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Fine Tuning of Pyrene Excimer Fluorescence in Molecular Beacons by Alteration of the Monomer Structure

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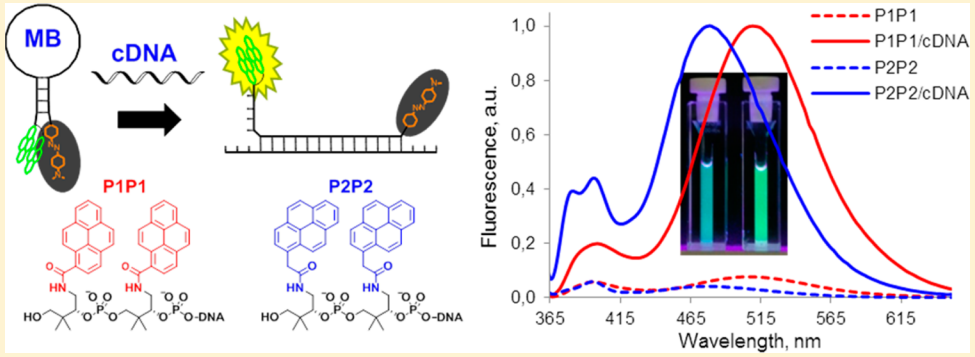
Supporting Information



ABSTRACT: Oligonucleotide probes labeled with pyrene pairs that form excimers have a number of applications in hybridization analysis of nucleic acids. A long excited state lifetime, large Stokes shift, and chemical stability make pyrene excimer an attractive fluorescent label. Here we report synthesis of chiral phosphoramidite building blocks based on (*R*)-4-amino-2,2-dimethylbutane-1,3-diol, easily available from an inexpensive D-(−)-pantolactone. 1-Pyreneacetamide, 1-pyrenecarboxamide, and DABCYL derivatives have been used in preparation of molecular beacon (MB) probes labeled with one or two pyrenes/quenchers. We observed significant difference in the excimer emission maxima (475−510 nm; Stokes shifts 125−160 nm or 7520−8960 cm^{-1}) and excimer/monomer ratio (from 0.5 to 5.9) in fluorescence spectra depending on the structure and position of monomers in the pyrene pair. The pyrene excimer formed by two rigid 1-pyrenecarboxamide residues showed the brightest emission. This is consistent with molecular dynamics data on excimer stability. Increase of the excimer fluorescence for MBs after hybridization with DNA was up to 24-fold.

INTRODUCTION

Molecular beacons (hairpins and related structured probes) based on pyrene excimer fluorescence (excimer molecular beacons, EMBs) are used in diverse analytical applications.[1] Excimer-forming pyrene pairs have some remarkable features making them valuable members of a panel of conventional fluorophores: large Stokes shift (>100 nm), long excited lifetime, temperature-independent emission, as well as considerable chemical stability.[2] Microenvironment, dynamics, and sequence of an oligonucleotide probe strongly influence the excimer brightness. Thus, rational design of multipyrene fluorophores for EMBs with enhanced brightness and increased excimer/monomer (E/M) ratio is a nontrivial issue. EMBs are usually labeled either with single pyrenes at 5′- and 3′-ends (Figure 1, I) or as a classic MB with excimer and quencher at the ends (Figure 1, IIa) of hairpin probe.[1a] The excimer fluorescence light-up occurs by inversion of E/M ratio or by overall fluorescence increase after the hairpin opening after capture of a target. Different interaction modes of pyrene residues with ssDNA vs dsDNA were used in quencher-free excimer hairpin probes (Figure 1, III,IV).[1a,i] Peculiar EMBs based on donor−acceptor complex formation between pyrenes and perylenediimides inside the stem (Figure 1, V,VI) were proposed by Häner et al.[1a,e,f] In spite of high E/M values for these probes, the introduction of chromophores only in the

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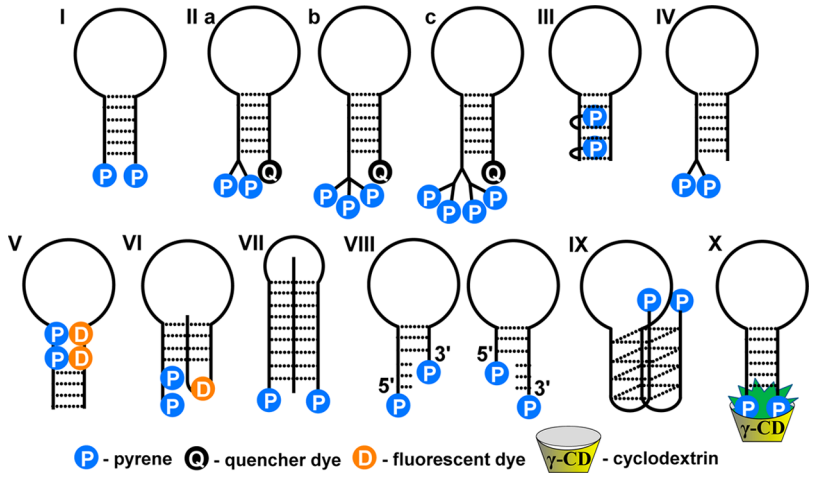

Figure 1. Pyrene excimer-forming molecular beacon probes.

middle of a probe limits their wide applicability. A straightforward way to improve E/M ratio relies on increasing of the number of pyrene moieties (Figure 1, IIb,c).[1a] However, high lipophility of multipyrene probes can result in nonspecific hydrophobic interactions, e.g. inside living cells. In most cases, E/M ratio for two-pyrene MBs is only 1.5−2, thus limiting wide use of pyrene EMBs. γ-Cyclodextrin can significantly amplify the E/M ratio by settling of a pyrene pair in the cavity (Figure 1, X).[1a,g,j] The excess of γ-cyclodextrin increases the sensitivity of hairpin probes when an excimer is formed during target recognition, however, this approach is applicable only for *in vitro* studies. EMBs were employed in homogeneous assays in complex biological fluids[1a] or for visualization in living cells.[1h] Time-resolved detection of EMB intracellular fluorescence in a time window of 60−110 ns allowed efficient decay of the strong green autofluorescence.[1a] A number of DNA aptasensors were developed based on excimer hairpin probes.[1a,l] Usually, the increase of fluorescence after EMB hybridization to a target was measured, and only few papers report evaluation of E/M ratios.[1a,k] However, the last parameter, reflecting specificity and sensitivity, is vital for most applications. Attachment of a pyrene pair to the 5′ end of MB probes is generally performed using flexible linkers.[1a]

The aim of our study was to develop and optimize a pyrene fluorophore capable of considerable light-up of excimer fluorescence within molecular beacon probes. In this study we suggested a chiral backbone, (*R*)-4-amino-2,2-dimethylbutane-1,3-diol (**2**), as a precursor of modifying reagents, and developed excimer molecular beacons with high E/M ratio and considerable fluorescence light-up upon hybridization.

RESULTS AND DISCUSSION

Synthesis of Monomers. Non-nucleoside surrogates[3] suitable for multiple incorporation into oligonucleotides usually contain a reactive group for functionalization and primary plus secondary (or two primary) hydroxyl groups on a backbone more or less mimicking nucleoside structure (Figure 2). Pyrene reagents were reported on a number of backbones: 1,2-diols,[4] 1-substituted 1,3-diols[5] (including chiral 2,4-dihydroxybutyramides[5b,d]), 2-substituted 1,3-diols[6] (including serinol[6b] and threoninol[6c−h]), 1,2-disubstituted 1,3-diols[7] (including pyrrolidine[7g] and tetrahydrofurans[6d,7a−f,h−l]), 1,4-diol (hydroxyprolinol),[8] 1,5-diols[9] (diethanolamine[9a,c] and 1,3-bis(hydroxymethyl)benzene[9b]), etc. 1,2-Diol reagents in 5′-terminal position usually suffer from partial cleavage during ammonia deprotection due to cyclophosphate formation. 1,3-Diols containing three carbons between hydroxyls are more suitable as nucleoside mimics. The presence of two primary hydroxyl groups in a backbone decreases the final yield of a reagent due to poor selectivity of tritylation. Racemic or prochiral (e.g., serinol) synthons are inconvenient in phosphoramidite DNA synthesis because they lead to diastereomeric oligodeoxynucleotides, thus complicating HPLC purification due to broadening or doubling of peaks.

