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Ends free and self-quenched molecular beacon with pyrene labeled pyrrolocytidine in the middle of the stem

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ABSTRACT

Pyrene labeled pyrrolocytidine was incorporated into an oligonucleotide to construct ends free and self-quenched molecular beacon in which the fluorophore containing pyrrolocytidine was placed in the middle of the stem and used for the detection of a target DNA with an excellent efficiency.

1. Introduction

Molecular beacons (MBs) (Fig. 1a) are widely used in fluorometric analysis of nucleic acids.[1] After publication of the idea of molecular beacon by Tyagi et al., numerous papers have appeared along with its modifications to afford more excellent sensitivity and selectivity in DNA detection. As a result of tremendous efforts, several conceptually new MB probes were developed,[2] including a most recent design of quencher-free molecular beacon.[3] However, all the recent advances in the design of quencher-free molecular beacon are based on the introduction of a fluorophore at the stem terminus. For efficient quenching of the hairpin state, these MBs require at least one guanosine (**G**) base as a quencher at the opposite stem terminus, thus limiting their generality of the design and the use for multiplex detection.[3] There has been no report so far for the quencher-free MBs having no terminal **G** but with the fluorophore in the middle of the stem and with two free ends (3′- and 5′-end) for introducing other functionalities. Therefore, we thought that it will be worthwhile and of greatest versatility if we develop such a new type of MBs having two free ends for further application and modification.

Our long term efforts in designing fluorescently labeled oligonucleotide probes[4] for high-throughput genetic analyses have led to a rational design of a novel self-quenched MB with two free ends (3′- and 5′-end) that require no terminal **G** for quenching at hairpin state[3b] and with the fluorophore attached to pyrrolocytidine (**pC**)[5] placed in the middle of the stem. We sought a new concept, in which the generation of signal 'off' hairpin state originates from the quenching of the fluorophore by the opposite base **G** of the **pC-G** base pair due to their close proximity (Fig. 1b).[3g] The unique features of our method are; (a) the generic design, which could be applicable irrespective of the position of the fluorophore in the stem and the sequence of the stem, (b) the full regeneration of fluorescence signal upon opening of the stem with optimum discrimination, and (c) the allowance of almost unrestricted access of fluorophores. Therefore, with its simplicity, sensitivity, and specificity, this strategy holds a great promise in applications such as in heterogeneous assay and high-throughput DNA analysis.

2. Results and discussion

To assess the sensitivity and specificity of our method, two molecular beacons (**MB2** and **MB3**) were designed in which labeled pyrrolocytidine (**pC**) was placed three base pairs apart from the 3′-terminus. We have first synthesized alkylamino substituted pyrrolocytidine (Fig. 1c) according to the reported synthetic protocol.[6] The synthesis of pyrrolo-dC derivative **5** and its cyanoethyl phosphoramidite **6** is shown in Scheme 1. Thus, 5-iodo-2′-deoxyuridine was coupling with *N*-trifluoroacetyl propynylamide in dry DMF under Sonogashira coupling protocols[7] to yield nucleoside **2**, which was cyclized to the furano derivative **3** by following the literature procedure.[8,9] 5′-Hydroxyl group was then protected with standard tritylation method to get nucleoside **4**. Next, the ammonia exchange method of Woo et al.[10] was employed to give 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-6-(*N*-methylamino)-pyrrolocytidine, which upon treatment with ethyl trifluoroacetate in methanol afforded amino protected pyrrolo-dC analogue **5**. All the synthesized nucleosides were well characterized by NMR and HRMS spectrometer. Phosphoramidite **6** was incorporated into DNA by automated DNA synthesizer. Finally, fluorophores were post-synthetically incorporated into **MB1** to afford **MB2** and **MB3**, with **PyI**- and **PyII**-labeled fluorophores, respectively (see Scheme S1, Supplementary data) and were used to evaluate their ability in sensing target DNA. As a target loop strand, we also synthesized ODN **9** (Table 1).

