Synthesis of 5- and 6-Hydroxymethylfluorescein Phosphoramidites

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The incorporation of nonradioactive reporter groups into oligonucleotide probes continues to be of wide interest (see Mattingly et al.[1] and references therein). Fluorescein derivatives, in particular, have received much attention both in the research lab and in general commerce. The oldest approach taken to incorporate the fluorophore has been the simple postsynthetic labeling of an amino-substituted oligonucleotide with fluorescein isothiocycanate (FITC).[2−5] In addition to the inconvenience of dealing with a large excess of unreacted FTIC during the purification of the oligo, FITC conjugates are notoriously unstable.[6] Recently, methods have been developed to randomly introduce thiol groups into oligonucleotides and nucleic acids,[7] allowing fluorescein to be introduced via its maleimide[8] derivative. Unfortunately, this random labeling approach is not useful for every application. Fluorescein-containing phosphoramidites address this deficiency by allowing the fluorescein group to be selectively placed during the solid-phase synthesis of oligonucleotides. Fluorescein phosphoramidites have been reported that explore such variables as the point of attachment to the fluorescein nucleus and the type of linker employed. Nelson et al.[9] reported the preparation of a FITC-based, 2-aminobutyl-1,3-propanediol-linked phosphoramidite. Others have coupled the widely available isomers of carboxyfluorescein to acyclic aminodiols that were subsequently converted into *O*-4,4′-dimethoxytrityl (DMT)-protected phosphoramidites.[10,11] More recently a 5(6)-carboxyfluorescein phosphoramidite employing a 4-aminocyclohexyl-1,1-dimethanol scaffolding was reported.[12] The authors suggested that this backbone minimized steric hindrance, leading to improved coupling efficiency. Because the scaffolding employed in these phosphoramidites would interrupt the base sequence in the synthetic oligonucleotide, they are most often used for either 5′ or 3′ labeling. Some researchers have labeled nucleoside phosphoramidites with fluorescein so that the label can be placed anywhere in the sequence without generating a base deletion. 5(6)-Bromomethylfluorescein was used to alkylate N^3 of pyrimidine nucleosides, which were subsequently converted into their 5′-DMT-3′-phosphoramidites.[13] Pfleiderer's group[14] prepared purine and pyrimidine phosphoramidites conjugated to 5-aminofluorescein via a urea linkage. C^5-Fluoresceinated dT derivatives have also been described[15] and are commercially available.[16]

The flexibility to place the fluorescein label anywhere in the oligonucleotide sequence can be advantageous at the research/design stage of oligonucleotide probe preparation. However, if only 3′-labeling is required at the production stage, it may be more economical to use a fluorescein-substituted solid support, rather than a phosphoramidite. If 5′-labeling is all that is required, a simpler, monofunctional phosphoramidite could be more cost-effective. For this purpose 5(6)-carboxyfluorescein was coupled to 6-aminohexanol, and the resulting amide was further elaborated to the phosphoramidite.[11,17] Likewise, a fluorescein phosphoramidite stemming from the xanthene face was prepared from fluorescein methyl ester after alkylation with 4-chloro-*O*-DMT-1-butanol. The 3-*O*-(4-hydroxybutyl)fluorescein methyl ester was deprotected, converted into the phosphoramidite, and efficiently (>90%) introduced into oligos at the 5′ end. However, the quantum efficiency of the fluorophore so attached was only 70% of the 5(6)-carboxy-linked fluorescein derivatives.

Recently, 5- and 6-hydroxymethylfluorescein-3′,6′-diacetate served as intermediates in the synthesis of 5- and 6-aminomethylfluorescein (AMF)[18] and 5- and 6-(*O*-fluoresceinylmethyl)hydroxylamine (OFMHA).[19,20] We thought that this versatile intermediate could easily be converted into a simple, monofunctional phosphoramidite (**9**, Scheme 1) for 5′-oligonucleotide labeling in one step at a reasonable scale.

In the original Mattingly report,[18] 5(6)-hydroxymethylfluorescein was isolated as the 3′,6′-diacetate after reduction of the corresponding ethyl carbonic anhydride of carboxyfluorescein (aqueous THF, pH 6, 0 °C) with excess sodium borohydride. The ease with which the acetate protecting groups are removed made this derivative too labile for the present purpose. All of the commercially available fluorescein phosphoramidites protect the 3′ and 6′ hydroxyl groups as pivalate esters, the increased steric hindrance providing enhanced stability over the corresponding acetates. Thus, as shown in