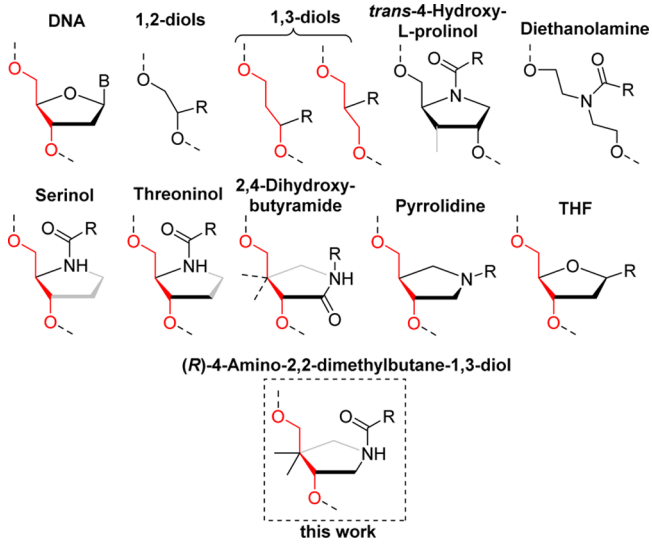

Figure 2. DNA (B = nucleobase) and some deoxyribose-mimicking backbones in pyrene-modified reagents (R = pyrene containing unit). 1,3-Diols (marked in red) maintain three-carbon distance of natural nucleosides.

Here we developed a chiral 1,3-diol backbone, (*R*)-4-amino-2,2-dimethylbutane-1,3-diol (**2**), easily available from an inexpensive precursor and convertible to various modifying reagents by robust and scalable procedures (Scheme 1). The key aminodiol **2**, previously described only in the form of protected derivatives,[10] was synthesized using two-step procedure from commercially available D-(−)-pantolactone with 75% overall yield in multigram scale. (*R*)-2,4-Dihydroxy-3,3-dimethylbutanamide (**1**)[11] was reduced with borane-dimethyl sulfide complex (BMS) in THF followed by sorption

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Scheme 1. Synthesis of Aminodiol 2, Pyrene, and DABCYL Reagents and Incorporation of Modifications into Oligonucleotides

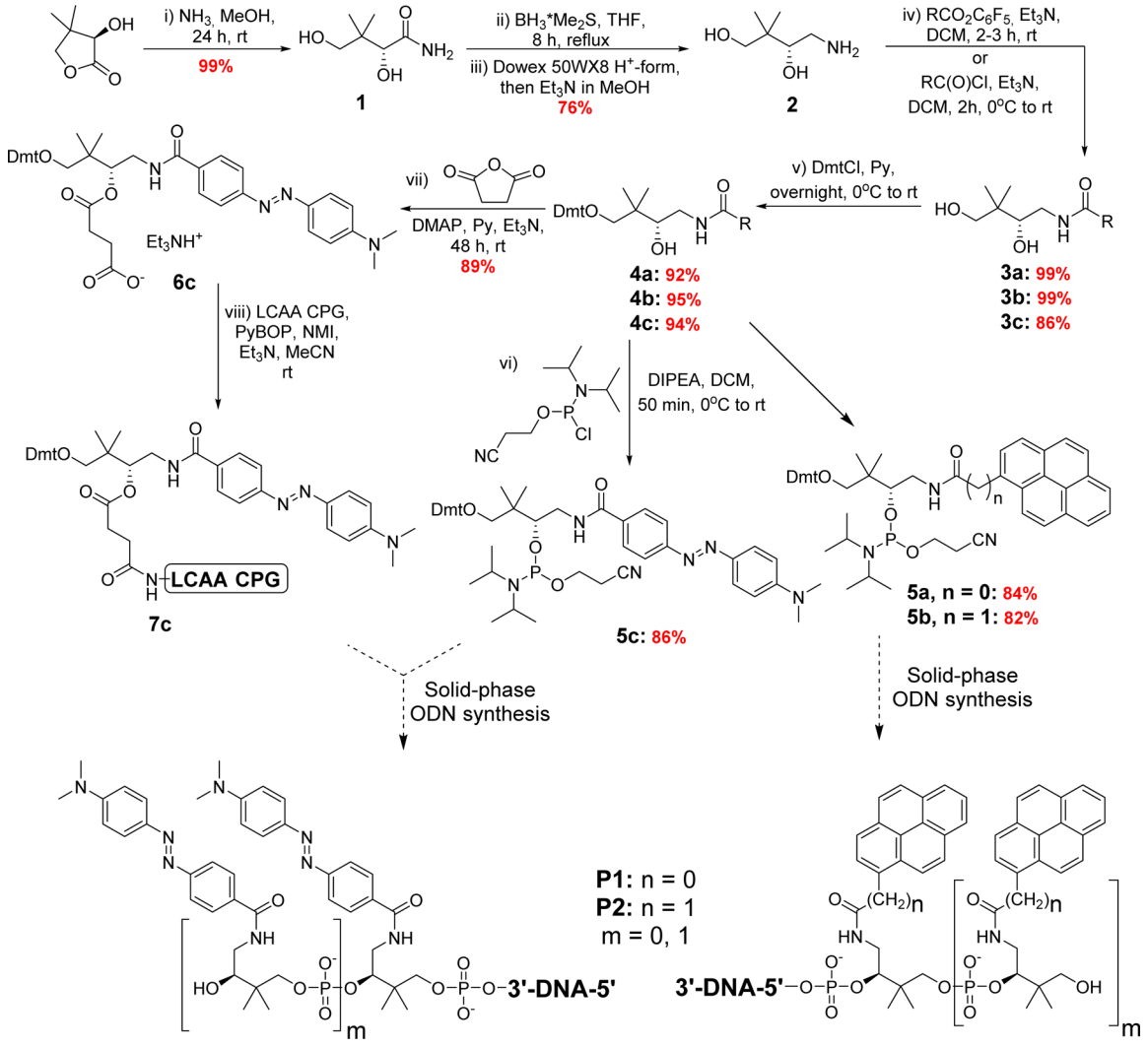

Table 1. Sequences of EMBs (Seq1 and Seq2) and Complementary DNA Target (Seq3)[a]

Seq1:	5′-F-**GCGGGGT**CATTCGAAACGCATTCATT**ACCCCGC**-Q-3′											
Probe	**MB1**	**MB2**	**MB3**	**MB4**	**MB5**	**MB6**	**MB7**	**MB8**	**MB9**	**MB10**	**MB11**	**MB12**
5′-F	**P1**	**P1P1**	**P2P1**	**P2**	**P1P2**	**P2P2**	**P1**	**P1P1**	**P2P1**	**P2**	**P1P2**	**P2P2**
3′-Q	Dabcyl						Double Dabcyl					
Seq2:	5′-F-**CCGGGGC**TCATTCGAAACGCATTCATTA**CCCCGG**-Q-3′											
Probe	**MB13**	**MB14**	**MB15**	**MB16**	**MB17**	**MB18**	**MB19**	**MB20**	**MB21**	**MB22**	**MB23**	**MB24**
5′-F	**P1**	**P1P1**	**P2P1**	**P2**	**P1P2**	**P2P2**	**P1**	**P1P1**	**P2P1**	**P2**	**P1P2**	**P2P2**
3′-Q	Dabcyl						Double Dabcyl					
Seq3:	5′-TTGAGCGGGGTAATGAATGCGTTTCGAATGAGGGAG-3′											

[a]Complementary parts in the target (**Seq3**) and MBs (**Seq1**, **Seq2**) are underlined. Stem self-complementary sequences in MBs are marked in bold. For structure of fluorophore (F) and quencher (Q) see Scheme 1 and Figure S1.

on an ion-exchange resin, wash out of admixtures and selective product elution with triethylamine in ethanol. This straightforward procedure can be extended to the synthesis and isolation of other amino polyols without additional protection.