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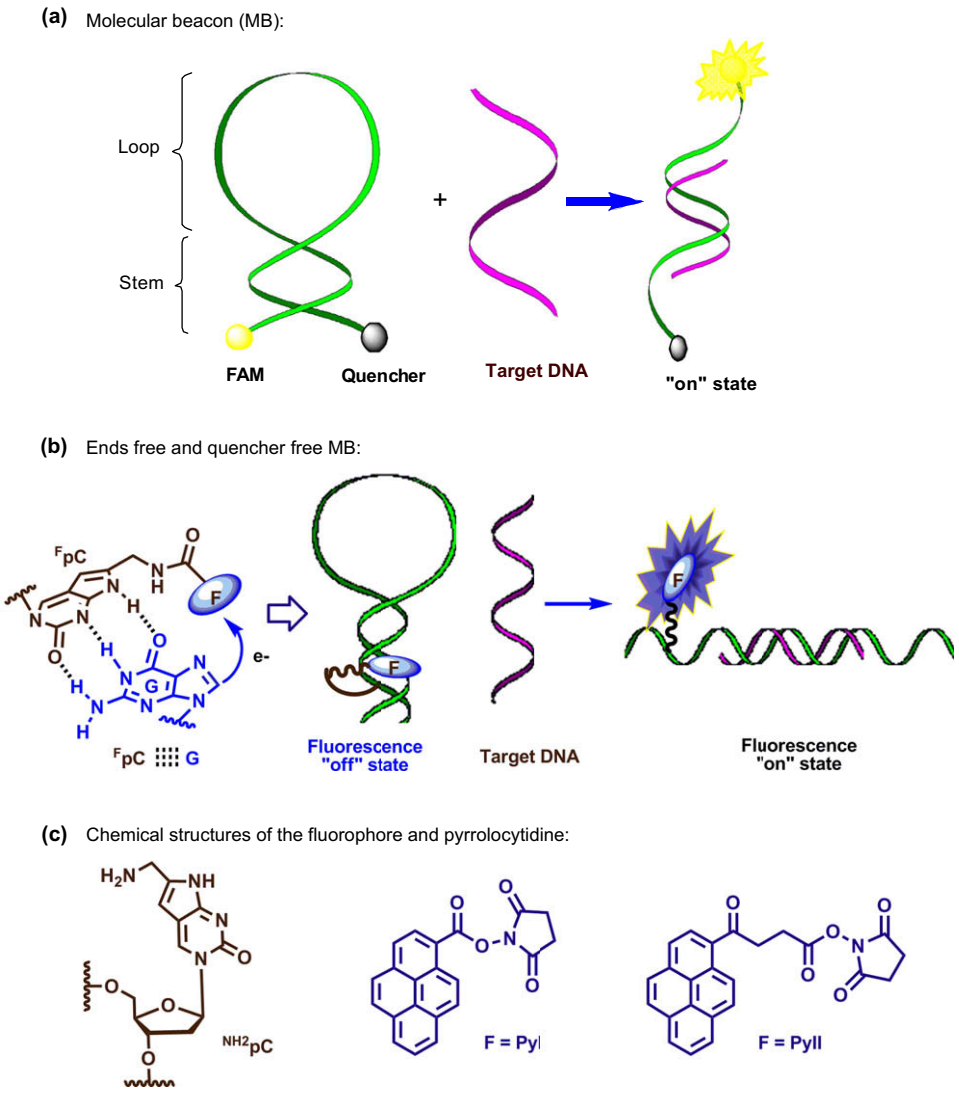
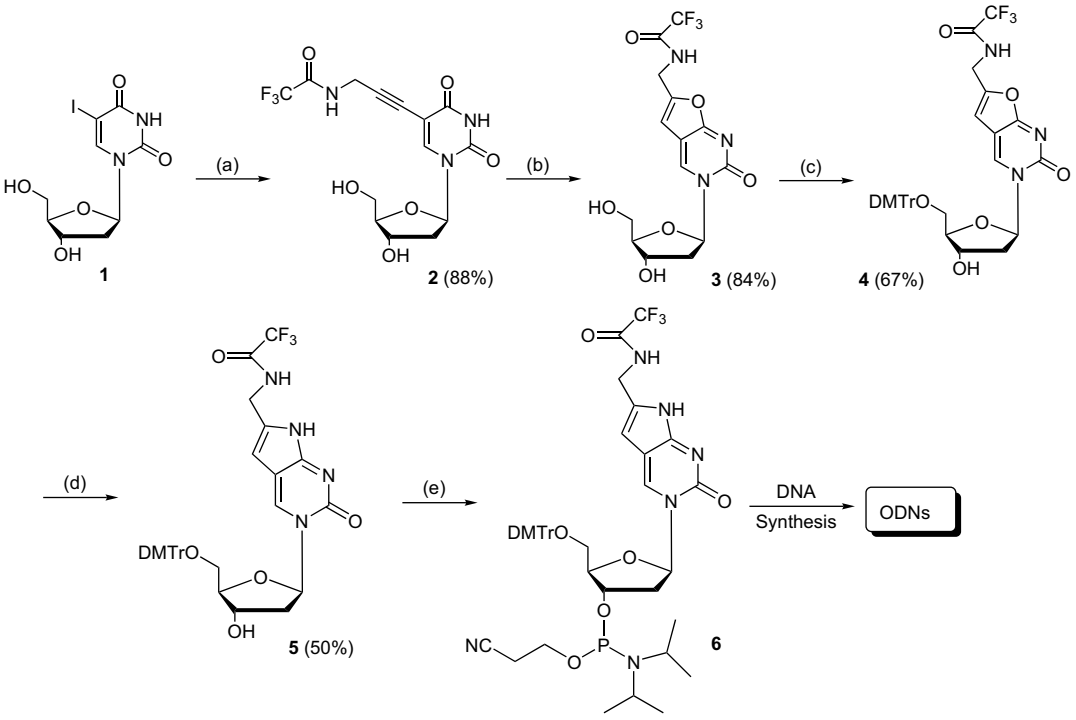


Figure 1. Illustration of (a) molecular beacon, (b) ends free and quencher-free MB, and (c) chemical structures of the fluorophores.

