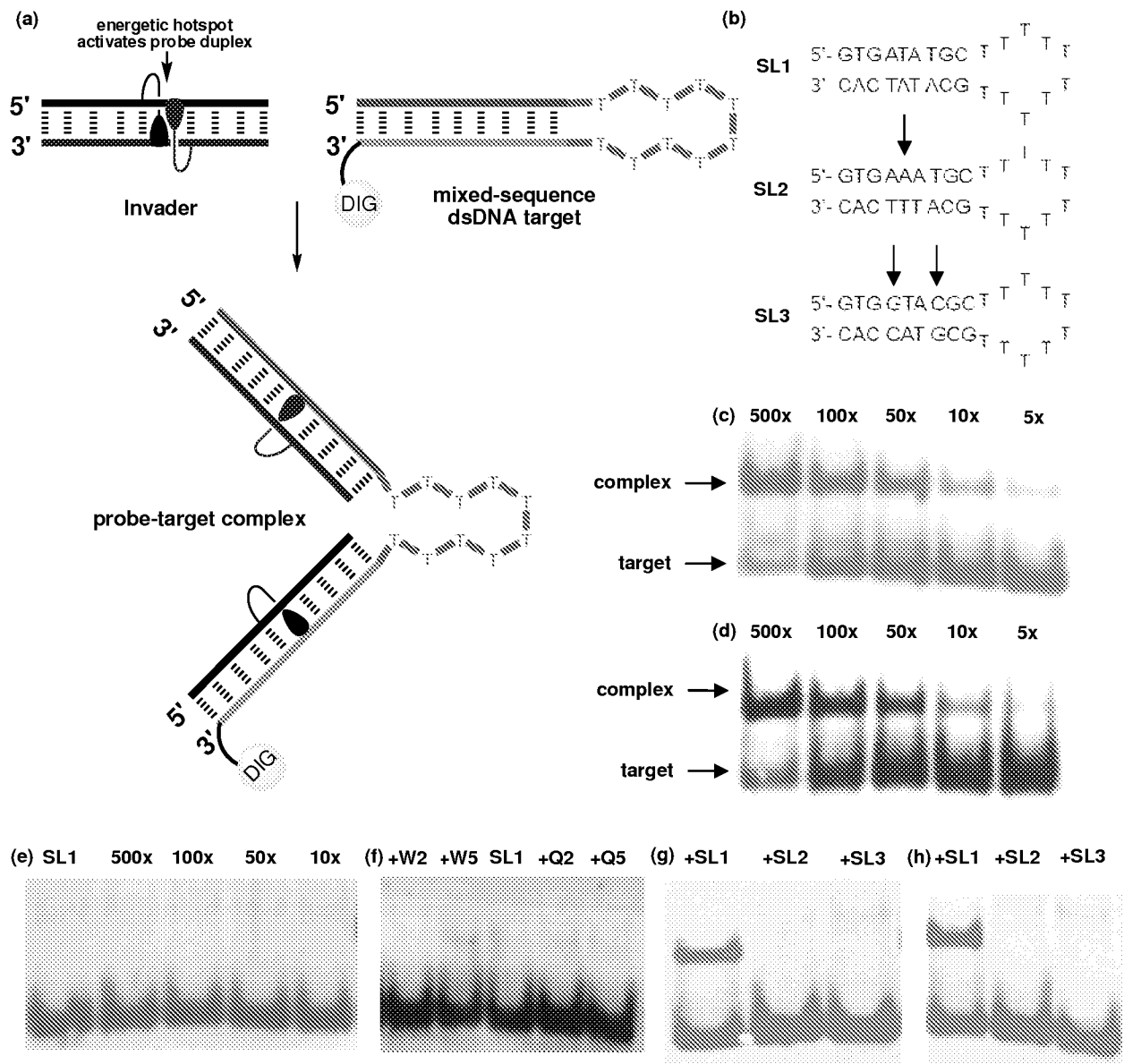


FIG. 30

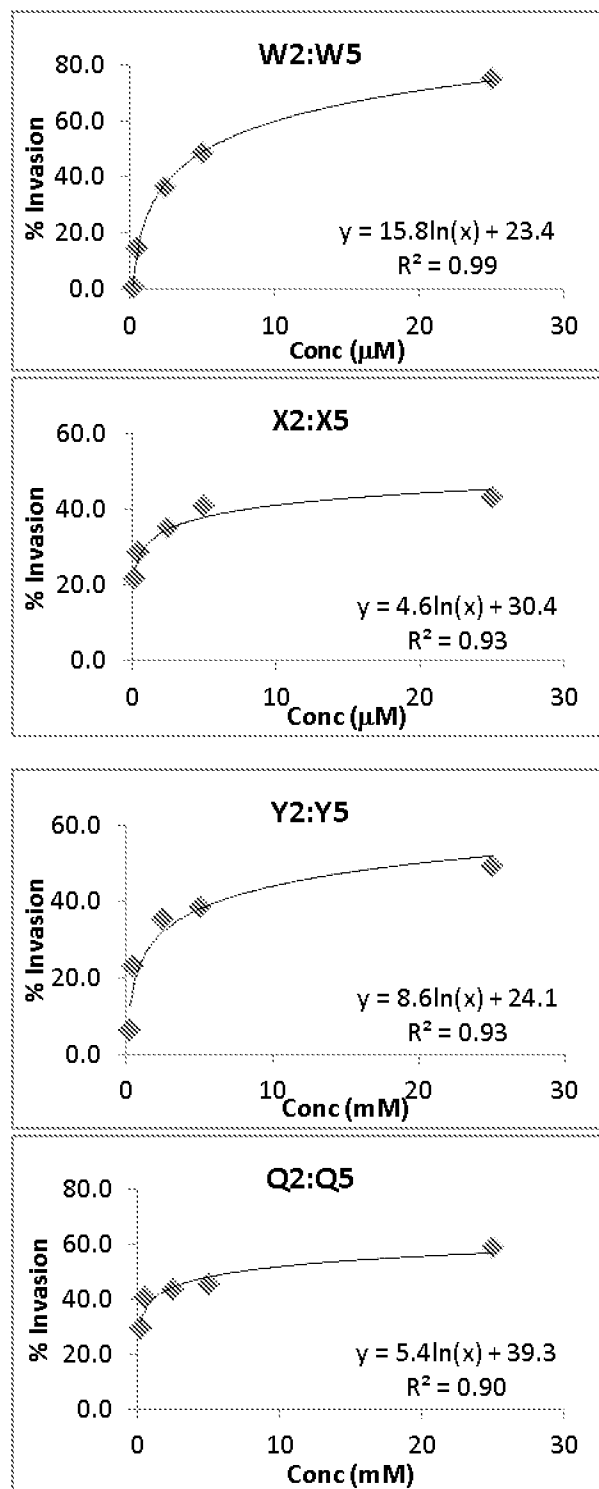