Amine **2** was acylated with carboxylic acid derivatives to amides **3a**–**c** followed by selective dimethoxytritylation of the primary hydroxyl and phosphitylation of the secondary one (Scheme 1). Three *O*-Dmt-protected phosphoramidite precursors, bearing residues of pyrenecarbocylic acid (**4a**), pyreneacetic acid (**4b**), or 4-[(4-dimethylaminophenyl)azo]-benzoic acid (DABCYL) (**4c**), have been converted into phosphoramidites **5a**–**c** and DABCYL CPG solid support **7c**. Since two methyl groups shield the secondary hydroxyl, thus making it less reactive, the synthesis of phosphoramidites from

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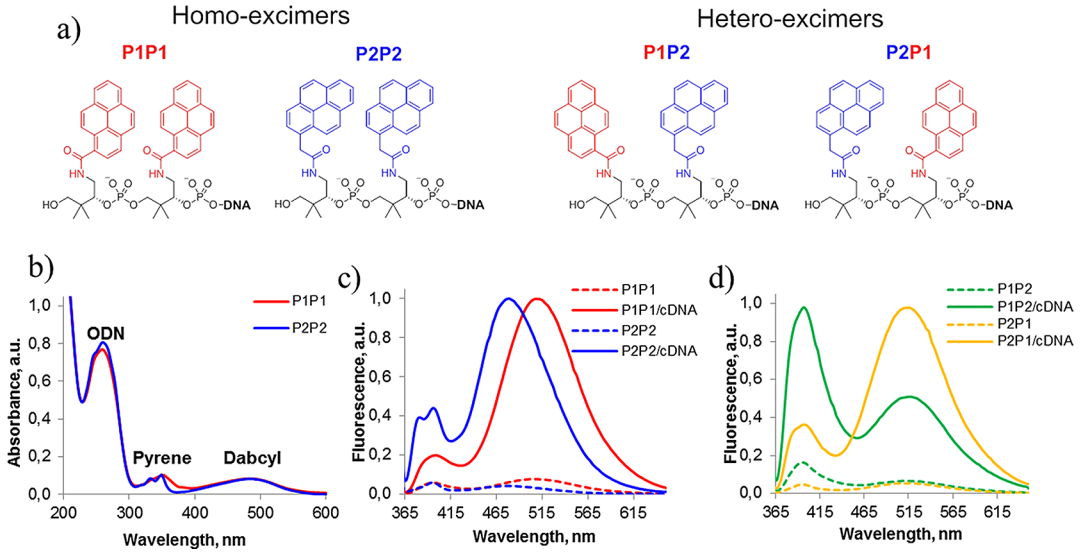

Figure 3. (a) Structures of four excimer-forming pyrene pairs; (b) absorbance spectra of typical hybrids **MB20** and **MB24** labeled with 5′-**P1P1**, 5′-**P2P2** excimer pairs, respectively, and 3′-DABCYL; (c) normalized fluorescence spectra (λ_{ex} 350 nm) of hairpins labeled with an excimer (dashed lines) and their duplexes with **Seq3** (solid line): **MB20** (**P1P1**) – red line, **MB24** (**P2P2**) – blue line, **MB21** (**P2P1**) – yellow line, **MB23** (**P1P2**) – green line.

corresponding precursors **4** was carried out with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite/DIPEA in DCM instead of common 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite. Acylation of **4c** with succinic anhydride was performed in dry pyridine using DMAP catalysis followed by attachment of the hemiester **6c** to the solid support, resulting in **7c** (Scheme 1).

MBs Design, Synthesis, and Spectral Properties. The modifying reagents **5a–c** and **7c** were used in automated oligonucleotide synthesis. A number of MBs labeled with single pyrene or with various combinations of pyrene pairs using 1-pyreneacetamide (**P1**) and 1-pyrenecarboxamide (**P2**) derivatives have been synthesized (Table 1). DABCYL CPG support **7c** and amidite **5c** were used in solid phase synthesis for attachment of single or double quenchers at 3′ end of MBs (see Table S1 and Figure S1) because the improved efficiency of multiple quenchers in fluorogenic DNA probes has been reported earlier.[12]

We used two MBs sequences (**Seq1**,**2**) that demonstrated maximal light-up of fluorescence after DNA hybridization in our previous studies (Table 1).[12e] The **Seq1** has a 7-bp stem with 5′-dG/3′-dC and **Seq2** has 6-bp stem with 5′-dC/3′-dG. Therefore, 5′-pyrene (pyrene pair) quenching by an adjacent/opposite neighbor guanine can be accurately evaluated. 5′-Stem sequences of MBs are not complementary to the target, thus preventing undesirable pyrene interaction with an overhanging target strand after hybridization. Indeed, forced interactions of a pyrene pair with DNA may result in fluorescence quenching and/or alteration of E/M ratio.[1d,g] A 36-mer ssDNA target sequence **Seq3** corresponds to the 255–290 region (noncoding strand) of translation elongation factor 1α gene from *F. avenaceum* (GeneBank accession no. JF278604). We retained several additional nucleotides at both ends of MB-capturing sequence in the target to simulate MBs interactions with a long ssDNA.

An anticipated fluorescence increase upon hybridization to the target was observed for all molecular beacons (**MB1–MB24**) labeled with one or two pyrenes (see ESI Figures S74–S97). Unexpectedly, the introduction of the second DABCYL quencher into MBs demonstrated neither reduced initial excimer fluorescence nor improved signal-to-background ratios (SBR) (Figures S74–S97). This contradicts the reported cases of positive influence of an additional quencher on MBs' SBR with pyrene and xantene dyes.[12] The doubled DABCYL quencher reduced monomer fluorescence in hairpins while excimer fluorescence remained the same or in some cases even higher than for single DABCYL quencher. This could be a result of tight interaction between single DABCYL moiety and pyrene pair that does not allow interactions with the second quencher that seems to be not in close proximity, probably due to the steric hindrance. Even so, we found out that the second quencher stabilizes the stem since MBs' hairpins enhanced with couple quenchers are denatured at several degrees higher temperature (Table S2). Apparently, the contact quenching mode prevails over the FRET-mediated one in the case of polyaromatic pyrene chromophores in stem-loop MBs. Therefore, we focused on further studies of mono DABCYL labeled MBs.

In the case of **P1** or **P1P1** labeled MBs, we observed smooth shaped absorbance and emission spectra (e.g., Figure 3a,c, red traces). In contrast, MBs with **P2** or **P2P2** showed vibronic patterns both in absorbance and emission spectra (e.g., Figure 3a,c, blue traces, for monomer emission). It should be noted that the localization of the spatial distributions of the frontier molecular orbitals in pyrene, as well as the lowest electronic excitation energy (the HOMO–LUMO energy gap), is strongly determined by the nature of substituents.[2i] This property can also influence the stability of excimers and the wavelength of excimer emission. Moreover, the observed fluorescence spectrum for a pyrene pair in a complex biomolecular system is usually a superposition of emissions from several monomeric and excimeric structures.

Hybridization of EMBs to the DNA target results in a strong light-up of excimer fluorescence (up to 24-fold). Remarkably, excimer fluorescence of **P1P1** pair is significantly red-shifted compared to **P2P2** one (505 nm vs 475 nm; Figure 3c). Hetero excimers from **P2P1** or **P1P2** pairs display additional bathochromic shift (to 510 nm; Figure 3c). Thus, wavelength

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Table 2. Fluorogenic Propertiers of EMBs upon Hybridization with DNA Seq 3[a]

	molecular beacons	5′-pyrene pair	excimer λ_{max}, nm	SBR[b] mono	SBR[b] exc	E/M ratio[c]	average distance, Å[d]	average overlapping[d]
Seq1	**MB2**	**P1P1**	505	3.2	4.6	5.9	7.1 ± 2.3	9.6
	MB3	**P2P1**	510	3.1	2.1	3.5	9.5 ± 3.6	5.7
	MB5	**P1P2**	510	5.6	4.6	1.0	10.9 ± 2.7	5.7
	MB6	**P2P2**	475	19.5	4.4	1.5	10.9 ± 2.9	5.0
Seq2	**MB14**	**P1P1**	505	2.2	23.1	5.4	6.2 ± 2.2	10.9
	MB15	**P2P1**	510	5.3	16.1	2.6	9.3 ± 3.1	9.2
	MB17	**P1P2**	510	3.8	3.4	0.5	9.1 ± 3.1	7.7
	MB18	**P2P2**	475	2.8	22.1	2.5	10.2 ± 3.6	3.1

[a]EMBs with 5′-couple pyrenes and single 3′-DABCYL. [b]SBR (signal-to-background ratios) were calculated as I_{flMBi}/I_{0MBi}, where I_{0MBi} – remaining fluorescence of self-quenched MB_i, I_{flMBi} – fluorescence of the duplex formed by MB_i with DNA target (**Seq3**) (see Figures S74–S97). [c]Relation of $I_{flMB(Exc)}/I_{flMBi(Pyr)}$ values for hybrids of EMBi with **Seq3**. [d]Calculated from molecular dynamic data set.

of fluorescent maxima from 475 to 510 nm can be rationally tuned by variation of excimer composition with **P1** and **P2** monomers. The feature is helpful for adjusting excimer fluorescence to the particular wavelength channel of a detection device, e.g., a qPCR-machine, or to a specific FRET acceptor dye, especially in case of multi probe analysis.