To prove our concept for the fluorescence quenching by the opposite guanosine, we have first synthesized 13 mer linear ODNs **1** and **2** with free alkylamino substituent in which the fluorophores were incorporated via post synthetic modification to generate pyrene labeled ODNs **3** and **4** (Table 1). We examined the thermal melting behavior, which showed an enhanced stability of the fully matched duplex, ODN **3**/**5** (N=**G**) and ODN **4**/**6** (N=**G**) as compared with the unmodified duplexes (ODN **7**/**5**, ODN **8**/**6**) (Table 2). We checked the fluorescence response of the ODNs upon duplex formation with the complementary sequences. Thus, from the fluorescence spectra (Fig. 2), it is clear that the emission from the pyrene-hooked pyrrolocytidine labeled probes is greatly decreased in the presence of complementary strand ODN **5** (N=**G**) or ODN **6** (N=**G**) containing guanosine base (**G**) opposite to the labeled nucleotide as compared with other complementary ODNs containing **A**, **T**, **C** mismatched nucleotides. Thus, our probes are able to sense the opposite matched base **G** from its target DNA by a drastic decrease of the fluorescence intensity. The close proximity of the fluorophore and the opposite **G** in the duplexes is responsible for the decrease in fluorescence intensity, which was again reflected from the higher duplex stability compared with the control duplexes ODN **7**/**5** (N=**G**) and ODN **8**/**6** (N=**G**) (Table 2).

With the finding that the fluorescence of the pyrene-hooked pyrrolocytidine is efficiently quenched by the opposite **G**, we turned out our attention to evaluate the sensitivity of the designed MBs in response to the duplex formation with the target DNA by comparing the signal generated from the fluorophores. Thus, in the resulting fluorescence spectra of **MB2** and **MB3**, we observed a large difference in the fluorescence of the hairpin MBs and their duplexes with complementary loop strands (**MB2**/ODN **9**; **MB3**/ODN **9**). For example, the hybridization of hairpin **MB2** with its complementary strand ODN **9** displayed a higher thermal stability (Table 2) and a 3.4-fold enhancement in emission intensity relative to that observed for hairpin state (Fig. 3a). **MB3** is exceptionally excellent, showing almost no background signal and thus allowing us to detect the target DNA with high efficiency (Fig. 3c). This is due to the pyrene carbonyl group, which is more sensitive to quenching by electron transfer from opposite **G**, leading to a negligible background signal.[4a] Therefore, efficient quenching of hairpin state lead to the fluorescence 'off' state and upon duplex formation strong fluorescence appeared at 'on' state due to no contact with **G**, to result in an efficient detection of the target DNA.

To assess whether the design of ^{PyI}, ^{PyII}**pC-G** base pair could be used irrespective of the stem sequence and the position in the stem,



Scheme 1. Synthesis of alkylamino substituted pyrrolocytidine and the incorporation into short oligonucleotides. Reagents and conditions: (a) DMF/Pd(PPh_3)$_4$/CuI/Et_3N/trifluoroacetylpropargylamider/rt, overnight; (b) CuI/MeOH/Et_3N/60 °C, 3 h; (c) pyridine/DMTrCl/DMAP/rt, 12 h; (d) (i) aq NH_3/MeOH/55 °C, 24 h, (ii) ethyltrifluoroacetate/MeOH, rt, 12 h; (e) 2-cyanoethyldiisopropylchlorophosphoramidite/CH_2Cl_2/Et_3N.

we have synthesized **MB4** and **MB7** in which the ^{NH2}**pC-G** pair was placed three and two bases apart, respectively, from the 3′-stem terminus and having no terminal G-C base pair.[4a] Thus, **MB5** and **MB6** were synthesized from **MB4** and **MB8** from **MB7** via post synthetic modification. The thermal stability (Table 2) and the fluorescence response in the presence of target DNA (Fig. 3b, d, e) was found to be quite similar to that of other stem sequences (Fig. 3a and c). The emissive colors of **MB6** with the target are depicted in Figure 3f. Thus, it is clear that our MBs with fluorophore labeled **pC-G** base pair are capable of detecting the target DNA with an excellent efficiency, irrespective of the sequence as well as the position in the stem.

