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The use of 6-carboxynaphthofluorescein phosphoramidite in the automated synthesis of quencher-dye oligonucleotide probes (QDOPs)

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ABSTRACT

Real-time detection and quantitation of specific amplicons have been achieved using quencher and dye-labeled oligonucleotides such as molecular beacons. The molecular beacon molecule has a fluorescent reporter dye at the 5′-end and a quencher at the 3′-end. When the closed molecular beacon is excited by irradiation, the reporter fluorescence is greatly reduced by quenching through the process of fluorescence resonance energy transfer. When the molecular beacon hybridizes to the target, the stem loop opens making the fluorophore and quencher spatially distinct, thus increasing the reporter dye fluorescence intensity. Labeling of dyes to 5′-end of oligonucleotides has been done typically using manual methods, it is possible to do manual coupling at the milligram scale. Described here is the development of a scalable process for oligonucleotide labeling, which is robust, and has been achieved for 6-carboxynaphthofluorescein by connecting it to a corresponding phosphoramidite [Theisen, P.; McCollum, C.; Upadhya, K.; Jacobson. K.; Vu, H.; Andrus, A.; *Tetrahedron Lett.* **1992**, *33*, 5033–5036].

1. Materials and methods

The chemical synthesis of bis-protected naphthofluorescein phosphoramidite, containing a piperidine-4-ethanol linker, is shown in Scheme 1. The 6-isomer products were isolated by silica gel column chromatography using solvent systems with dichloromethane and methanol, and were characterized by ^{1}H NMR based on the chemical shifts of the protons (*ortho*-, *meta*-, *para*-) on the benzene ring to which the linker was attached. The mixed isomers of 5-(6)-carboxynaphthofluorescein (**1**, Marker Gene Tech.) appear as a purple powder, which has an absorbance of Ex 602 nm and Em 670 nm. This compound was used as a starting material for synthesis. Compound **1** was protected with trimethylacetyl chloride (**2**, Aldrich) in anhydrous pyridine (Aldrich) to provide a mixture of protected 5-, 6-carboxynaphthofluorescent lactones (**3a**, 5-isomer, and **3b**, 6-isomer). The two lactones were determined by thin layer chromatography (TLC, solvent system: CH_2Cl_2/methanol; 9:1). The 6-isomer was isolated from the reaction mixture by silica gel column chromatography using the following solvent systems: CH_2Cl_2, CH_2Cl_2/methanol, 98:2, 97:3, and 95:5, to yield a white powder product with a yield of 33.2% and with isomeric purity of 99.5%. The carboxylic group of lactone **3b** was then coupled to piperidine-4-ethanol (**4**) using HBTU (O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, Fluka), HOBT (1-hydroxybenzotriazole hydrate, Aldrich), and 4-ethylmorpholine (Aldrich) in anhydrous DMF (Aldrich).[1,2,4] The reaction was monitored using TLC (CH_2Cl_2/methanol; 9.5:0.5). The crude mixture was purified by silica gel column chromatography using solvent systems CH_2Cl_2, CH_2Cl_2/methanol, 99:1, 98:2, and 97:3 to yield bis-trimethylacetyl-6-(*N*-piperidine-4-ethanol)-naphthofluorescein carboxamide (**5**), with a yield of 45.9% and with isomeric purity of 99.8%. Compound **5** was phosphorylated using 2-cyanoethyltetraisopropyl phosphorodiamidite **6** (ChemGenes) and 5-ethylthiotetrazole (ChemGenes) in anhydrous acetonitrile.[3] The crude mixture was purified by silica gel column chromatography, using solvent systems: *n*-hexane/ethylacetate, 1:4, 1:1, and then ethylacetate to give the target compound, 6-carboxynaphthofluorescein phosphoramidite **7**, with the yield of 63% and with isomeric purity of 99.9% by ^{1}H NMR (Fig. 1). ^{31}P NMR of phosphoramidite (Fig. 2) was shown to be only one isomer of phosphoramidite at 148.03 ppm with 97.41% purity. The remaining 2.59% of material is an hydrolyzed product, which is thought to be the H-phosphonate derivative with a chemical shift of 9 ppm. The synthesized naphthofluorescein phosphoramidites, mixed isomers and single isomer, were then diluted in anhydrous acetonitrile (10 ppm water). These were used for labeling at the 5′-end of 9 quencher-dye oligonucleotide probes (QDOPs) on a large-scale DNA synthesizer, model AKTA Oligopilot 100 (G&E HealthCare Biosciences), and on the small-scale DNA synthesizer Expidite (Applied Biosystem, Inc.).