FIG. 31

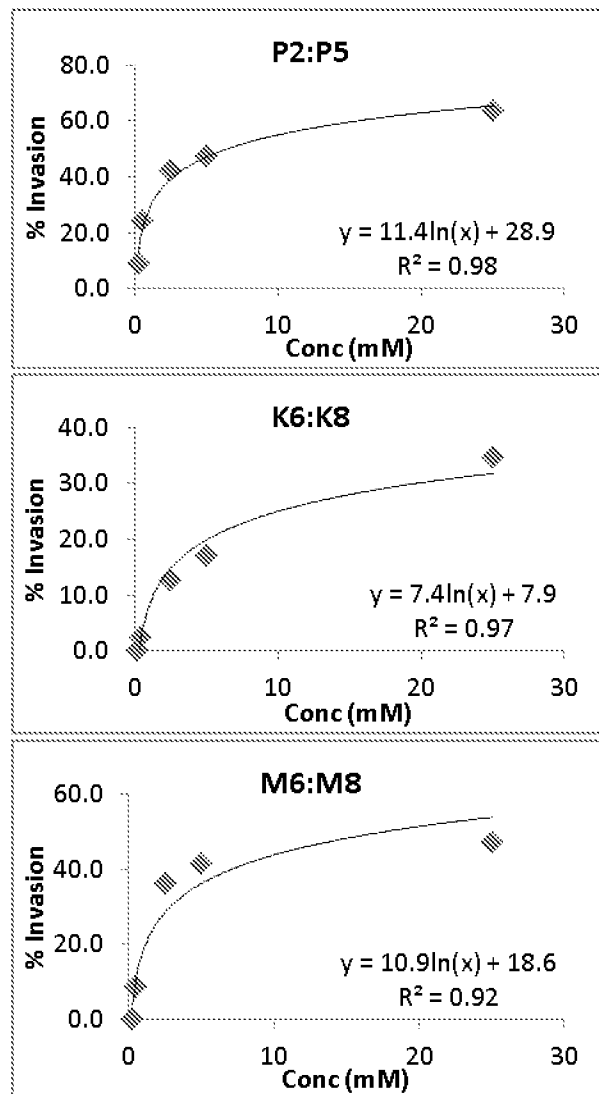


FIG. 31 (cont.)