3. Conclusion

In conclusion, we have developed conceptually new quencher-free MBs. We were able to detect the target DNA irrespective of sequences and the length of the stem with high efficiency. The designed ends free MBs shown here produced excellent 'on'/'off' signals that are thus widely usable as a sensitive probe. **MB3**, **MB6**, and **MB8** are of particular importance with almost no background signals and thus capable of sensing specific target DNA sequence with an excellent selectivity and sensitivity. Our designed MBs may be used in heterogeneous assay and on microarray via immobilization on a solid surface through the free ends.

Table 1
DNA sequences used in this study

ODNs	Sequences
1	5′-d(CGCAAT^{NH2}**pC**TAACGC)-3′
2	5′-d(CGCAAC^{NH2}**pC**CAACGC)-3′
3	5′-d(CGCAAT^{Pyl}**pC**TAACGC)-3′
4	5′-d(CGCAAC^{Pyl}**pC**CAACGC)-3′
5	5′-d(GCGTTA **N** ATTGCG)-3′ [**N**=A, T, G, C]
6	5′-d(GCGTTG **N** GTTGCG)-3′ [**N**=A, T, G, C]
7	5′-d(CGCAAT**C**TAACGC)-3′
8	5′-d(CGCAAC**C**CAACGC)-3′
9	5′-d(TTCAATCTTGGATAC)-3′
10 (**MB1**)	5′-d(GAGGG*GTATCCAAGATTGAAC*^{NH2}**pC**CTC)-3′
11 (**MB2**)	5′-d(GAGGG*GTATCCAAGATTGAAC*^{Pyl}**pC**CTC)-3′
12 (**MB3**)	5′-d(GAGGG*GTATCCAAGATTGAAC*^{Pyl}**pC**CTC)-3′
13 (**MB4**)	5′-d(TGAGGA*GTATCCAAGATTGAATC*^{NH2}**pC**TCA)-3′
14 (**MB5**)	5′-d(TGAGGA*GTATCCAAGATTGAATC*^{Pyl}**pC**TCA)-3′
15 (**MB6**)	5′-d(TGAGGA*GTATCCAAGATTGAATC*^{Pyl}**pC**TCA)-3′
16 (**MB7**)	5′-d(TGAGGA*GTATCCAAGATTGAATCT*^{NH2}**pC**CA)-3′
17 (**MB8**)	5′-d(TGAGGA*GTATCCAAGATTGAATCT*^{Pyl}**pC**CA)-3′

4. Experimental

4.1. General

^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (100 MHz) were measured with Bruker Avance 400F spectrometer. Coupling constant (*J* value) are reported in hertz. The chemical shifts are shown in parts per million downfield from tetramethylsilane, using residual chloroform (δ 7.26 in ^{1}H NMR, δ 77.2 in ^{13}C NMR) and dimethyl sulfoxaide (δ 2.50 in ^{1}H NMR, δ 39.5 in ^{13}C NMR) as an internal standard. ESI-TOF masses were recorded on a *Mariner (5076)* spectrometer, Applied Biosystems.

The reagents for DNA synthesis were purchased from Glen Research. Mass spectra of oligodeoxynucleotides were determined with a MALDI-TOF MS (Shimadzu AXIMA-LNR, positive mode)

Table 2
Thermal melting properties and the fluorescence quantum yields

Duplexes	T_m (°C)	Φ_F	Duplexes	T_m (°C)	Φ_F
ODN **3**	—	0.030	ODN **3**/**5** [N=**G**]	56.0	0.009
ODN **4**	—	0.023	ODN **4**/**6** [N=**G**]	63.0	0.004
ODN **7**/**5** [N=**G**]	53.7	—	ODN **8**/**6** [N=**G**]	61.1	—
MB2	50.4	0.018	**MB2**/ODN **9**	52.8	0.048
MB3	45.7	0.001	**MB3**/ODN **9**	47.4	0.037
MB5	46.7	0.020	**MB5**/ODN **9**	53.9	0.061
MB6	47.5	0.008	**MB6**/ODN **9**	50.4	0.130
MB8	49.5	0.005	**MB8**/ODN **9**	52.2	0.120