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(1) Mattingly, P. G.; Fino, J. R.; Ray, K. *Bioconjugate Chem.* **1996**, *7*, 274−80.
(2) De Bellis, G.; Manoni, M.; Pergolizzi, R.; Luzzana, M. *Nucleic Acids Res.* **1990**, *18*, 4951−2.
(3) Snitman, D. L.; Stroupe, S. D. U.S. Patent 5,273,882.
(4) Snitman, D. L.; Stroupe, S. D. U.S. Patent 5,641,630.
(5) Hagmar, P.; Bailey, M.; Tong, G.; Haralambidis, J.; Sawyer, W. H.; Davidson, B. E. *Biochim. Biophys. Acta* **1995**, *1244*, 259−68.
(6) Banks, P. R.; Paquette, D. M. *Bioconjugate Chem.* **1995**, *6*, 447−58.
(7) Westling, M. E.; Daniel, S. G. U.S. Patent 5,700,921.
(8) Haugland, R. P. U.S. Patent 4,213,904.
(9) Nelson, P. S.; Kent, M.; Muthini, S. *Nucleic Acids Res.* **1992**, *20*, 6253−9.
(10) Jadahav, V. R.; Barawkar, D. A.; Natu, A. A.; Ganesh, K. N. *Nucleosides Nucleotides* **1997**, *16*, 107−14.
(11) Theisen, P.; McCollum, C.; Upadhya, K.; Jacobson, K.; Vu, H.; Andrus, A. *Tetrahedron Lett.* **1992**, *33*, 5033−6.
(12) Su, S.-H.; Iyer, R. S.; Aggarwal, S. K.; Kalra, K. L. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1639−44.
(13) Holletz, T.; Moeller, U.; Knaf, A.; Reinhardt, R.; Cech, D. *Liebigs Ann. Chem.* **1993**, 1051−6.
(14) Sigmund, H.; Maier, T.; Pfleiderer, W. *Nucleosides Nucleotides* **1997**, *16*, 685−96.
(15) Ruth, J. L. U.S. Patent 4,948,882.
(16) Glen Research, Sterling, VA.
(17) Brush, C. K. U.S. Patent 5,583,236.
(18) Mattingly, P. G. *Bioconjugate Chem.* **1992**, *3*, 430−1.
(19) Adamczyk, M.; Chen, Y.-Y.; Grote, J.; Mattingly, P. G. *Steroids* **1999**, *64*, 283−90.
(20) Adamczyk, M.; Mattingly, P. G.; Moore, J. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3599−602.

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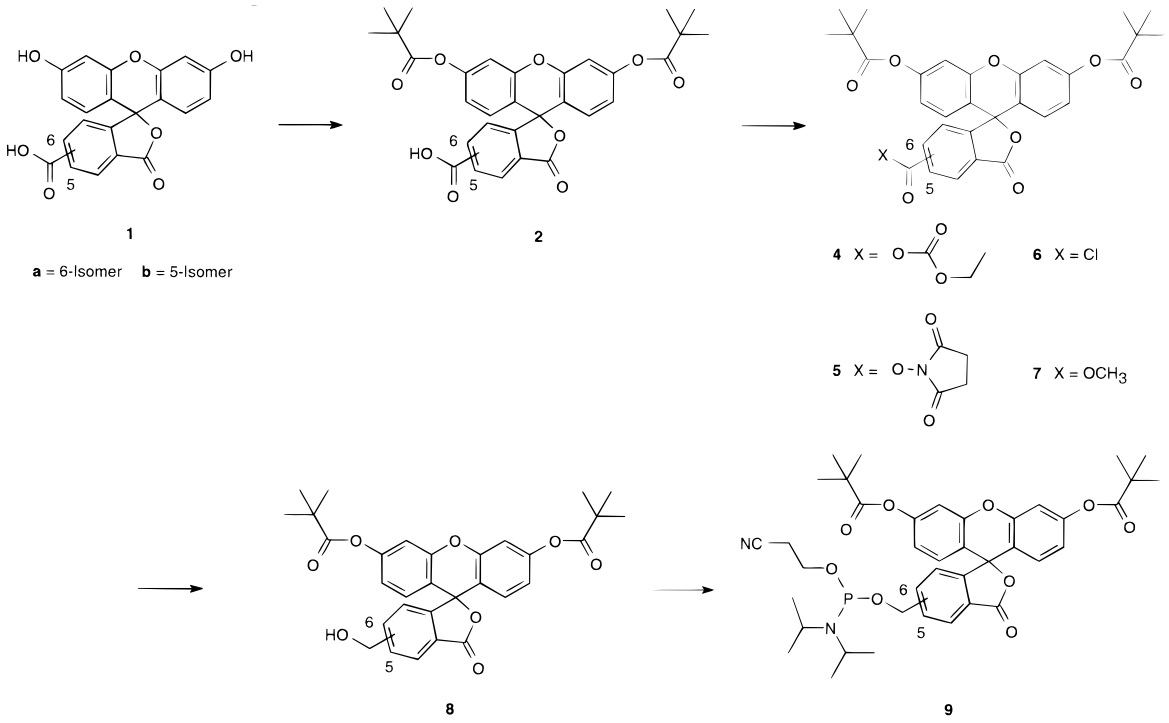
Scheme 1



Scheme 1, a mixture of 5(6)-carboxyfluorescein was converted to the bis-pivalate ester mixture **2** (93%). The literature[21] suggested that the 5(6)-isomers could be separated at this point by selective crystallization of the 6-isomer as its diisopropylamine salt from ethanol, and the 5-isomer could be recovered from the supernatant and recrystallized from nitromethane. We were never successful in obtaining the 5-isomer in pure form via this route. The isomers could be separated by preparative reversed-phase HPLC, but it was found that separation was more efficient at a later stage of the synthesis.