The optimal coupling time was 4 min for the dye-labeling process. Synthesis was performed at 0.1–15 μmol scales using 2′-*O*-methyl-nucleoside phosphoramidite monomers (Pierce), spacer phosphoramidite 9 (Glen Research), Dabcyl-CPG (Prime Synthe-

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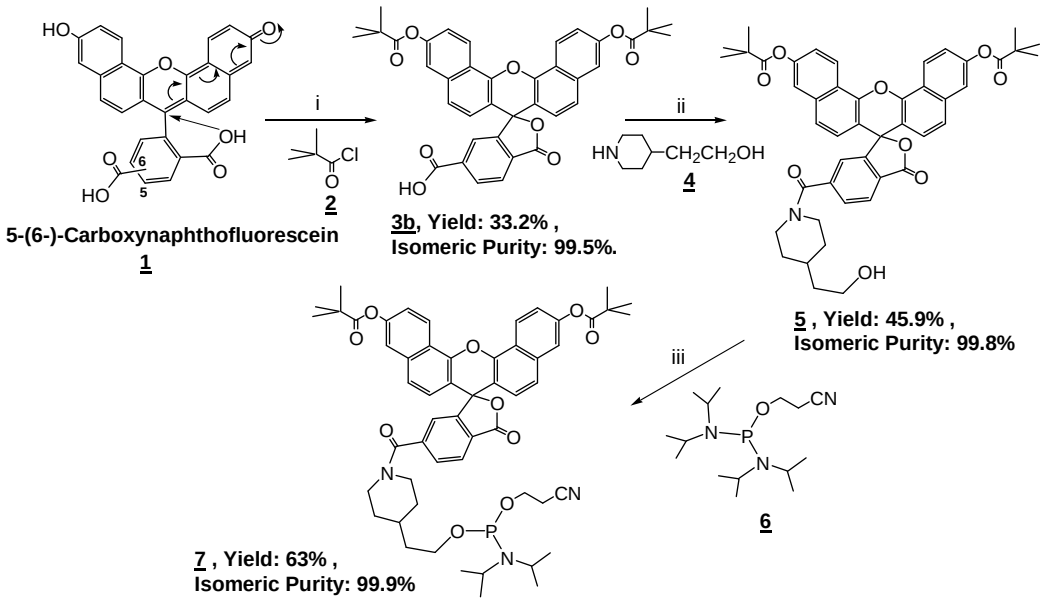

Scheme 1. Total synthesis of 6-carboxynaphthofluorescein phosphoramidite, containing piperidine-4-ethanol linker. Reagents: (i) anhydrous pyridine; (ii) HBTU, HOBT, 4-ethylmorpholine, anhydrous DMF; (iii) anhydrous acetonitrile, 5-ethylthiotetrazole.

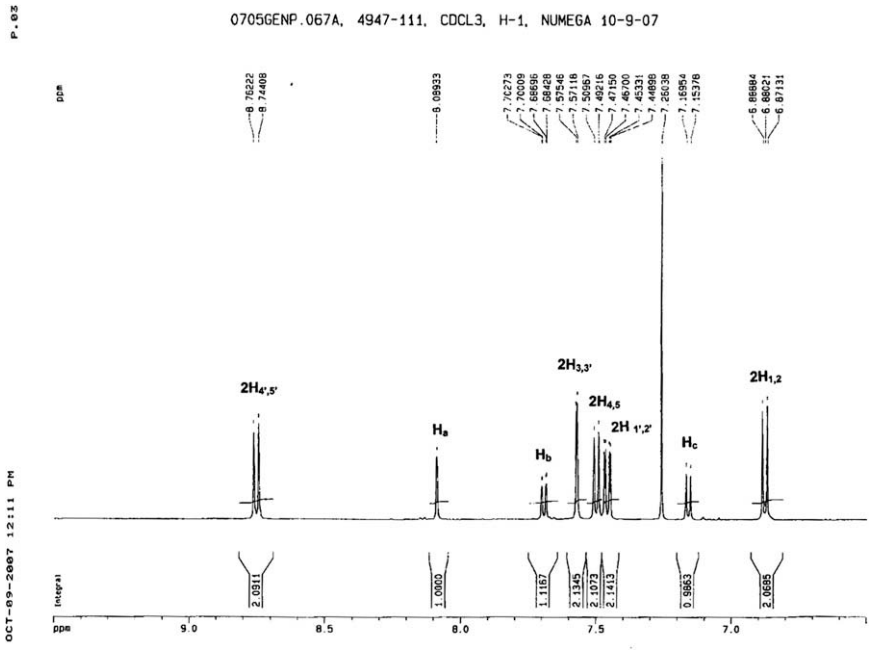

Figure 1. Expanded ^{1}H NMR spectrum of naphthofluorescein phosphoramidite **7** showed chemical shifts of H_a, H_b, and H_c, at 6–10 ppm of aromatic protons area. Isomeric purity of the 6-isomer was estimated to be 99.9%, based on integrated peak areas.