For most probes, we have observed efficient excimer formation. However, in the case of **P1P2** pair, the monomer fluorescence was predominant. The most stable excited complex is formed in **P1P1** labeled probes, according to maximal E/M fluorescence ratio (Table 2). **P1P1** labeled EMBs demonstrated also the brightest excimer fluorescence after hybridization to the target (Figures S74–S97). We compared background fluorescence, increase of fluorescence intensity (Figure 3), and SBR (Table 2) for **Seq1** and **Seq2**-based EMBs. The resulting fluorescence intensities and E/M ratios are similar for each particular pyrene pair composition (e.g., for **P2P1** in **MB3** and **MB15**) within EMB/target hybrids. The observed background excimer fluorescence of MBs with **Seq1** was higher in comparison to these of **Seq2** (Figure 4), probably

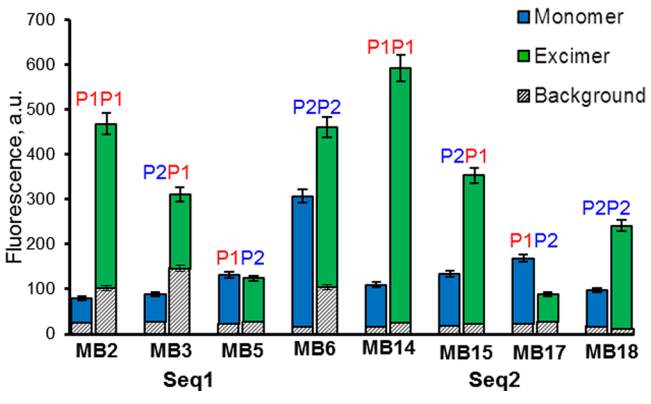

Figure 4. Monomer and excimer fluorescence of hairpin EMBs (I_{0MBi}, dashed bars). Increase of emission intensity (I_{flMBi}–I_{0MBi}) for pyrene monomer (blue bars) and excimer (green bars) fluorescence upon EMBs hybridization with a **Seq3**.

due to short-range interactions of neighbor pyrene with terminal GC pair in stem. Guanine in opposite chain appeared to be a more efficient coquencher vs adjacent guanine in the same chain. This is consistent with a reported case of quenching of single pyrene chromophore on a hairpin stem with adjacent/opposite neighbor dG.[13]

Using molecular dynamics, we have calculated the distribution of probability of two pyrenes overlapping if they are attached to 5′-end of 6-mer oligonucleotide. A number of states with stacked pyrenes for **P1P1** in **MB2** and in **MB14** (Figure 5,

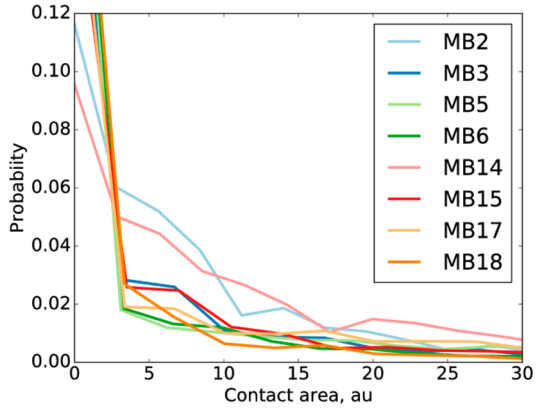

Figure 5. Probability distribution of pyrenes overlapping in molecular beacons. Contact area calculated in contacts of atoms for opposite pyrene residues. Overlapping of pyrene monomers was considered only if distances between centers of pyrene were less or equal to 4.0 Å.

light blue and rose lines) are significantly higher than for probes with **P1P2**, **P2P1**, and **P2P2** 5′-pyrene pairs (Figures S2–S9). Thus, 5′-conjugated **P1P1** pair with highest E/M ratios revealed superior stacking interactions and an average overall surface between two pyrenes that confirm our experimental data (Table 2).

CONCLUSION

In summary, we developed a chiral three-carbon backbone for introduction of modifications into oligonucleotides. The key block, (*R*)-4-amino-2,2-dimethylbutane-1,3-diol, is easily available from natural D-(−)-pantolactone and can be modified with various small molecules. We synthesized DABCYL, 1-pyrenecarboxamide, and 1-pyreneacetyl reagents and used them for the synthesis of pyrene excimer molecular beacons. The resulting hairpin probes with different compositions of pyrene pairs showed a 40 nm range for excimer fluorescence maxima (from 475 to 510 nm), high excimer/monomer emission ratio, and considerable excimer fluorescence light-up upon hybridization to complementary DNA target. These valuable features pave the way for a design of excimer molecular

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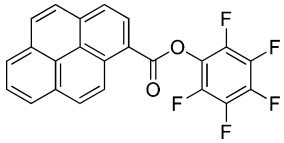
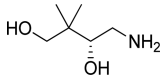
beacons with a tuned fluorescence, e.g., acting as donor in various FRET applications.

EXPERIMENTAL SECTION

General. All solvents and reagents were purchased from commercial suppliers and used as received, unless otherwise stated. Pentafluorophenyl 1-pyreneacetate[14] and DABCYL chloride[15] were prepared as described. The assignments of signals in ^{1}H and ^{13}C NMR spectra were done using 2D ^{1}H−^{1}H COSY, ^{1}H−^{1}H NOESY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC experiments (Figures S98−S100). High-resolution mass spectra (HRMS) were registered using electrospray ionization (ESI) using quadrupole mass analyzer. Analytical thin-layer chromatography was performed on silica gel precoated aluminum plates. Silica gel column chromatography was performed using Kieselgel 60 0.040−0.063 mm.

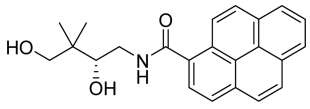


Pentafluorophenyl Pyrene-1-carboxylate. To a stirred, ice-cooled suspension of 1-pyrenecarboxylic acid (2.12 g, 8.6 mmol) and pentafluorophenol (1.75 g, 9.5 mmol) in dry DCM (100 mL) a solution of DCC (1.88 g, 9.1 mmol) in DCM (20 mL) was added in one portion and the mixture was left overnight with stirring. The precipitate of DCU was filtered off and washed with small amount of cold DCM. The combined solution was concentrated under reduced pressure, the residue was dissolved in ethyl acetate (200 mL); washed with water (100 mL), saturated aqueous $NaHCO_3$ (2 × 150 mL), and brine (50 mL); dried over sodium sulfate; and evaporated. The crude residue was purified by column chromatography on silica gel eluting with DCM/ethyl acetate (98:2) to afford yellow powder (3.30 g, 93%). R_f 0.74 (v/v DCM/*n*-hexane = 1:1); ^{1}H NMR (600.13 MHz, $CDCl_3$) δ 9.20 (d, J = 9.5 Hz, 1H), 8.85 (d, J = 8.1 Hz, 1H), 8.28−8.16 (m, 5H), 8.09−8.05 (m, 2H); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 163.2 (C═O), 135.7, 132.5, 130.8, 130.7, 130.6, 130.1, 129.3, 127.0, 127.0, 126.9, 126.6, 124.7, 124.1 (2C), 123.7, 118.8; ^{19}F NMR (564.68 MHz, $CDCl_3$) δ − 152.23 (m, 2F), − 158.05 (t, J = 21.7 Hz, 1F), − 162.31 (m, 2F); HRMS (ESI) m/z $[M+Na]^+$ calcd for $C_{23}H_9F_5NaO_2$, 435.0415; found 435.0422.