The 5(6)-isomer mixture was carried forward and converted to the ethyl carbonic anhydride **4** quantitatively. Reduction under the previous conditions[18] gave the alcohol **8** in inconsistent yields at best. A survey of the reaction byproducts suggested that reduction and/or hydrolysis of the pivalate group was competitive with reduction of the carbonic anhydride.

To avoid hydrolysis of the pivalate groups we investigated anhydrous reducing conditions using lithium borohydride. Nonselective reduction was addressed by careful examination of the effect of temperature on the reduction of **4** and other activated carboxyfluorescein derivatives. Thus, the *N*-hydroxysuccinimide (NHS) active ester **5** was prepared from the acid **3** by the action of EDC/NHS in 97% yield after flash chromatography on silica. The carboxyfluorescein acid chloride **6** was prepared by reaction with oxalyl chloride in the presence of catalytic DMF.

The results of the reduction study are listed in Table 1. The reduction of NHS ester **5** was much more sluggish than that of either the carbonic anhydride or the acid chloride. It was completely unreactive at −60 °C. At higher temperatures consumption of the activated ester was slow, generating nearly equal amounts of the desired alcohols and the undesired depivalated byproducts. The acid chloride **6** was much more easily reduced. The slow addition of lithium borohydride in THF at −60 °C gave the desired alcohol mixture **8** in 70−85% yield. The carbonic anhydride **4** performed similarly.

Table 1. Reduction of Activated Carboxyfluoresceins with Lithium Borohydride

		products (%)		
compound	temp (°C)	alcohol **8**	unreacted	other
5	−20	12.8	77.3	9.9
	−40	15	70.5	14.5
	−60	0	100	0
6	−60	85.6	1	13.4
4	−60	83.6	4.9	11.8

The key hydroxymethylfluorescein derivatives **8a** and **8b** were conveniently separated by silica gel chromatography at this stage and then converted to the corresponding phosphoramidites (**9a** and **9b**) under standard conditions.[1]

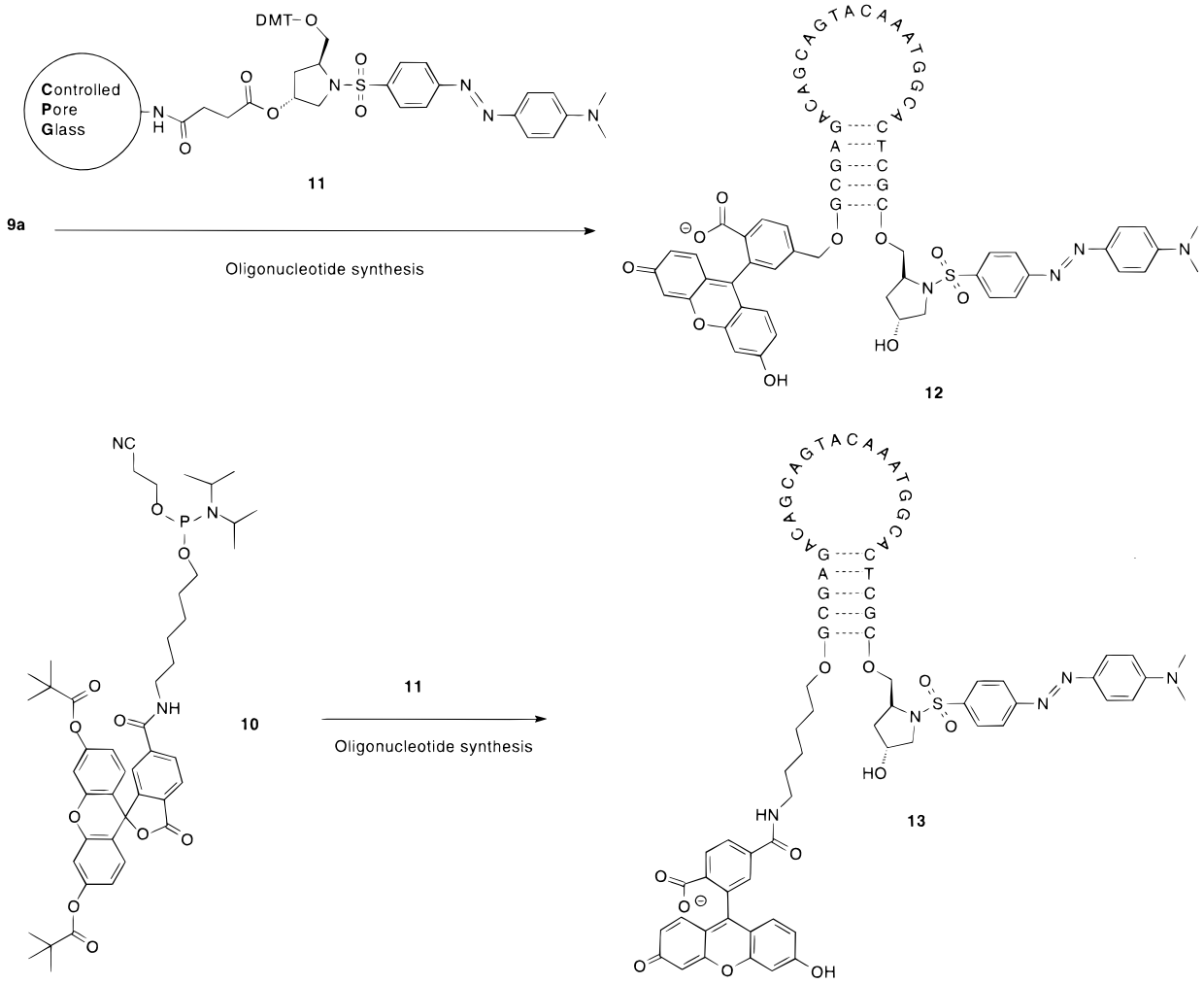
The efficacy of the hydroxymethylfluorescein-derived phosphoramidites **9a** and **9b** were evaluated in a molecular beacon format [22,23] and compared to the commercially available amidite **10** (Scheme 2). The molecular beacon format is a relatively new, homogeneous nucleic acid detection technology that employs single-stranded oligonucleotide probes in a stem−loop−stem arrangement. The stems are alternately labeled with a fluorophore and quencher and are complementary, while the loop carries a sequence complementary to the target nucleic acid. In the absence of target, the stems form a duplex, and the fluorescence signal is quenched. In the presence of the target nucleic acid, the fluorophore and quencher cannot adopt this conformation, and the fluorescent signal is restored. Likewise, heating the probe above its melt