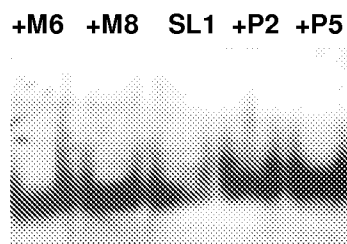


FIG. 32

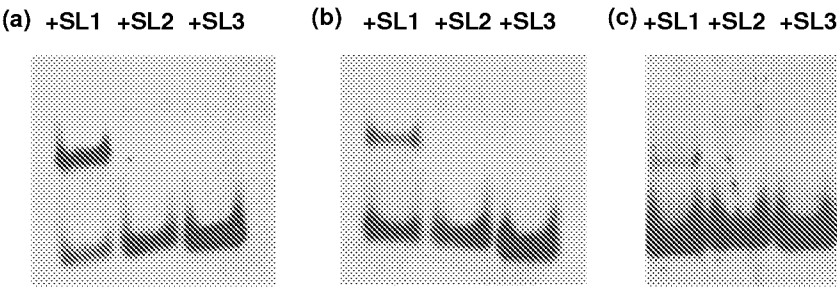


FIG. 33

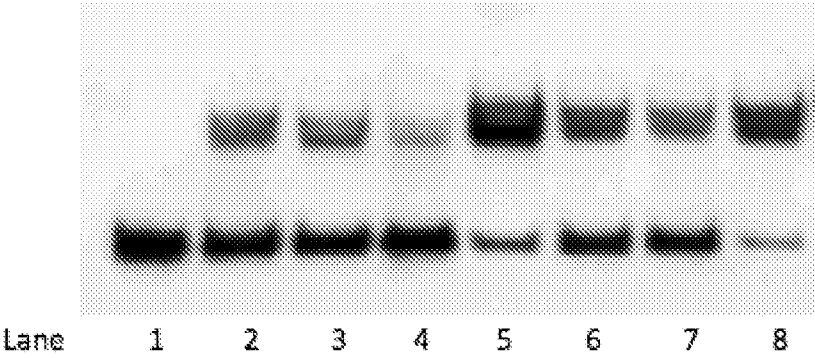


FIG. 34

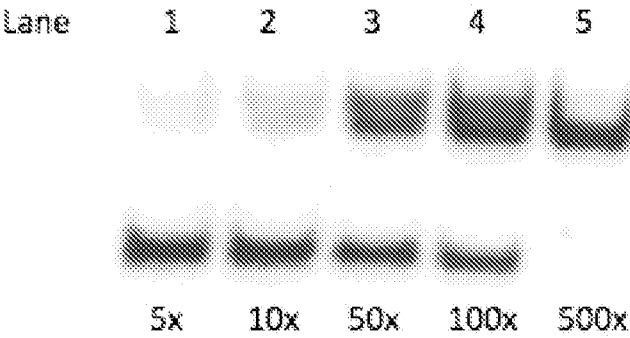


FIG. 35