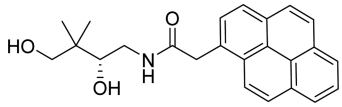


*(R)-4-Amino-2,2-dimethylbutane-1,3-diol (**2**).* One L three necked round bottomed flask equipped with a mechanical stirrer, a reflux condenser, and a dropping funnel was purged with argon and charged with (*R*)-2,4-dihydroxy-3,3-dimethylbutanamide (**1**)[11] (29.4 g, 0.2 mol) in absolute THF (600 mL). Borane dimethyl sulfide complex (BMS) (57.0 mL, 0.6 mol) was transferred via cannula into a dropping funnel, then solution in THF was cooled to 0−5 °C and BMS was added dropwise to amide solution for 40 min with stirring. The reactor was purged with argon after addition, and reaction was refluxed for 8 h in inert atmosphere. The mixture was cooled to room temperature and quenched with water (50 mL) followed by Dowex-50WX in H^+-form (1.7 mmol/mL, 200 mL of swollen resin in water). Dimethyl sulfide was condensed in a Drexel bottle filled with 10% hydrogen peroxide in water. The suspension was heated to 50 °C for 30 min to decompose borane-amine complex and then cooled to room temperature. Dowex resin was collected by filtration, rinsed with water (2 L) followed by ethanol (800 mL). Amine was eluted with the mixture methanol/triethylamine = 19:1 (1800 mL) and solution was concentrated *in vacuo*. Residual yellowish oil was evacuated with heating at 40 °C to give a white crystalline compound (20.3 g, 76%), mp 212−214 °C (96% EtOH), $[\alpha]^{27}_D$ −23.0 (c 1.0, 96% EtOH). ^{1}H NMR (600.13 MHz, CD_3CN) δ 3.33 (d, J = 11.0 Hz, 1H, C$\underline{H}_2$OH), 3.28 (dd, J = 7.7, 3.6 Hz, 1H, C$\underline{H}$OH), 3.22 (d, J = 11.0 Hz, 1H, C$\underline{H}_2$OH), 2.80 (dd, J = 12.5, 3.6 Hz, 1H, NHC$\underline{H}_2$), 2.52 (dd, J = 12.5, 7.7 Hz, 1H, NHC$\underline{H}_2$), 0.83 (s, 3H, CH_3), 0.82 (s, 3H, CH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 76.5 (CHOH), 67.0 (CH_2OH), 42.6 (CH_2NH), 38.6 ($(CH_3)_2\underline{C}$), 21.3 (CH_3), 20.6 (CH_3); HRMS (ESI) m/z $[M+H]^+$ calcd for $C_6H_{16}NO_2$, 134.1176; found 134.1162.

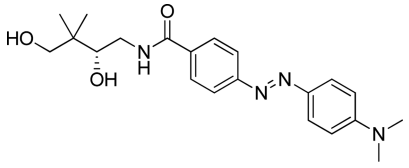
*General Procedure A: Acylation of Compound (**2**) with Pentafluorophenyl Esters.* To a stirred suspension of aminodiol **2** (880 mg, 6.6 mmol) in DCM (20 mL), the corresponding PFP ester (6 mmol) and triethylamine (830 μL, 6.6 mmol) were added and the stirring was continued for 2−3 h at room temperature until the reaction is complete (monitoring by TLC). Then DCM was removed *in vacuo* and the residue was triturated with water (50 mL). The crystalline compound was separated by filtration, washed with 5% aqueous potassium carbonate and water, dried in air and in desiccator with P_2O_5. The compounds were obtained quantitatively and used for the next step without purification.



*(R)-N-(2,4-Dihydroxy-3,3-dimethylbutyl)pyrene-1-carboxamide (**3a**).* Compound **3a** was prepared according to general procedure A as a colorless powder (2.14 g; 99%); scale: 6 mmol. R_f 0.45 (v/v DCM/methanol = 95:5); mp 183−185 °C (toluene); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.60 (d, J = 9.4 Hz, 1H, H10, pyrene), 8.53 (t, J = 5.4 Hz, 1H, NH), 8.34−8.32 (m, 3H, H3, H6, H8, pyrene), 8.27−8.20 (m, 4H, H2, H4, H5, H9, pyrene), 8.11 (t, J = 7.6 Hz, 1H, H7, pyrene), 4.80 (d, J = 4.1 Hz, 1H, CHO$\underline{H}$), 4.58 (m, 1H, $CH_2O\underline{H}$), 3.79−3.72 (m, 1H, C$\underline{H}$OH), 3.70 (m, 1H, C$\underline{H}_2$NH), 3.41−3.25 (m, 3H, C$\underline{H}_2$NH, C$\underline{H}_2$OH), 0.95 (s, 3H, CH_3), 0.93 (s, 3H, CH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 169.0 (C═O), 132.2 (C1, pyrene), 131.5 (C3a, pyrene), 130.7 (C5a, pyrene), 130.2 (C8a, pyrene), 128.2 (C5, pyrene), 127.9 (C9, pyrene), 127.8 (C10a, pyrene), 127.2 (C4, pyrene), 126.5 (C7, pyrene), 125.7 (C6, pyrene), 125.5 (C8, pyrene), 125.3 (C2, pyrene), 124.9 (C10, pyrene), 124.4 (C3, pyrene), 123.8 (C10b, pyrene), 123.7 (C10c, pyrene), 73.9 (CHOH), 68.2 (CH_2OH), 42.4 (CH_2NH), 38.8 ($\underline{C}(CH_3)_2$), 21.2 (CH_3), 20.00 (CH_3); HRMS (ESI) m/z $[M+H]^+$ calcd for $C_{23}H_{24}NO_3$, 362.1751; found 362.1758.



*(R)-N-(2,4-Dihydroxy-3,3-dimethylbutyl)-2-(pyren-1-yl)acetamide (**3b**).* Compound **3b** was prepared according to General procedure A as a yellow powder (2.23 g; 99%); scale: 6 mmol. R_f 0.41 (v/v DCM/methanol = 95:5); mp 182−184 °C (toluene). ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.41 (d, J = 9.2 Hz, 1H, H10, pyrene), 8.29−8.20 (m, 4H, H3, H6, H8, H9, pyrene), 8.15 (s, 2H, H4, H5, pyrene), 8.10−8.02 (m, 3 H, NH, H2, H7, pyrene), 4.60 (d, J = 5.4 Hz, 1H, CHO$\underline{H}$), 4.45 (t, J = 5.4 Hz, 1H, $CH_2O\underline{H}$), 4.23 (s, 2H, C(O)CH_2), 3.46−3.41 (m, 1H, C$\underline{H}$OH), 3.38−3.35 (m, 1H, C$\underline{H}_2$NH), 3.26−3.13 (m, 2H, C$\underline{H}_2$OH), 2.88−2.82 (m, 1H, C$\underline{H}_2$NH), 0.76 (s, 6H, 2 × CH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 170.3 (C═O), 131.1 (C5a, pyrene), 130.8 (C3a, pyrene), 130.4 (C8a, pyrene), 129.7 (C1, pyrene), 129.0 (C10a, pyrene), 128.7 (C2, pyrene), 127.4 (C4, pyrene), 127.2 (C9, pyrene), 126.8 (C5, pyrene), 126.1 (C7, pyrene), 125.0 (C6, pyrene), 124.9 (C8, pyrene), 124.7 (C3, pyrene), 124.2 (C10, pyrene), 124.1 (C10b, pyrene), 123.9 (C10c, pyrene), 74.0 (CHOH), 68.1 (CH_2OH), 41.6 (CH_2NH), 40.1 (C(O)$\underline{C}H_2$), 38.6 ($\underline{C}(CH_3)_2$), 21.0 (CH_3), 19.8 (CH_3); HRMS (ESI) m/z $[M+H]^+$ calcd for $C_{24}H_{26}NO_3$, 376.1907; found 376.1911.



*(R,E)-N-(2,4-Dihydroxy-3,3-dimethylbutyl)-4-((4-(dimethylamino) phenyl)diazenyl)benzamide (**3c**).* The suspension of 4-((4-(dimethylamino)phenyl)diazenyl)benzoyl chloride hydrochloride[15]

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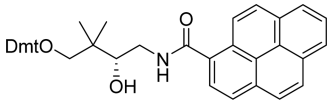
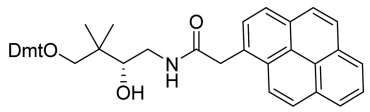
(3.24 g, 10 mmol) in dry DCM (100 mL) was cooled to 0−5 °C under stirring followed by dropwise addition of triethylamine (3.05 mL, 22 mmol). The orange solution was transferred into a dropping funnel and added to the solution of compound **2** (1.46 g, 11 mmol) in DCM (100 mL). The mixture was stirred for 1 h at room temperature and then concentrated *in vacuo*. The residue was dissolved in ethyl acetate (100 mL) and water (100 mL), organic layer was separated and washed with water (2 × 100 mL), saturated sodium bicarbonate solution (2 × 50 mL), and brine (50 mL); dried over sodium sulfate; and evaporated. Crude product was purified by column chromatography on silica gel, eluted with DCM/MeOH from 95:5 to 90:10 to obtain red crystalline compound (3.28 g, 86%). R_f 0.31 (v/v DCM/methanol = 95:5); mp 194−196 °C (toluene); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.39 (t, J = 5.3 Hz, 1H, NH), 8.01 (d, J = 8.5 Hz, 2H, Ar in DABCYL), 7.82 (m, 4H, Ar in DABCYL), 6.84 (d, J = 9.2 Hz, 2H, Ar in DABCYL), 4.70 (d, J = 5.0 Hz, 1H, CHOH), 4.47 (t, J = 5.3 Hz, 1H, CH_2OH), 3.60 (ddd, J = 13.4, 5.8, 2.2 Hz, 1H, CHOH), 3.54 (ddd, J = 9.0, 5.0, 2.3 Hz, 1H, CH_2NH), 3.33−3.23 (m, 2H, CH_2OH), 3.10 (m, 1H, CH_2NH), 3.07 (s, 6H, N(CH_3)$_2$), 0.86 (s, 3H, CCH_3), 0.85 (s, 3H, CCH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6), δ 165.8 (C═O), 153.9 (Ar in DABCYL), 152.8 (Ar in DABCYL), 142.6 (Ar in DABCYL), 135.00 (Ar in DABCYL), 128.4 (2C, Ar in DABCYL), 125.1 (2C, Ar in DABCYL), 121.5 (2C, Ar in DABCYL), 111.6 (2C, Ar in DABCYL), 73.7 (CHOH), 68.1 (CH_2OH), 42.3 (CH_2NH), 38.7 (C(CH_3)$_2$), 21.2 (CCH_3), 19.9 (CCH_3); HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{21}H_{29}N_4O_3$, 385.2234; found 385.2229.