(21) Rossi, F. M.; Kao, J. P. Y. *Bioconjugate Chem.* **1997**, *8*, 495−7.

(22) Marras, S. A.; Kramer, F. R.; Tyagi, S. *Genet. Anal.* **1999**, *14*, 151−6.

(23) Leone, G.; van Schijndel, H.; van Gemen, B.; Kramer, F. R.; Schoen, C. D. *Nucleic Acids Res.* **1998**, *26*, 2150−5.

Scheme 2



temperature also disrupts the stem duplex, and fluorescence returns.

Thus, as shown for **9a**, an oligonucleotide was prepared starting with a DNA synthesis support labeled with a fluorescence-quenching dye (dabsyl-CPG, **11**). A short sequence of five bases (5′-CGCTC-3′) was added via automated synthesis, followed by a longer 18-base target sequence.[24] The last five bases added were complementary to the beginning sequence (3′-GAGCG-5′). The molecular beacon was completed by the addition of **9a**, then removed from the solid support, and purified under standard conditions to afford **12**. The same procedure was followed to prepare the **10**-derived molecular beacon **13**. Both beacons were prepared in high yield and purity. The integrity of the fluorescein and dabsyl labels was confirmed by ESI/MS.

The fluorescence characteristics of the molecular beacons were investigated in both the closed and open conformations. At ambient temperature the complementary five-base stem regions of the molecular beacon hybridize, bringing the fluorescein and dabsyl moieties close enough to result in fluorescence quenching. This closed form of the molecular beacons **12** and **13** exhibited a very low level of fluorescence (1.5% and 3.2%, respectively) relative to the open conformer obtained after hybridization to the complementary oligonucleotide target (data not shown). The excitation/emission profiles recorded for of each of the probes under these conditions (Figure 1) were nearly superimposable (**12**, λ $492_{ex}/520_{em}$ nm; **13**, λ $493_{ex}/520_{em}$ nm).

Figure 2 shows the similarity of the fluorescence quenching profiles of the two molecular beacon oligonucleotides as a function of temperature. In the absence of the synthetic target (Figure 2A) both beacons show increasing fluorescence as the temperature increases above 50 °C, corresponding to the open conformation. In the presence of an excess of the synthetic target (Figure 2B), beacons **12** and **13** are in the open conformation regardless of the temperature. However, because the fluorescence efficiency of the fluorescein label is lessened at higher temperature, the relative fluorescence of the beacons increases as the temperature decreases.

In conclusion, the use of the pivaloyl group to protect the phenolic hydroxyl groups of fluorescein offered some advantage in stability over the previously employed acetyl groups in the preparation hydroxymethylfluorescein derivatives. However, partial deprotection was still observed. Reduction of **6** and the previously unreported carboxyfluorescein acid chloride derivative **4** at low temperature mitigated the problem and allowed for good yields of **8**. Conversion of this intermediate was convenient for the preparation of fluorescein phosphoramidite **9**. No deleterious effects were caused by the short linker

(24) Kroeger, P. E.; Abravaya, K.; Esping, C. A. C.; Gorzowski, J. J.; Hoenle, R. J.; Moore, J. J. PCT Int. Appl. WO 9858086.