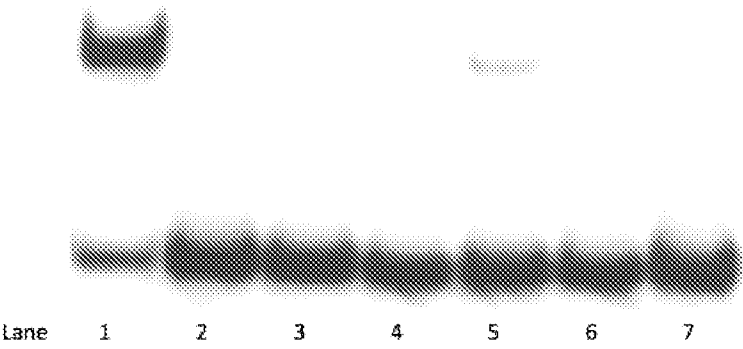


FIG. 36

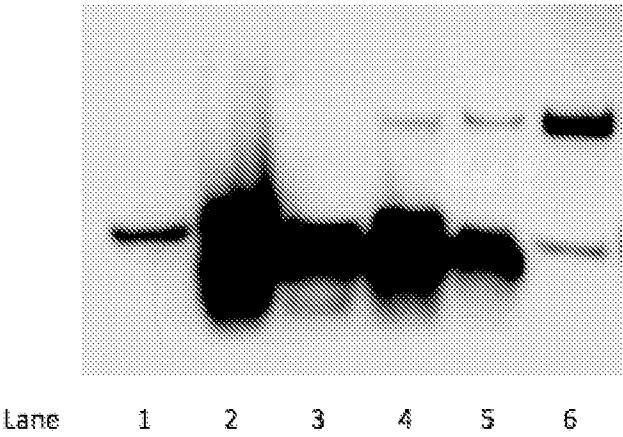


FIG. 37

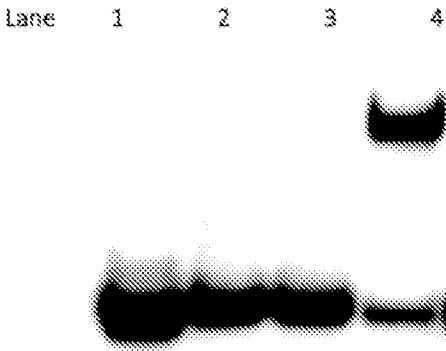


FIG. 38

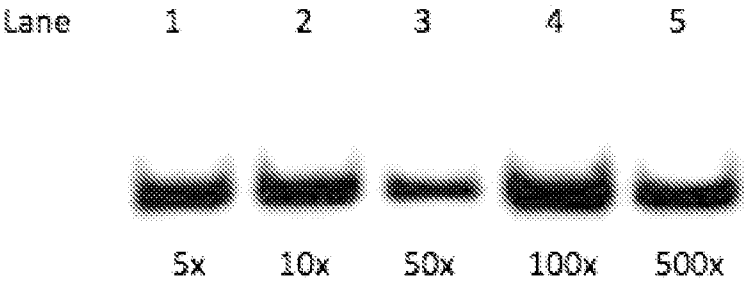


FIG. 39