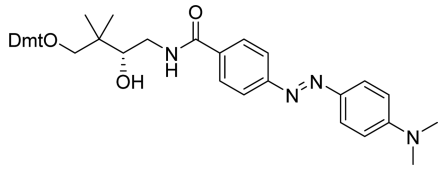
*General Procedure B: Preparation of the Compounds **4a−c**.* Compound **3a−c** (5 mmol) was coevaporated with anhydrous pyridine (2 × 20 mL) and dissolved in anhydrous pyridine (30 mL). The solution was cooled to 0 °C and solution of dimethoxytrityl chloride (1.86 g, 5.5 mmol) in anhydrous pyridine (5 mL) was injected with a syringe. The reaction mixture was left overnight under stirring with slow warming to room temperature. Reaction mixture was diluted with ethyl acetate (300 mL); washed with water (3× 300 mL), 5% citric acid (2 × 150 mL), and brine (50 mL); dried with sodium sulfate; and concentrated *in vacuo*. The residue was applied onto a column with silica gel and eluted with toluene/ethyl acetate/triethylamine (70:30:1). The isolated product was evaporated twice with DCM to obtain solid foam of the desired compound.



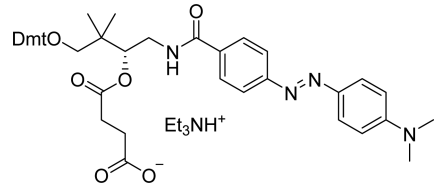
*(R)-N-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxy-3,3-dimethylbutyl)pyrene-1-carboxamide (**4a**).* Compound **4a** was prepared according to general procedure B as a colorless solid foam (3.15 g; 92%); scale: 5 mmol. R_f 0.61 (v/v/v toluene/ethyl acetate/triethylamine, 70:30:1); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.59 (d, J = 9.3 Hz, 1H, H10, pyrene), 8.50 (t, J = 5.3 Hz, 1H, NH), 8.34−8.31 (m, 3H, H3, H6, H8, pyrene), 8.25−8.19 (m, 4H, H2, H4, H5, H9, pyrene), 8.11 (t, J = 7.6 Hz, 1H, H7, pyrene), 7.46 (d, J = 7.3 Hz, 2H, Ar in Dmt), 7.33−7.30 (m, 6H, Ar in Dmt), 7.24 (m, 1H, Ar in Dmt), 6.92 (d, J = 8.9 Hz, 4H, Ar in Dmt), 4.80 (d, J = 5.6 Hz, 1H, OH), 3.77−3.72 (m, 1H, CHOH), 3.75 (s, 6H, 2 × CH_3 in Dmt) 3.67−364 (m, 1H, CH_2NH), 3.21−3.16 (m, 1H, CH_2NH), 3.02 (d, J = 8.6 Hz, 1H, CH_2O), 2.91 (d, J = 8.6, 1H, CH_2O), 0.99 (s, 3H, CCH_3), 0.96 (s, 3H, CCH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 169.00 (C═O), 157.1 (2C, Ar in Dmt), 145.3 (Ar in Dmt), 136.0 (Ar in Dmt), 136.00 (Ar in Dmt), 132.1 (C1, pyrene), 131.5 (C3a, pyrene), 130.7 (C5a, pyrene), 130.2 (C8a, pyrene), 129.8 (4C, Ar in Dmt), 128.2 (C5, pyrene), 127.9 (C9, pyrene), 127.8 (2C, Ar in Dmt), 127.7 (2C, Ar in Dmt), 127.2 (C4, pyrene), 126.5 (2C, C7, pyrene and Ar in Dmt), 125.7 (C6, pyrene), 125.5 (C8, pyrene), 125.3 (C2, pyrene), 124.8 (C10, pyrene), 124.3 (C3, pyrene), 123.8 (C10b, pyrene), 123.6 (C10c, pyrene), 113.0 (4C, Ar in Dmt), 85.0 (Ph$_3$C), 73.7 (CHOH), 68.6 (CH_2O), 55.0 (2C, 2 × CH_3 in Dmt), 42.2 (CH_2NH), 38.6 (C(CH_3)$_2$), 21.7 (CCH_3), 20.9 (CCH_3); HRMS (ESI) m/z [M+Na]$^+$ calcd for $C_{44}H_{41}NO_5Na$, 686.2877; found 686.2873.



*(R)-N-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxy-3,3-dimethylbutyl)-2-(pyren-1-yl)acetamide (**4b**).* Compound **4b** was prepared according to general procedure B as a green solid foam (3.32 g; 95%); scale: 5 mmol. R_f 0.44 (v/v/v toluene/ethyl acetate/triethylamine, 70:30:1); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.42 (d, J = 9.2 Hz, 1H, H10, pyrene), 8.27−8.03 (m, 9H, NH, H2, H3, H4, H5, H6, H7, H8, H9, pyrene), 7.40 (d, J = 7.7 Hz, 2H, Ar in Dmt), 7.26−7.28 (m, 6H, Ar in Dmt), 7.19 (t, J = 7.3 Hz, 1H, Ar in Dmt), 6.85−6.83 (m, 4H, Ar in Dmt), 4.61 (d, J = 5.6 Hz, 1H, OH), 4.24 (s, 2H, CH_2CO), 3.69 (s, 3H, CH_3 in Dmt), 3.68 (s, 3H, CH_3 in Dmt), 3.51 (m, 1H, CHOH), 3.38 (m, 1H, CH_2NH), 2.91−2.80 (m, 3H, CH_2NH, CH_2O), 0.88 (s, 3H, CCH_3), 0.82 (s, 3H, CCH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 170.2 (C═O), 157.9 (2C, Ar in Dmt), 145.2 (Ar in Dmt), 136.0 (Ar in Dmt), 135.8 (Ar in Dmt), 130.8 (C3a, pyrene), 130.3 (C8a, pyrene), 129.7 (4C, Ar in Dmt), 129.7 (2C, C1, C5a, pyrene), 129.0 (C10a, pyrene), 128.6 (C2, pyrene), 127.7 (2C, Ar in Dmt), 127.6 (2C, Ar in Dmt), 127.4 (C4, pyrene), 127.1 (C9, pyrene), 126.7 (C5, pyrene), 126.4 (2C, Ar in Dmt), 126.1 (C7, pyrene), 125.0 (C6, pyrene), 124.8 (C8, pyrene), 124.7 (C3, pyrene), 124.1 (C10, pyrene), 124.1 (C10b, pyrene), 123.9 (C10c, pyrene), 113.0 (4C, Ar in Dmt), 85.0 (Ph$_3$C), 74.0 (CHOH), 68.5 (CH_2O), 54.9 (2C, 2 × CH_3 in Dmt), 41.5 (C(CH_3)$_2$), 40.1 (CH_2C(O)), 38.5 (C(CH_3)$_2$), 21.5 (CCH_3), 20.8 (CCH_3); HRMS (ESI) m/z: [M+Na]$^+$ calcd for $C_{45}H_{43}NO_5Na$, 700.3033; found 700.3029.