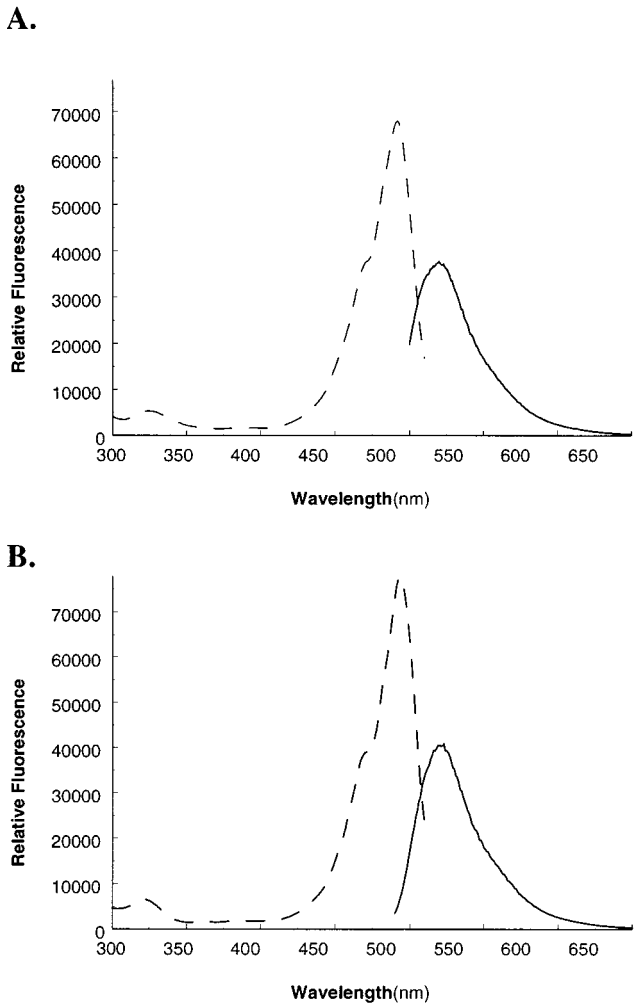


Figure 1. Fluorescence excitation and emission spectra of molecular beacons **12** (A) and **13** (B). Probes were hybridized with a 5-fold excess of synthetic oligonucleotide target (100 mM Tris, 10 mM $MgCl_2$, pH 7.5) Excitation spectra (---) were recorded with a fixed emission wavelength of 520 nm; emission spectra used an excitation wavelength of 470 nm.

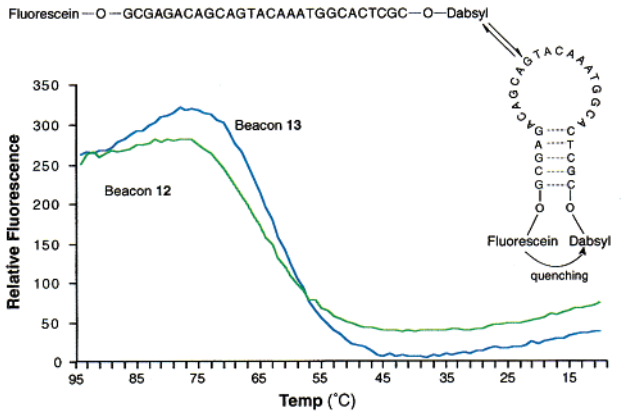
between the fluorescein nucleus and the reactive phosphoramidite groups in either the synthesis of oligonucleotides using **9** or the performance of those oligonucleotides in a molecular beacon format.

Experimental Section

Reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise noted. ^{1}H (300 MHz), ^{13}C (75 MHz), and ^{31}P NMR (121 MHz) spectra were recorded in deuteriochloroform. Chemical shifts are reported in ppm (δ) using tetramethylsilane (TMS, ^{1}H, ^{13}C) as the internal reference; coupling constants (J) are in Hz; ^{31}P NMR was performed with phosphoric acid as the reference. Mass spectrometry analyses were carried out using electrospray ionization (ESI/MS) in the positive ion mode unless otherwise noted. Elemental analyses were performed by Robertson Microlit Laboratories, Inc. (Madison, NJ). IUPAC names of new compounds were obtained using the ACD/ILab Web service version 3.5 at http://www.acdlabs.com/ilab.

3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-5(6)-carboxylic Acid (2). 5(6)-Carboxyfluorescein (Arcos Organics, Pittsburgh, PA, 100 g, 266 mmol) was dried at 100 °C for 48 h under reduced pressure (25−30 mmHg) over phosphorus pentoxide to afford **1** (97 g). To the dried 5(6)-carboxyfluorescein **1** (30 g, 79.5 mmol) in DMF (188 mL) were added diisopropylethylamine (55.5 mL, 318 mmol) and finally trimethylacetic anhydride (32.7 mL, 175 mmol). The

A. Fluorescence intensity in the absence of hybridization with the target sequence as a function of temperature.



B. Fluorescence intensity from hybridization with the target sequence as a function of temperature.

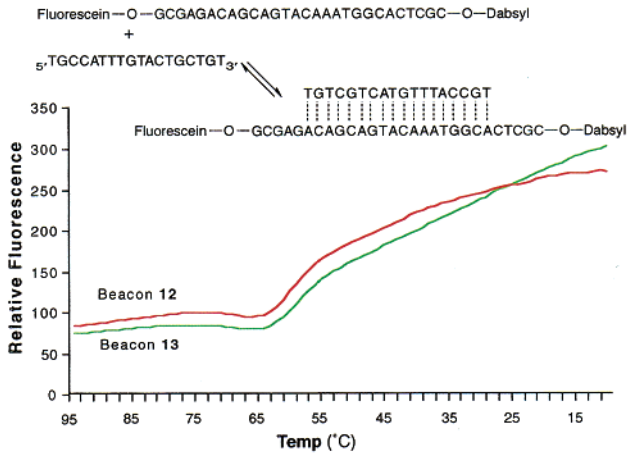


Figure 2. Comparison of the performance of **12** and **13** 5′-fluorescein/3′-dabsyl-labeled oligonucleotides.