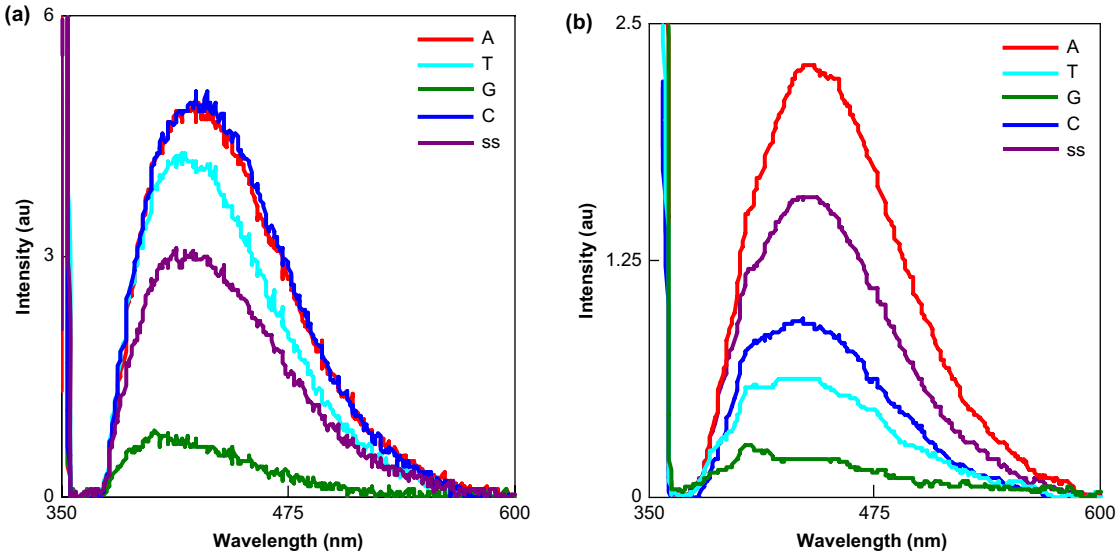


Figure 2. Fluorescence spectra of ODN **3** (a) and ODN **4** (b) (2.5 μM) and the duplexes formed by hybridization with ODN **5** and **6** (**N**=A, T, G, C), respectively (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, rt). Excitation wavelength was 346 nm. 'ss' denotes single stranded ODN.

with 2′,3′,4′-trihydroxyacetophenone as a matrix. Calf intestinal alkaline phosphatase (Promega), Crotalus adamanteus venom phosphodiesterase I (USB), and *Penicillium citrinum* nuclease P1 (Roche) were used for the enzymatic digestion of ODNs. All aqueous solutions utilized purified water (Millipore, Milli-Q sp UF). Reversed-phase HPLC was performed on CHEMCOBOND 5-ODS-H columns (10×150 mm, 4.6×150 mm) with a JASCO Chromatograph, Model PU-2080, using a UV detector, Model UV-2075 plus at 260 nm.

4.2. Procedure for the synthesis of compounds 2–6

*4.2.1. 5-[3-(Trifluoroacetamido)propynyl]-2′-deoxyuridine (**2**)*

5-Iodo-2′-deoxyuridine (**1**, 1.0 g, 2.8 mmol) was dissolved in dry DMF (20 ml). *N*-Trifluoroacetyl propynylamide (676.8 mg, 1.5 mmol), $Pd(PPh_3)_4$ (970.6 mg, 0.84 mmol), CuI (160.0 mg, 0.84 mmol), and Et_3N (1.2 ml, 8.46 mmol) were added to the reaction mixture and was stirred at room temperature under argon for overnight.[11] After the reaction is over, the reaction mixture was evaporated and purified by silica gel column chromatography ($CHCl_3$/MeOH=8:1) to yield **2** (938.1 mg, 2.49 mmol, 88%) as a colorless solid. ^{1}H NMR (CD_3OD, 400 MHz) δ_H 8.32 (s, 1H), 6.22 (dd, *J*=6.5, 6.6 Hz, 1H), 4.38 (ddd, *J*=3.7, 6.6, 9.6 Hz, 1H), 4.26 (s, 2H), 3.93 (ddd, *J*=3.0, 5.1, 6.6 Hz, 1H), 3.80 (dd, *J*=3.0, 12.0 Hz, 1H), 3.72 (dd, *J*=5.1, 12.0 Hz, 1H), 2.30 (ddd, *J*=3.7, 6.5, 13.6 Hz, 1H), 2.19 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ_C 29.5, 40.4, 61.4, 70.9, 76.7, 85.9, 87.1, 87.7, 98.5, 116.2 (q, *J*=284.9 Hz), 144.5, 149.9, 157.3 (q, *J*=37.2 Hz), 163.4; ESI-MS *m*/*z* 378.1 $[M+H]^+$; HR-ESIMS *m*/*z* 400.0745 $[M+Na]^+$ calcd for $C_{14}H_{14}F_3N_3O_6Na$, 400.0732.