*(R,E)-N-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxy-3,3-dimethylbutyl)-4-((4-(dimethylamino)phenyl)diazenyl)-benzamide (**4c**).* Compound **4c** was prepared according to general procedure B as an orange solid foam (3.42 g; 94%); scale: 5.2 mmol. R_f 0.56 (v/v/v toluene/ethyl acetate/triethylamine, 70:30:1); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.38 (t, J = 5.3 Hz, 1H, NH), 8.03 (d, J = 8.5 Hz, 2H, Ar in DABCYL), 7.83 (m, 4H, Ar in DABCYL), 7.43 (d, J = 7.5 Hz, 2H, Ar in Dmt), 7.31 (m, 6H, Ar in Dmt), 7.21 (t, J = 7.3 Hz, 1H, Ar in Dmt), 6.90 (d, J = 8.9 Hz, 4H, Ar in Dmt), 6.84 (d, J = 9.2 Hz, 2H, Ar in DABCYL), 4.76 (d, J = 5.2 Hz, 1H, OH), 3.73 (s, 6H, 2 × CH_3 in Dmt), 3.65 (m, 1H, CHOH), 3.57 (m, 1H, CH_2NH), 3.07 (s, 6H, (CH_3)$_2$N in DABCYL), 3.07 (m, 1H, CH_2NH), 2.97 (d, J = 8.5 Hz, 1H, CH_2O), 2.85 (d, J = 8.5 Hz, 1H, CH_2O), 0.94 (s, 3H, CH_3C), 0.92 (s, 3H, CH_3C); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 165.9 (C═O), 157.9 (2C, Ar in Dmt), 153.9 (Ar in DABCYL), 152.8 (Ar in DABCYL), 145.3 (Ar in Dmt), 142.6 (Ar in DABCYL), 136.03 (Ar in Dmt), 135.95 (Ar in Dmt), 134.9 (Ar in DABCYL), 129.8 (2C, Ar in Dmt), 128.4 (2C, Ar in DABCYL), 127.8 (2C, Ar in Dmt), 127.6 (2C, Ar in Dmt), 126.5 (2C, Ar in Dmt), 125.0 (2C, Ar in DABCYL), 121.4 (2C, Ar in DABCYL), 113.0 (4C, Ar in Dmt), 111.5 (2C, Ar in DABCYL), 85.0 (Ph$_3$C), 73.6 (CHOH), 68.4 (CH_2O), 55.0 (2C, 2 × CH_3 in Dmt), 42.2 (CH_2NH), 39.8 (2C, N(CH_3)$_2$ in DABCYL), 38.5 (C(CH_3)$_2$), 21.8 (CCH_3), 20.6 (CCH_3); HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{42}H_{47}N_4O_5$, 687.3541; found 687.3527.



*Triethylammonium (R,E)-4-((4-(Bis(4-methoxyphenyl)-(phenyl)-methoxy)-1-(4-((4-(dimethylamino)phenyl)-diazenyl)benzamido)-3,3-dimethylbutan-2-yl)oxy)-4-oxobutanoate (**6c**).* To a solution of compound **4c** (1.37 g, 2.0 mmol) in dry pyridine (10 mL)

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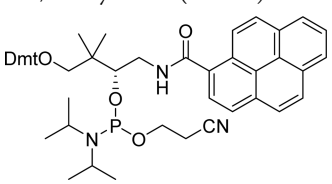
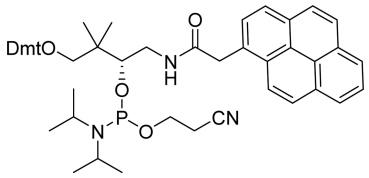
triethylamine (0.55 mL, 4.0 mmol), DMAP (250 mg, 2.0 mmol) and freshly recrystallized from toluene succinic anhydride (1.0 g, 10 mmol) were added. The reaction was kept under stirring at room temperature for 48 h. The mixture was diluted with EtOAc (200 mL) and vigorously shaken with saturated aqueous $NaHCO_3$ (300 mL) for 30 min. Organic layer was separated and washed again with saturated $NaHCO_3$ (2 × 100 mL), 5% citric acid (3 × 100 mL), water (200 mL), and brine (50 mL); dried over sodium sulfate; and evaporated to dryness after addition of several drops of triethylamine. Crude product was purified by column chromatography on silica gel eluting with DCM/methanol/triethylamine (95:5:0.5). Pure product was dissolved in dry DCM (50 mL), filtered through a cotton plug, and evaporated to dryness after addition of several drops of triethylamine to give an orange solid foam (1.58 g, 89%). R_f 0.61 (v/v/v DCM/methanol/triethylamine, 95:5:0.5); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.52 (t, J = 5.6 Hz, 1H, NH), 7.93 (d, J = 8.4 Hz, 2H, Ar in DABCYL), 7.80 (m, 4H, Ar in DABCYL), 7.41 (d, J = 7.5 Hz, 2H, Ar in Dmt), 7.32 (t, J = 7.7 Hz, 2H), 7.28 (m, 4H, Ar in Dmt), 7.21 (t, J = 7.3 Hz, 1H, Ar in Dmt), 6.90 (d, J = 7.3 Hz, 4H, Ar in Dmt), 6.84 (d, J = 9.2 Hz, 2H, Ar in DABCYL), 5.11 (dd, J = 9.5, 1.6 Hz, 1H, C<u>H</u>OH), 3.76 (m, 1H, C<u>H$_2$</u>NH), 3.73 (s, 6H, 2 × CH_3 in Dmt), 3.24 (m, 1H, C<u>H$_2$</u>NH), 2.85 (m, 2H, C<u>H$_2$</u>O), 2.58 (q, J = 7.2 Hz, 5.3 H, triethylamine), 2.41−2.31 (m, 4H, CH_2CH_2 in succinate), 0.97 (t, J = 7.2 Hz, 8H, triethylamine; s, 3H, CCH_3), 0.92 (s, 3H, CCH_3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 174.2 (CO_2^-), 171.8 (<u>C</u>O$_2$CH), 165.8 (C(O)NH), 158.0 (Ar in Dmt), 153.9 (Ar in DABCYL), 152.8 (Ar in DABCYL), 144.9 (Ar in Dmt), 142.7 (Ar in DABCYL), 135.6 (Ar in Dmt), 135.6 (Ar in Dmt), 134.9 (Ar in DABCYL), 129.8 (4C, Ar in Dmt), 128.3 (2C, Ar in DABCYL), 127.8 (2C, Ar in Dmt), 127.7 (2C, Ar in Dmt), 126.5 (Ar in Dmt), 125.0 (2C, Ar in DABCYL), 121.4 (2C, Ar in DABCYL), 113.0 (4C, Ar in Dmt), 111.5 (2C, Ar in DABCYL), 85.3 (Ph$_3$<u>C</u>), 76.0 (<u>C</u>HOC(O)), 67.7 (CH_2O), 55.0 (2C, 2 × CH_3 in Dmt), 45.3 (2.67 C, 3 × CH_3 in triethylamine), 39.8 (2C, N(CH_3)$_2$ in DABCYL), 37.7 (CH_2NH), 30.6 (<u>C</u>(CH$_3$)$_2$), 29.9 (CH_2 in succinate), 29.7 (CH_2 in succinate), 21.8 (C<u>C</u>H$_3$), 21.1 (C<u>C</u>H$_3$), 10.5 (2.67 C, 3 × CH_3 in triethylamine); HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{46}H_{51}N_4O_8$, 787.3701; found 787.3688.

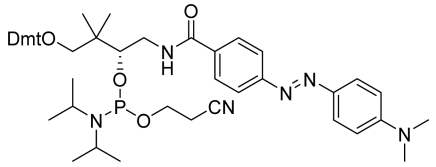
*General Procedure C: Synthesis of Amidites **5a−c**.* Dmt-protected precursor **4** (1 mmol) was dried in a evacuated vacuum desiccator over P_2O_5 overnight, transferred to a flame-dried Schlenk flask with a stir bar followed by freshly distilled DCM (10 mL) and DIPEA (260 μL, 1.5 mmol). The flask was purged with argon, plugged with a rubber septum, and cooled to 0−5 °C. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (270 μL, 1.2 mmol) was injected dropwise. The reaction mixture was kept 10 min at 0−5 °C and 40 min at room temperature, then diluted with toluene (80 mL) and washed with saturated $NaHCO_3$ (2 × 100 mL), water (100 mL), and brine (50 mL). Organic phase was filtered through a cotton plug and concentrated *in vacuo*. The residue was evaporated twice with DCM to obtain a solid foam. Crude product was purified by column chromatography with silica gel (approximately 30 g per mmol of Dmt-precursor) with the linear gradient of toluene/ethyl acetate/triethylamine from 88:10:2 to 68:30:2. Pure compounds **5a−c** were dissolved in anhydrous benzene and lyophilized to obtain a solid material. TLC: toluene/ethyl acetate/triethylamine (55:40:5).