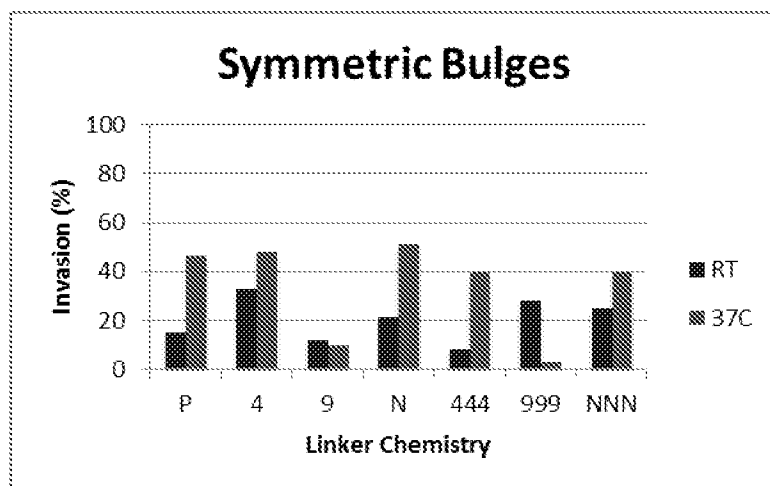


FIG. 40

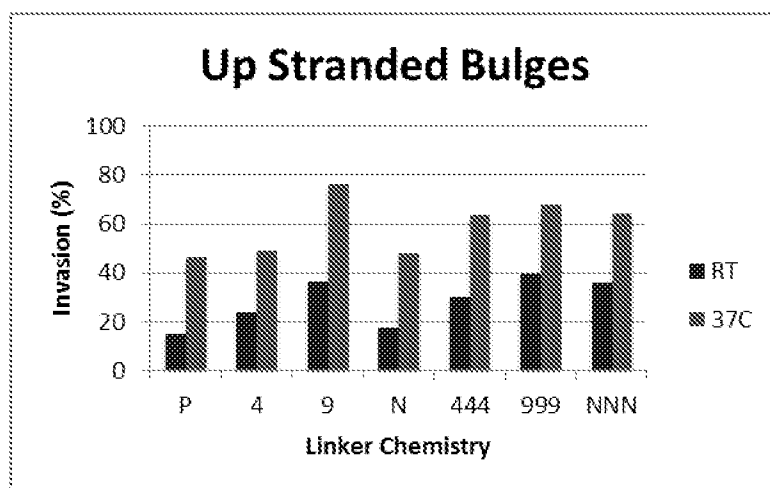


FIG. 41

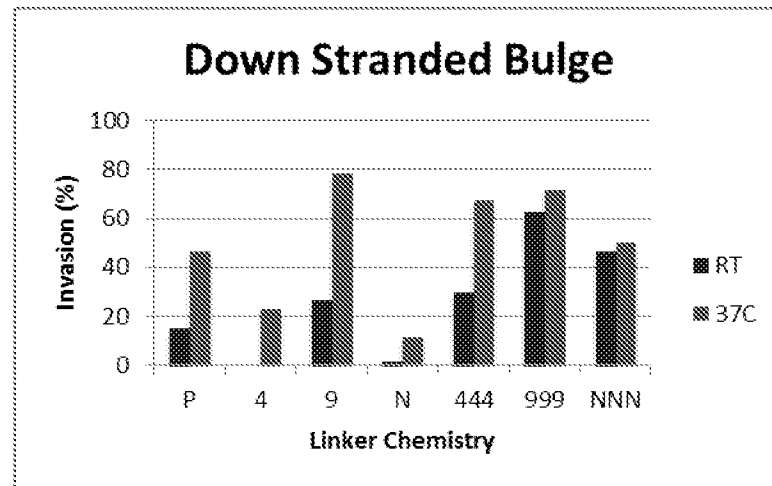


FIG. 42

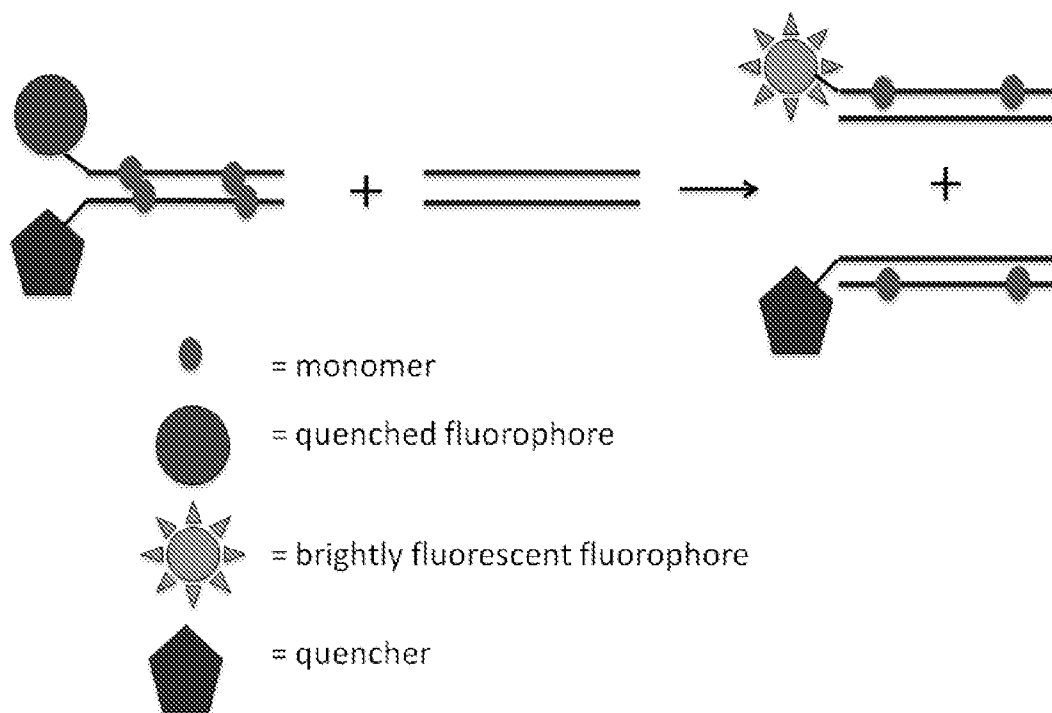


FIG. 43