sis), Black Hole Quencher 2 (BHQ) -CPG (Biosearch Technology), and DNA liquid reagents (GE HealthCare Biosciences, and Burdick & Jackson). Upon successful completion of oligonucleotide synthesis, cleavage/deprotection of the QDOPs containing naphthofluorescein was optimized. The naphthofluorescein was found to be hydrolyzed in concentrated NH_4OH solution at room temperature. Therefore, a milder condition, with *tert*-butylamine/methanol/water (1:1:2) at 56 °C overnight, was used for deprotection. The product was found to be cleaved and deprotected completely under this condition.[5] Seven QDOPs, containing the linker Spacer 9, naphthofluorescein, and dabcyl quencher, were test-synthesized using phosphoramidite lots with mixtures of 5- and 6-carboxynaphthofluorescein phosphoramidites. After cleavage/deprotection, one crude mixture of the 22-mer was analyzed by ion-exchange analytical HPLC to give two peaks at 39.5 min and 41.2 min. MALDI mass spectrometry of this crude material showed a single and sharp peak at 10,343 Da, which was consistent with the theoretical molecular weights of the two isomers (Fig. 3).

The crude material of the two syntheses, with different sequences, were gel-purified by polyacrylamide gel electrophoresis (PAGE). As shown in Figure 4, a dark product band of oligonucleotide probe, which contained the 5- and 6-carboxynaphthofluorescein, and dabcyl, was observed, which demonstrated that the dye

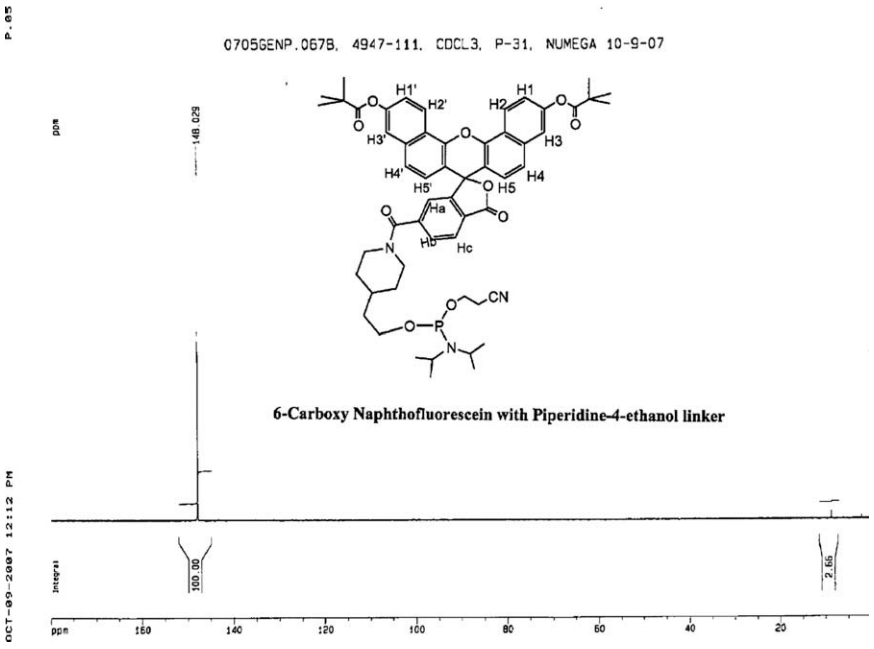

Figure 2. ^{31}P NMR of Naphthofluorescein phosphoramidite **7** showed a single peak at 148.03 ppm.