reaction solution was stirred under nitrogen at ambient temperature for 72 h protected from light. The solvent was removed under reduced pressure to afford a brown oil. This residue was dissolved in dichloromethane (200 mL) and then further diluted with ethyl acetate (400 mL). The solution was then washed with aqueous sodium phosphate buffer (1 M, pH 7.0, 3 × 400 mL) followed by aqueous sodium chloride (5 M, 400 mL). After drying over anhydrous sodium sulfate and filtering, the solvent was removed in vacuo to give **2** as a tan, glassy foam (40 g, 73.5 mmol, 92.5%). ^{1}H NMR δ 9.52 (1 H, bs), 8.78 (0.6 H, s, 5-isomer), 8.37 (1 H, dt, J = 1.5, 8.1), 8.14 (0.4 H, d, J = 8.0, 6-isomer), 7.89 (0.4 H, s, 6-isomer), 7.28 (0.6 H, d, J = 8.1, 5-isomer), 7.10−7.08 (4 H, m), 6.96−6.77 (8 H, m), 1.36 (18 H, s). ^{13}C NMR δ 176.46 (5), 176.42 (6), 168.85 (6), 168.59 (5), 168.19 (6), 168.09 (5), 157.09 (5), 153.02 (6), 152.69 (5), 152.67 (6), 151.48 (6), 151.38 (5), 136.63 (5), 136.42 (6), 132.16 (6), 131.67 (5), 129.73 (6), 128.68 (5,6), 128.13, 127.42 (5), 126.48 (5), 125.67 (6), 125.34 (6), 124.29 (5), 117.83 (5), 117.79 (6), 115.39 (6), 115.32 (5), 110.41 (5,6), 82.13 (6), 81.89 (5), 39.13 (5,6), 26.97 (5,6). ESI/MS m/z 545 $(M + H)^+$. HPLC [Waters NovaPak C_{18}, 3.9 mm × 150 mm, 15:60:15:10 acetonitrile/methanol/water/5% aqueous

acetic acid, 1 mL/min, 230 nm] 6-isomer (**2a)** (9.1 min, 38.5%), 5-isomer (**2b)** (10.5 min, 57.8%).

3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-5(6)-carboxylic Acid Chloride (6). Compound **2** (40 g, 73.5 mmol) was dissolved in dry dichloromethane (900 mL). DMF (0.27 mL, 3.7 mmol) was added. Oxalyl chloride (2 M in dichloromethane, 54.8 mL, 110 mmol) was then added over 10 min via syringe. After 45 min of stirring under nitrogen, the solvent was removed in vacuo. The residue was taken up in dichloromethane (900 mL) and then evaporated in vacuo to give **6** as a glassy foam. ^{1}H NMR δ 8.81 (0.5 H, s, 5-isomer), 8.37 (1 H, d, J = 8.0), 8.18 (0.5 H, d, J = 8.4, 6-isomer), 7.88 (0.5 H, s, 6-isomer), 7.33 (0.5 H, d, J = 8.1, 5-isomer), 7.12−7.08 (4 H, m), 6.90−6.72 (8 H, m), 1.36 (18 H, s). ^{13}C NMR δ 176.40 (5), 176.33 (6), 167.32 (6), 167.26 (5), 167.19 (6), 166.98 (5), 158.52 (5), 153.36 (6), 152.88 (5), 151.46 (6), 151.40 (6), 151.32 (5), 139.17 (6), 136.99 (5), 135.40, 132.53, 131.14, 128.88, 128.54, 128.13, 127.01, 126.68, 125.86 (6), 125.34 (6), 124.88 (5), 117.98 (5, 6), 114.86 (6), 114.75 (5), 110.57 (5,6), 82.23 (6), 81.99 (5), 39.14 (5,6), 26.98 (5,6). Anal. Calcd for $C_{31}H_{27}ClO_8$: C, 66.13; H, 4.83 Cl, 6.30. Found: C, 66.17; H, 4.72; Cl, 5.93. For HPLC analysis an aliquot was quenched with methanol. The resulting mixture of methyl esters **7a** and **7b** was analyzed [Waters NovaPak C_{18}, 3.9 mm × 150 mm, 15:60:15:10 acetonitrile/methanol/water/5% aqueous acetic acid, 1 mL/min, 230 nm] **2a** (2.4%), **2b** (3%), **7a**, (16.9 min, 47.1%), and **7b** (17.4 min, 32.8%).