*4.2.2. 2′-Deoxy-6-(N-trifluoroacetyl methylamido)-furanouridine (**3**)*

A round-bottomed flask was charged with 5-(*N*-trifluoroacetyl-propynylamido)-2′-deoxyuridine (**2**, 151.2 mg, 0.4 mmol) and MeOH (3 ml), and CuI (15.3 mg, 0.08 mmol) was then added along with Et_3N (2 ml). This mixture was heated at 60 °C for 3 h until the cyclization reaction was completed as monitored by TLC. The solvent was removed and the residue was dried under vacuum for 1 h. The resulting residue was purified by silica gel column chromatography using gradient elution with $CHCl_3$/MeOH (8:1 to 5:1) to yield desired compound **3** (116.7 mg, 0.309 mmol, 77%) as a colorless foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 10.13 (t, *J*=5.6 Hz, 1H), 8.80 (s, 1H), 6.66 (s, 1H), 6.15 (dd, *J*=6.1, 6.1 Hz, 1H), 5.12 (t, *J*=5.3 Hz, 1H), 4.46 (d, *J*=5.6 Hz, 2H), 4.23 (m, 1H), 3.93 (m, 1H), 3.70–3.59 (complex, 2H), 2.41 (ddd, *J*=4.2, 6.1, 13.5 Hz, 1H), 2.04 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 36.8, 41.8, 61.4, 70.4, 88.3, 88.9, 103.3, 106.2, 116.4 (q, *J*=286.2 Hz), 139.2, 152.5, 154.3, 157.1 (q, *J*=36.5 Hz), 171.8; ESI-MS *m*/*z* 378.1 $[M+H]^+$; HR-ESIMS *m*/*z* 400.0743 $[M+Na]^+$ calcd for $C_{14}H_{14}F_3N_3O_6Na$, 400.0732.

*4.2.3. 2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-6-(N-trifluoroacetyl methylamido)-furanouridine (**4**)*

To a solution of **3** (635.6 mg, 1.686 mmol) and *N*,*N*-dimethylaminopyridine (catalytic amount) in anhydrous pyridine (10 ml) was added 4,4′-dimethoxytrityl chloride (685.5 mg, 2.02 mmol) and stirred at room temperature for overnight. After completion of the reaction, MeOH (1.0 ml) was added to the reaction mixture. The solvent was then evaporated and the crude mixture was purified by silica gel column chromatography ($CHCl_3$/MeOH/Et_3N=40:1:1) to yield **4** (960.9 mg, 1.41 mmol, 84%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 10.11 (s, 1H), 8.62 (s, 1H), 7.40–7.38 (complex, 2H), 7.32–7.21 (complex, 8H), 6.94–6.89 (complex, 4H), 6.15 (dd, *J*=5.0, 6.3 Hz, 1H), 6.02 (s, 1H), 5.42 (d, *J*=4.8 Hz, 1H), 4.42 (s, 2H), 4.33 (m, 1H), 4.03 (m, 1H), 3.74 (s, 6H), 3.35–3.27 (complex, 2H), 2.46 (m, 1H), 2.19 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 36.7, 46.3, 55.7 (2C), 60.4, 63.4, 69.8, 86.6, 88.2, 102.6, 106.1, 113.9 (4C), 116.4 (q, *J*=286.2 Hz), 127.4, 128.2 (2C), 128.6 (2C),130.4 (2C), 130.4 (2C), 135.9, 136.1, 138.7, 145.5, 152.7, 154.2, 157.3 (q, *J*=36.5 Hz), 158.8 (2C), 171.7; ESI-MS *m*/*z* 680.3 $[M+H]^+$; HR-ESIMS *m*/*z* 702.2066 $[M+Na]^+$ calcd for $C_{35}H_{32}F_3N_3O_8Na$, 702.2039.

*4.2.4. 2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-6-(N-trifluoroacetyl-methylamido)-pyrrolocytidine (**5**)*

A two necked round-bottomed flask equipped with a reflux condenser was charged with 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-6-(*N*-trifluoroacetyl methylamido)-furanouridine (**4**, 96.2 mg, 0.142 mmol), concentrated NH_4OH (3 ml), and MeOH (4 ml), and then stirred at 55 °C. Progress of the reaction was followed by TLC. After completion of the reaction (24 h), the solvent was removed under vacuum and dried. The crude reaction mixture was treated with 5 ml of MeOH containing ethyl trifluoroacetate (72.2 mg, 23 μl, 0.191 mmol) and was stirred for 12 h at room temperature. After completion of the reaction, the solvent was removed and the residue was further dried under vacuum. The resulting residue was