*(R)-4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-3,3-dimethyl-1-(pyrene-1-carboxamido)butan-2-yl (2-cyanoethyl) diisopropylphosphoramidite (**5a**).* Compound **5a** was prepared according to general procedure C as a colorless solid foam (800 mg; 84%); scale: 1.1 mmol. R_f 0.63, 0.81−diastereomers (v/v/v toluene/ethyl acetate/triethylamine, 55:40:5); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.63−8.55 (m, 1H), 8.41 (m, 0.4 H), 8.37−8.31 (m, 3H), 8.28−8.09 (m, 5H), 8.00 (m, 0.4H), 7.44 (m, 2H), 7.34−7.25 (m, 7H), 6.88 (m, 4H), 4.07−3.99 (m, 1H), 3.81−3.40 (m, 12H), 3.15−3.08 (m, 1H), 2.97−2.91 (m, 1H), 2.59 (t, J = 6.0 Hz, 1H), 2.22−2.05 (m, 1H), 1.14 (s, 1H), 1.07−0.97 (m, 15H); ^{31}P NMR (242.93 MHz, DMSO-d_6) δ 149.35 (0.47P), 148.29 (0.53P), diastereomers; HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{53}H_{59}N_3O_6P$, 864.4136; found 864.4143.



*(R)-4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-3,3-dimethyl-1-(2-(pyren-1-yl)acetamido)butan-2-yl (2-cyanoethyl) diisopropylphosphoramidite (**5b**).* Compound **5b** was prepared according to general procedure C as a green solid foam (1.22 g, 82%); scale 1.7 mmol. R_f 0.54, 0.74 diastereomers (v/v/v toluene/ethyl acetate/triethylamine, 55:40:5); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.36−8.20 (m, 4H), 8.17−8.13 (m, 3H), 8.07−8.03 (m, 1H), 7.99−7.96 (m, 1H), 7.61 (t, J = 5.5 Hz, 0.4H), 7.36−7.34 (m, 1H), 7.28−7.16 (m, 7 H), 6.84−6.80 (m, 4H), 4.21 (m, 1H), 4.16 (m, 0.65 H), 3.76−3.60 (m, 7H), 3.47−3.35 (m, 2H), 3.30−3.14 (m, 3H), 2.93 (m, 1H), 2.79−2.58 (m, 2H), 2.41 (t, J = 6.0 Hz, 0.7 H), 1.18−0.77 (m, 18H); ^{31}P NMR (242.93 MHz, DMSO-d_6) δ 149.48 (0.59P), 148.30 (0.41P), diastereomers; HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{54}H_{61}N_3O_6P$, 878.4292; found, 878.4295.



*(R)-4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-1-(4-((E)-(4-(dimethylamino)phenyl)diazenyl)benzamido)-3,3-dimethyl-butan-2-yl (2-cyanoethyl) diisopropylphosphoramidite (**5c**).* Compound **5c** was prepared according to general procedure C as an orange solid foam (700 mg, 86%); scale: 0.9 mmol. R_f 0.61, 0.74 diastereomers (v/v/v toluene/ethyl acetate/triethylamine, 55:40:5); ^{1}H NMR (600.13 MHz, DMSO-d_6) δ 8.29 (m, 0.4 H), 7.97−7.93 (m, 2.4 H), 7.85−7.79 (m, 4H), 7.43−7.40 (m, 2H), 7.31−7.19 (m, 7H), 6.89−6.84 (m, 6H), 3.97−3.90 (m, 1H), 3.73 (s, 6H), 3.70−3.32 (m, 6H), 3.10−3.04 (m, 1H), 3.08 (s, 6H), 2.89 (m, 1H), 2.60−2.51 (m, 2H), 1.08−0.91 (m, 18H); ^{31}P NMR (242.93 MHz, DMSO-d_6) δ 149.77 (0.58P), 148.63 (0.42P), diastereomers; HRMS (ESI) m/z [M+H]$^+$ calcd for $C_{51}H_{64}N_6O_6P$, 887.4619; found 887.4632.

*DABCYL CPG Solid Support (**7c**).* **7c** was prepared as described[3c] using PyBOP as a coupling reagent. Scale: 16.3 g of native amino LCAA CPG 500 Å, loading of dimethoxytritylated DABCYL: 54 μmol per 1 g of CPG. After preparation modified CPG (**7c**) was dried *in vacuo* and stored at +4 °C.

Oligonucleotide Synthesis, Purification, and Characterization. Synthesis of oligonucleotides was performed at 1 μmol scale on DNA/RNA synthesizer with the phosphoramidite method according to the manufacturer's recommendations. In the case of DABCYL (**5c**) and pyrene (**5a**,**b**) phosphoramidites condensation time was increased to 5 min. Oligonucleotide probes were deprotected using concentrated aqueous ammonia at 55 °C overnight and double purified by denaturing PAGE followed by RP-HPLC. The denaturing gel electrophoresis of oligonucleotides was performed in 15% PAGE containing 7 M urea in Tris-borate buffer (50 mM Tris HCl, 50 mM boric acid, 1 mM EDTA, pH 8.3). Oligonucleotides were recovered from the gel by electroelution in Tris-borate buffer (5 mM Tris HCl, 5 mM boric acid, pH 8.3). The HPLC purification of oligonucleotides was carried out on a 4.6 × 250 mm C18 column (5 μm); buffer A: 0.05 M ammonium acetate (pH 7), 5% MeCN; buffer B: 0.03 M ammonium acetate, 80% MeCN, pH 7; a gradient of B: 0 → 15% (1 CV), 15 → 60% (10 CV); a flow rate of 1 mL/min; temperature 45 °C. The HPLC analysis of oligonucleotides was carried out on a 4.6 × 250 mm C18 column (5 μm); buffer A: 0.05 M ammonium acetate

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(pH 7), 5% MeCN; buffer B: 0.03 M ammonium acetate, 80% MeCN, pH 7; a linear gradient of B: 0 → 100% (8 CV); a flow rate of 1 mL/min; temperature 45 °C. ESI-MS spectra for oligonucleotides were obtained using a q-TOF system (Table S1).

Spectral Studies. Spectroscopic study of MB1−24 was carried out for 1.2 nM solution in 1 × PBS, pH 7.4. For duplex formation, MBs (1.2 nmol) were mixed with DNA **Seq3** (1.32 nmol) in PBS pH 7.4 and adjusted to 1 mL volume, heated to 95 °C and cooled to 25 °C during 4 h. Fluorescent melting experiments of 20 μM **MB1−MB24** hairpins and their duplexes with DNA matrix (see Table S2) were performed in 25 μL (including 5 μL PCR-mix-2-FRT and 176 μM dNTPs). Melting studies were performed in the qPCR machine under the following conditions: heating from 30 to 80 °C (2 °C min^{-1}) followed by cooling from 80 to 30 °C (2 °C min^{-1}). The fluorescence measurements were recorded every 30 s. Fluorescent signal was detected in "blue" channel: excitation wavelength −365 ± 20 nm, emission −460 ± 20 nm.

Molecular Dynamics.[16] Modified oligonucleotide's geometry was optimized at B3LYP 6-31G(3d,2f) level followed by point atomic charge calculation from ESP as reported earlier.[16a] The last one was derived on same level of theory with R.E.D. tool based on resp approach. Molecular topology with optimized coordinates was built by Acpype. Initial structures of all single-stranded DNA were prepared with the pmx[16b] package for Python. Each molecule was subjected to simulated annealing in vacuum, and ten conformations for each molecule were used for molecular dynamics simulation in explicit solvent with trajectory length from 90 to 100 ns. Classical geometry optimization was performed for each system individually. Explicit solvent simulations in parmbsc0 force field were performed at T = 300 K under control of velocity rescaling thermostat, with isotropic constant-pressure boundary conditions under the control of the Berendsen algorithm of pressure coupling with application of Ewald particle mesh method for long-range electrostatics interactions (PME). A triclinic box of the TIP4P water molecules was added around the DNA to a depth 15 Å on each side of the solute. Negative charges of systems were neutralized by addition of sodium cations, resulting in ∼0.15 M concentration of the ions. We used two temperature coupling groups in the simulations. The first one is modified DNA molecule and the second one is water with ions. Analysis was carried out by tools from GROMACS 5.0 software package and with python scripts. Pyrene overlap values were calculated as number of contacts of each carbon in a pyrene moiety with carbons in a neighbor pyrene in distance ≤4 Å. Average distance and overlap of pyrene pair were calculated from ten experiments for each MBs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b01451.

Characterization of oligodeoxynucleotide probes (mass-spectra, HPLC, melting curves) and NMR spectra of organic compounds (pentafluorophenyl pyrene-1-carboxylate and **2**−**7**) (PDF)

Jmol files (ZIP)

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Notes

The authors declare no competing financial interest.

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