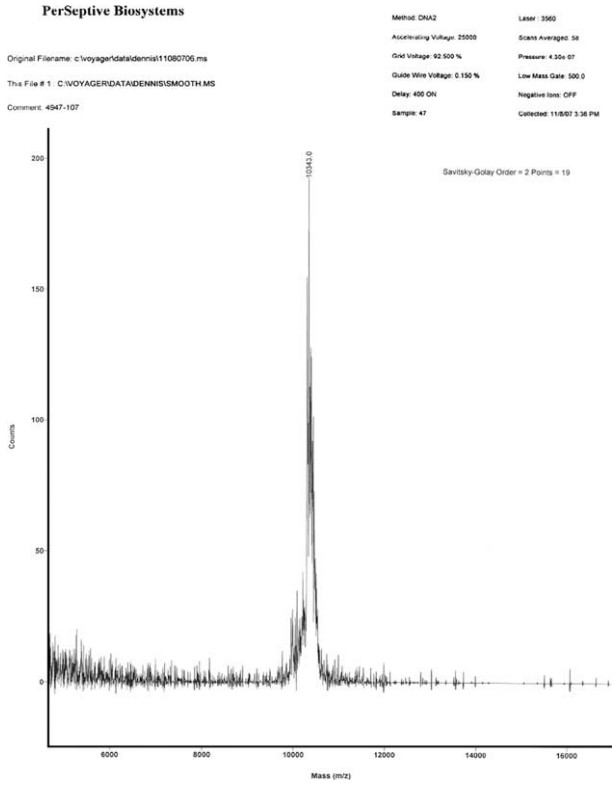

Figure 3. MALDI mass spectrometry of the crude QDOP, 22-mer, synthesized from mixed 5- and 6-isomers of naphthofluorescein phosphoramidite. The mass was 10,343 Da, which was consistent with the theoretical molecular weights of the two isomers.

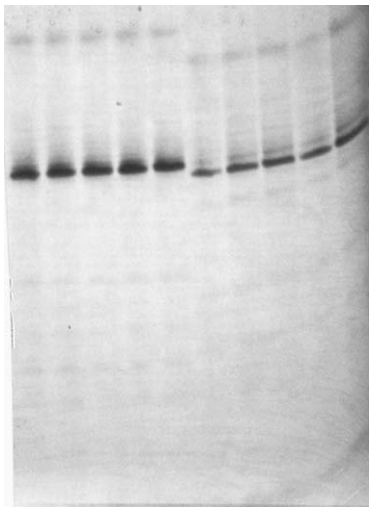

Figure 4. Gel electrophoresis purification of two crude QDOPs synthesized on the Expedite small-scale DNA synthesizer. The dark bands were products that contained 5- and 6-carboxynaphthofluorescein.

was incorporated efficiently on the synthesizer. The dark bands were collected separately and analyzed by reverse-phase HPLC. The results show two peaks for two QDOPs that contained the 5- and 6-carboxynaphthofluorescein. Another two QDOPs were synthesized, using single isomer naphthofluorescein phosphoramidite **7**, on dabcyl-CPG (sequence 1) and on BHQ-2-CPG (sequence 2). Sequences are described below:

Sequence 1:

5′-Naphthofluorescein-ccc uca auu ccg agg cux gag gg-Dabcyl-3′

Sequence 2:

5′-Naphthofluorescein-ccc uca auu ccg agg cux gag gg-Black Hole-3′

With lower case were 2′-*O*-methyl-nucleosides, and x is linker Spacer 9.

After cleavage/deprotection, the crude mixture of sequence 1 was purified by PAGE to eliminate N-minus and N-plus species. The QDOP containing 6-carboxynaphthofluorescein was then purified by reverse-phase HPLC (Beckman System Gold, using Amberchrome Profile XT20). The HPLC chromatogram of the crude QDOPs, with naphthofluorescein and dabcyl, is shown in Figure 5. The QDOP containing the 5-isomer, which migrated

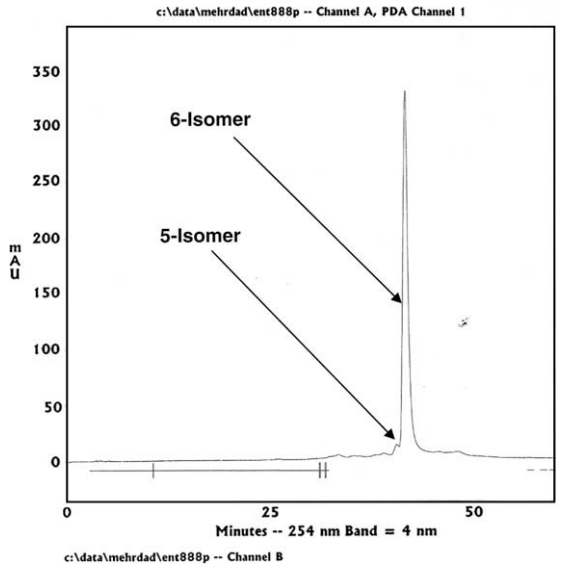

Figure 5. Reverse-phase HPLC chromatogram showing the QDOP with 6-carboxynaphthofluorescein and dabcyl. The QDOP containing the 5-carboxy was found as a minor component of the crude mixture.

slower than the 6-isomer, was found as a minor product in the mixture.

The main peak, identified as the oligonucleotide probe containing the 6-carboxynaphthofluorescein, was collected, then ethanol-precipitated at −20 °C for 30 min and centrifuged. The supernatant was decanted, and the pellet was washed with ethanol and re-dissolved in water to give a pure QDOP of sequence 1. Sequence 2 was processed the same way. The analytical gel electrophoresis showed a single band for both samples.

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