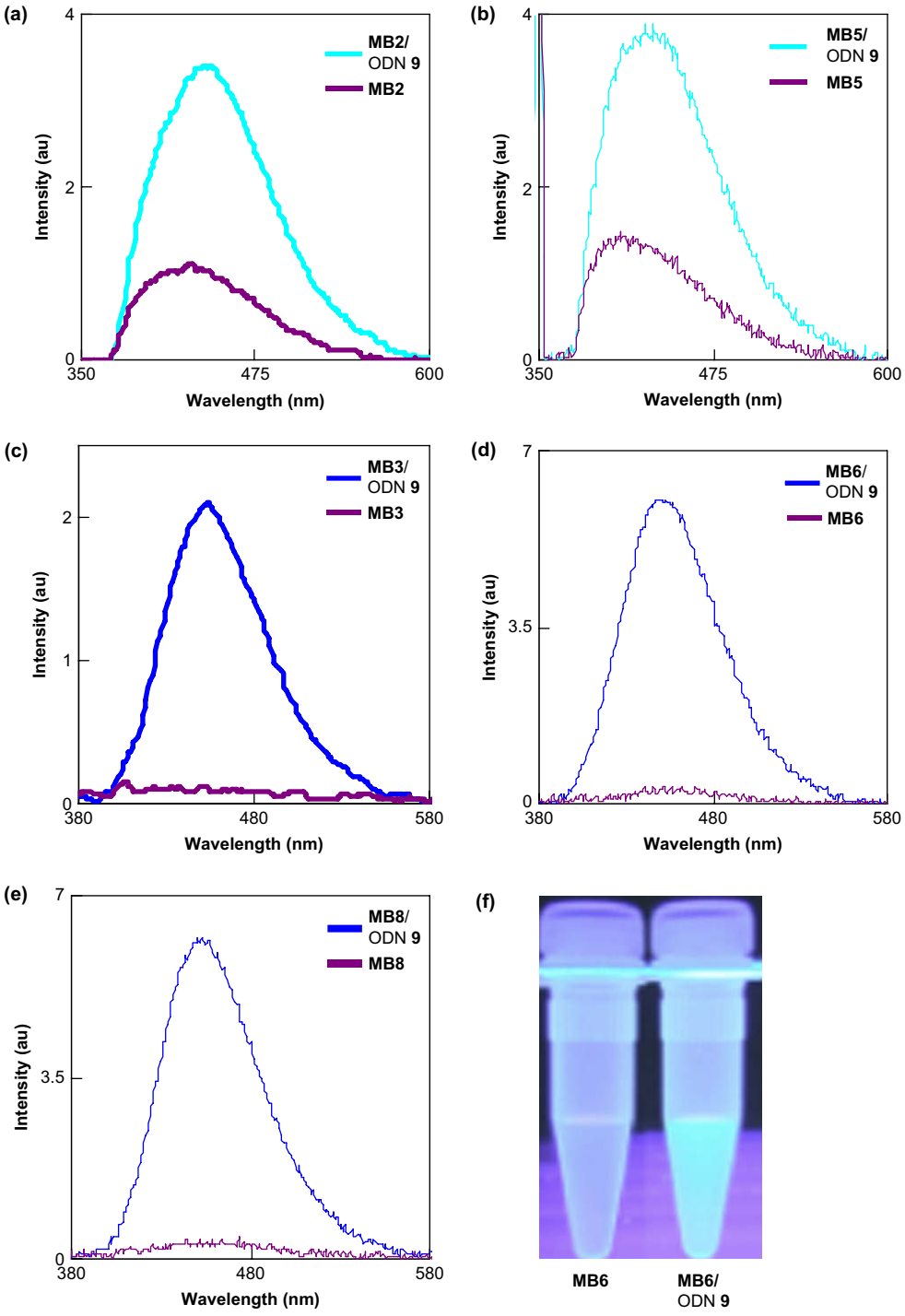


Figure 3. Fluorescence spectra of hairpin **MB2** (a), **MB5** (b), **MB3** (c), **MB6** (d), and **MB8** (e) and the duplexes formed by hybridization with ODN **9** (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, rt). Excitation wavelength was 346 nm for **MB2** and **MB5** and was 369 nm for **MB3**, **MB6**, and **MB8**. '**MB**' denotes hairpin state. (f) Photograph displaying the emission behavior at 25 °C of hairpin **MB6** alone and of the duplex with target ODN **9** (2.5 μM) upon illumination at 302 nm.

purified by silica gel column chromatography using $CHCl_3$/MeOH/Et_3N=100:10:1 to yield desired compound **5** (48.1 mg, 0.071 mmol, 50%) as a colorless foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 11.30 (br, 1H), 9.90 (br, 1H), 8.46 (s, 1H), 7.42–7.40 (complex, 2H), 7.32–7.22 (complex, 8H), 6.93–6.88 (complex, 4H), 6.26 (dd, J=5.9, 6.1 Hz, 1H), 5.38 (d, J=4.2 Hz, 1H), 4.34 (complex, 3H), 4.00 (dd, J=4.4, 7.7 Hz, 1H), 3.73 (s, 6H), 3.29 (complex, 2H), 2.40 (m, 1H), 2.11 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 37.0, 46.3, 55.7 (2C), 63.7, 70.1, 86.3, 86.6, 87.3, 98.0, 108.8, 113.9 (4C), 116.5 (q, J=286.4 Hz), 124.5, 128.3 (2C), 128.4 (2C), 130.4 (4C), 135.8, 135.9, 137.8, 145.5, 154.3, 157.1 (q, J=36.1 Hz), 158.8 (2C), 159.8, 172.7; ESI-MS m/z 679.2 $[M+H]^+$; HR-ESIMS m/z 701.2206 $[M+Na]^+$ calcd for $C_{35}H_{33}F_3N_4O_7Na$, 701.2199.