3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-5(6)-hydroxymethyl-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (8). The acid chloride (**6**) was dissolved in THF (1 L) and then cooled to −60 °C under nitrogen. Lithium borohydride (2 M in THF, 74 mmol, 37 mL) was added via a syringe pump at a rate of about 350 μL/min. Stirring was continued at −60 °C for 2 h after the addition was completed, and then glacial acetic acid (7 mL, 111 mmol, 350 μL/min) was added. After an additional 15 min aqueous HCl (1 N, 400 mL) was added. The aqueous layer was retained, and the organic layer was washed with aqueous HCl (1 N, 400 mL). The combined aqueous layers were extracted with ethyl acetate (500 mL). The organic layers were combined and washed with aqueous sodium chloride (5 M, 400 mL), dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to afford mixed the 5(6)-hydroxymethylfluorescein **8 (**28.1 g, 53.0 mmol, 72%). HPLC [Waters NovaPak C_{18}, 3.9 mm × 150 mm, 15:60:15:10 acetonitrile/methanol/water/5% aqueous acetic acid, 1 mL/min, 230 nm] 6-isomer (**8a**) (6.9 min, 40.2%), 5-isomer (**8b**) (8.1 min, 55.7%).

The 5(6)-isomers were conveniently separated at this stage by flash chromatography. The alcohol mixture **8** (20 g, 37.7 mmol) was dissolved in dichloromethane (200 mL) and added to the sample injection module (SIM) of the Biotage system, which had been loaded with flash-grade silica gel (150 g). The chromatography was then carried out using a Biotage Flash75L column (75 mm × 300 mm, silica gel), eluting with dichloromethane/diethyl ether (90:10, 200 mL/min). The 6-hydroxymethylfluorescein isomer (**8a**) eluted first and was followed closely by the 5-hydroxymethylfluoescein isomer (**8b**). Each isomer was isolated as a foamy glass after evaporation of the homogeneous fractions in vacuo.

6-Isomer (8a): 6.2 g, 11.7 mmol, 31%. ^{1}H NMR δ 7.94 (1 H, d, J = 7.8), 7.55 (1 H, d, J = 7.2), 7.11 (1 H, s), 7.03 (2 H, d, J = 2.1), 6.84−6.72 (4 H, m), 4.67 (2 H, d, J = 4.8), 2.65−2.56 (1 H, m), 1.34 (18 H, s). ^{13}C NMR δ 176.53, 169.20, 153.59, 152.39, 151.33, 149.64, 128.77, 128.07, 125.06, 124.60, 121.23, 117.60, 116.04, 110.18, 81.52, 63.90, 39.07, 26.93. ESI/MS m/z 531 (M + H)$^+$. HPLC <1% 5-isomer (**8b)**. Anal. Calcd for $C_{31}H_{30}O_8$: C, 70.18; H, 5.70. Found: C, 69.91; H, 5.39.

5-Isomer (8b): 8.6 g, 16.2 mmol, 43%. ^{1}H NMR δ 8.03 (1 H, d, J = 0.5), 7.66 (1 H, dd, J = 1.4, 8.0), 7.12 (1 H, d, J = 8.0), 7.04 (2 H, d, J = 1.9), 6.83−6.72 (4 H, m), 4.81 (2 H, d, J = 5.1), 1.91 (1 H, t, J = 5.5), 1.34 (18 H, s). ^{13}C NMR δ 176.51, 169.24, 152.44, 151.87, 151.47, 143.82, 133.90, 128.80, 126.25, 123.90, 122.92, 117.60, 116.05, 110.20, 81.79, 63.87, 39.08, 26.95. ESI/MS m/z 531 (M + H)$^+$. HPLC <2% 6-isomer (**8a)**. Anal. Calcd for $C_{31}H_{30}O_8$: C, 70.18; H, 5.70. Found: C, 69.91; H, 5.55.

Preparation of 5- and 6-Fluoresceinylmethyl Phosphoramidites. General Method. Hydroxymethylfluorescein **8** (2.0 g, 3.8 mmol) was dissolved in dry dichloromethane (50 mL). Diisopropylethylamine (2.6 mL, 15.1 mmol) and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (1.2 mL, 5.2 mmol) were added simultaneously via separate syringes to the vigorously stirred reaction solution at ambient temperature. After 1 h, ethyl acetate was added, and the solution was washed with water and aqueous sodium chloride (5 M). The organic solution was dried over anhydrous sodium sulfate and filtered, and the solvent was removed in vacuo. The residue was dissolved in a minimum of dichloromethane and purified by flash chromatography on a silica gel column that had been pretreated with dichloromethane/triethylamine (9:1) and then washed with dichloromethane. The sample was applied and then eluted with ethyl acetate/dichloromethane/hexanes (10:15:75). Appropriate fractions were combined, and the solvent was removed in vacuo to give a colorless glassy foam.