*4.2.5. 2′-Deoxy-3′-(2-cyanoethyldiisopropylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-6-(N-trifluoroacetylmethylamido)-pyrrolocytidine (**6**)*

To a solution of **5** (77.2 mg, 0.114 mmol) and Et_3N (1 ml) in anhydrous CH_2Cl_2 (3 ml) was added 2-cyanoethyldiisopropylchlorophosphorodiamidate (80 μl) under argon atmosphere. The resulting mixture was stirred at room temperature for 1 h and was

then evaporated to dryness. The residue was dissolved in EtOAc and was washed with aq $NaHCO_3$ and brine. The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated to dryness and was subsequently used for oligodeoxynucleotide synthesis without further purification.

4.3. Oligonucleotide synthesis and characterization

All the reagents for DNA synthesis were purchased from Glen Research. ODNs were synthesized by a conventional phosphoramidite method by using an Applied Biosystems 3400 DNA/RNA synthesizer. ODNs were purified by reverse phase HPLC on a 5-ODS-H column (10×150 mm, elution with 50 mM ammonium formate buffer (AF), pH 7.0, linear gradient over 45 min from 3% to 40% acetonitrile at a flow rate 2.0 ml/min). ODNs containing modified nucleotides were fully digested with calf intestine alkaline phosphatase (50 U/ml), and P1 nuclease (50 U/ml) at 37 °C for 12 h. Digested solutions were analyzed by HPLC on a CHEMCOBOND 5-ODS-H column (4.6×150 mm, elution with a solvent mixture of 50 mM AF buffer, pH 7.0, flow rate 1.0 ml/min). The concentration of each ODNs was determined by comparing peak areas with a standard solution containing dA, dC, dG, and dT at a concentration of 0.1 mM. Mass spectra of ODNs purified by HPLC were determined with a MALDI-TOF mass spectrometer.

4.3.1. *Synthesis of modified **G**-quenched molecular beacons (**MB**)*

The fluorophores were post-synthetically incorporated into alkylamino substituted **MB** to get desired molecular beacons, **MB2**, **3**, **5**, **6**, and **8**. Thus, active esters (1.0 mg) were dissolved in a small amount of dry DMF (20 μl) and added to amino modified **MBs** (20 μl) in a total volume of 150 μl of 1.0 M $NaHCO_3$ and incubated for 8 h at 37 °C. Purification and the characterization of the products were performed according to the standard procedure as described above.

4.3.2. *Melting temperature (T_m) measurements*

All T_ms of the ODNs (2.5 μM, final duplex concentration) were taken in 50 mM sodium phosphate buffers (pH 7.0) containing 100 mM sodium chloride. Absorbance versus temperature profiles were measured at 260 nm using a Shimadzu UV-2550 spectrophotometer equipped with a Peltier temperature controller using 1 cm path length cell. The absorbance of the samples was monitored at 260 nm from 4 °C to 90 °C with a heating rate of 1 °C/min. From these profiles, first derivatives were calculated to determine T_m values.

4.3.3. *UV absorption measurements*

ODN solutions were prepared as described in T_m measurement experiment. Absorption spectra were obtained using a Shimadzu UV-2550 spectrophotometer at room temperature using 1 cm path length cell.

4.3.4. *Fluorescence measurements*

ODN solutions were prepared as described in T_m measurement experiment. Fluorescence spectra were obtained using a Shimadzu RF-5300PC spectrophotometer at 25 °C using 1 cm path length cell. The excitation and the emission bandwidth was 1.5 nm.

4.3.5. *MALDI-TOF mass spectral data for the ODNs*

See Table 3.

Table 3
MALDI-TOF mass spectral data for the ODNs

ODNs	MALDI-TOF mass calcd $[M+H]^+$	MALDI-TOF mass found $[M+H]^+$
ODN **1**	3957.68	3956.97
ODN **2**	3927.66	3928.47
ODN **3**	4185.93	4186.72
ODN **4**	4155.90	4156.27
ODN **10 MB1**	7745.15	7745.14
ODN **11 MB2**	7973.40	7972.97
ODN **12 MB3**	8041.48	8041.12
ODN **13 MB4**	8361.57	8361.04
ODN **14 MB5**	8589.83	8590.02
ODN **15 MB6**	8657.90	8658.83
ODN **16 MB7**	8361.57	8361.62
ODN **17 MB8**	8657.90	8657.09

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Supplementary data

Some photophysical spectra, copies of ^1H, ^{13}C NMR spectra of selected compounds. This material is available free of charge via the Internet at http://elsevier.com. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.10.093.

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