2,2-Dimethyl-propanoic Acid, 6-[3-[Bis(1-methylethyl)-amino]-6-cyano-2,4-dioxa-3-phosphahex-1-yl]-3-oxospiro-[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-3′,6′-diyl Ester (9a). Yield: 1.3 g, (47%). ^{1}H NMR (CD_3CN) δ δ7.96 (1 H, d, J = 8.5), 7.65 (1 H, d, J = 7.2), 7.22 (1 H, s), 7.10 (2 H, d, J = 2.2), 6.93−6.81 (4 H, m), 4.82−4.68 (2 H, m), 3.73−3.63 (2 H, m), 3.60−3.48 (2 H, m), 2.54−2.50 (2 H, m), 1.32 (18 H, s), 1.10−1.20 (12 H, m). ^{13}C NMR (CD_3CN) δ 176.42, 169.62, 154.51, 153.59, 153.79, 152.28, 149.54, 149.44, 130.46, 129.98, 129.70, 125.85, 125.66, 122.69, 119.14, 118.26, 118.05, 117.42, 111.27, 81.98, 65.59, 65.34, 59.60, 59.34, 43.99, 43.83, 39.77, 27.21, 24.84, 24.74, 20.94, 20.84. ^{31}P NMR (CD_3CN) δ 150.42. Anal. Calcd for $C_{40}H_{47}N_2O_9P$: C, 65.74; H, 6.48; N, 3.83; P, 4.24. Found: C, 65.46; H, 6.41; N, 3.82; P, 4.37.

2,2-Dimethyl-propanoic Acid, 5-[3-[Bis(1-methylethyl)-amino]-6-cyano-2,4-dioxa-3-phosphahex-1-yl]-3-oxospiro-[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-3′,6′-diyl Ester (9b). Yield: 1.3 g, (47%). ^{31}P NMR δ 150.38. ^{1}H NMR δ 8.00 (1 H, s), 7.66 (1 H, dd, J = 1.4, 8.0), 7.14 (1 H, d, J = 7.8), 7.04 (2 H, d, J = 2.2), 6.84−6.75 (4 H, m), 4.94−4.66 (2 H, m), 3.98−3.80 (2 H, m), 3.74−3.62 (2 H, m), 2.67 (2 H, t, J = 6,32), 1.35 (18 H, s), 1.22 (12 H, m). ^{13}C NMR (CD_3CN) δ 177.38, 169.55, 153,76, 152.30, 143.55, 143.45, 136.80, 135.44, 135.36, 130.45, 129.98, 127.10, 125.61, 124.72, 124.65, 123.85, 123.74, 119.10, 118.21, 118.02, 117.35, 111.26, 82.13, 65.37, 65.12, 59.68, 59.42, 44.05, 43.88, 39.75, 27.21, 24.97, 24.94, 24.87, 24.84, 21.05, 20.96. Anal. Calcd for $C_{40}H_{47}N_2O_9P$: C, 65.74; H, 6.48; N, 3.83; P, 4.24. Found: C, 65.53; H, 6.53; N, 3.85; P, 4.12.

Oligonucleotide Preparation and Purification. 5′-(**9a**)-GCGAG-ACAGCAGTACAAATGGCA-CTCGC-3′(dabsyl) (**12**) and 5′-(**10**)-GCGAG-ACAGCAGTACAAATGGCA-CTCGC-3′-(dabsyl) (**13**) were synthesized using the standard 1 μmol scale protocol starting from dabsyl-labeled controlled pore glass (**11**). The coupling time for both fluorescein phosphoramidites **9a** and **10** was extended to 15 min. After synthesis was completed, cleavage and deprotection were carried out at 55 °C for 8 h with concentrated NH_4OH. The crude oligonucleotides were purified by ion-exchange chromatography [Dionex, DNA pack semi-preparative column, 9 mm × 250 mm, eluted with a linear gradient of 0.2−1.0 M NaCl in 20 mM NaOH, pH 12, 4 mL/min, UV detection 230−650 nm]. The fractions containing both the DNA and fluorescein absorptions were collected and analyzed with ion-exchange HPLC and by gel-filled capillary electrophoresis. The fractions with greater than 98% purity were combined and desalted. The concentration of the purified oligonucleotides was determined by UV absorption. Each molecular beacon was further characterized by ESI/MS (negative ion mode): **12** m/z 9509 (calcd 9505.5); **13** m/z 9623 (calcd 9618.9).

Molecular Beacon Evaluation. The fluorescence excitation and emission profiles (Figure 1) of the purified, fluorogenic probes **12** and **13** (100 nM in 100 mM Tris, 10 mM $MgCl_2$, pH 7.5) were measured in the presence of a 5-fold excess of the complementary synthetic target [5′TGCCATTTGTACTGCT3′]. Solutions of each probe and the complement were heated at 85 °C for 5 min and then rapidly cooled in ice. Excitation spectra were then recorded using a fixed emission wavelength of 520 nm (8 nm slit width), and the emission spectra were recorded using an excitation wavelength of 470 nm.

The fluorescence quenching profile of the purified, fluorogenic probes **12** and **13** were measured alone and with a complementary synthetic target as a function of temperature (Figure 2). A solution of each probe was prepared (100 uL in 10 mM Tris, 1 mM $MgCl_2$, pH 7.5). A second set of solutions containing the

fluorogenic probes and a 5-fold molar excess of synthetic target [5′TGCCATTTGTACTGCT3′] were prepared. The samples were placed in an Applied Biosystem 7700 sequencer, and the fluorescence of each solution was monitored (excitation, 480 nm; emission, 520 nm) as a function of temperature (95−25 °C in 1 °C per step cycle) with readings taken at each step.

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