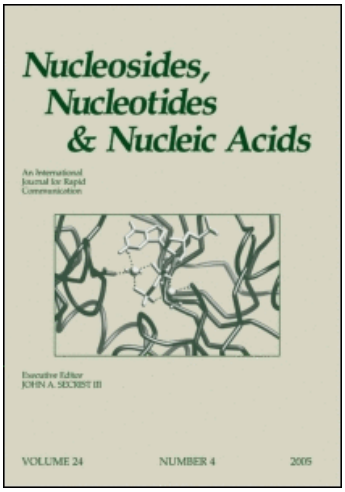

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Oligonucleotide Labeling Methods 4. Direct Labeling Reagents with a Novel, Non-Nucleosidic, Chirally Defined 2-Deoxy-β-D-Ribosyl Backbone.

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To cite this Article Smith, Thomas H. , Kent, Mark A. , Muthini, Sylvester , Boone, Steven J. and Nelson, Paul S.(1996) 'Oligonucleotide Labeling Methods 4. Direct Labeling Reagents with a Novel, Non-Nucleosidic, Chirally Defined 2-Deoxy-β-D-Ribosyl Backbone.', Nucleosides, Nucleotides and Nucleic Acids, 15: 10, 1581 — 1594
To link to this Article: DOI: 10.1080/07328319608002458
URL: http://dx.doi.org/10.1080/07328319608002458

NUCLEOSIDES & NUCLEOTIDES, 15(10), 1581-1594 (1996)

OLIGONUCLEOTIDE LABELING METHODS 4. DIRECT LABELING REAGENTS WITH A NOVEL, NON-NUCLEOSIDIC, CHIRALLY DEFINED 2-DEOXY-β-D-RIBOSYL BACKBONE.

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Abstract. Novel solid supports and CE-phosphoramidite reagents have been prepared featuring a unique 2'-deoxyribosyl backbone. These chirally pure reagents form the basis of an oligonucleotide labeling system which provides diastereomerically pure oligonucleotides.

Labeled or chemically modified oligonucleotides prepared via automated synthesis have found great utility in many area of biological and biochemical research. Historically the most common approach to these molecules has been the intoduction of an alkylamino functionality to the 5'-terminus of the oligonucleotide via a phosphoramidite or H-phosphonate reagent [1-4] during the final step on the synthesizer. Although useful for many applications, this approach is limited in that only one labeling or modifying moiety can be introduced and only at the 5'-terminus. The development of reagents incorporating a non-nucleosidic, bifunctional moiety [5-7] permitted the introduction of multiple labeling moieties into oligonucleotides. These and other methodologies for the introduction of alkylamino groups as well as other labeling or modifying functionalities into oligonucleotides have been the subject of a recent review.[8]

We recently introduced solid supports and CE-phosphoramidite reagents for use in automated DNA synthesis to directly modify or label oligonucleotides featuring a novel 2-aminobutyl-1,3-propanediol (ABPD) backbone.[9] These offered numerous advantages over the previously cited earlier methods [5-7] including the capability of labeling oligonucleotides

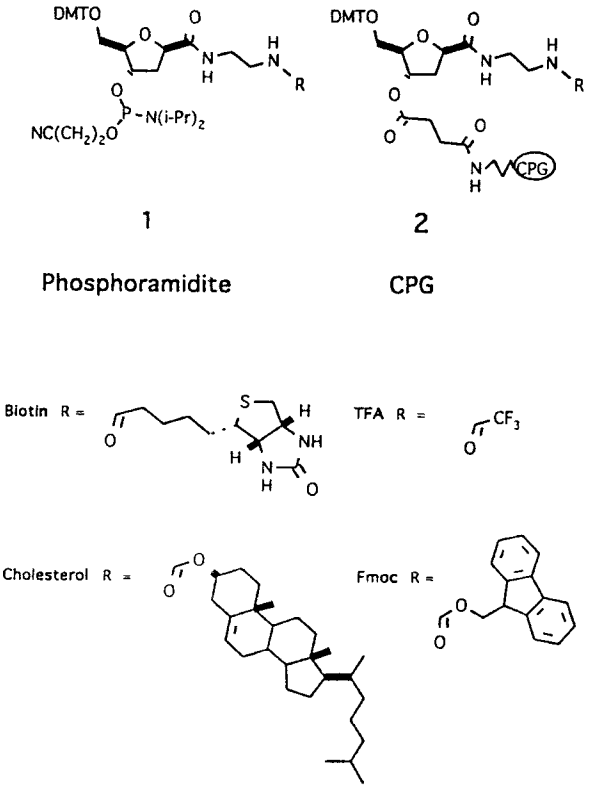

FIGURE 1. Structures of 2-deoxyribosyl reagents for the incorporation of labeling or modification moieties into synthetic oligonucleotides.

at any position (5', internal, 3'), with any number of labels, and the maintenance of the correct internucleotide distance when used internally. However, the ABPD backbone, as well as the other non-nucleosidic methods, contains an unresolved chiral center. Incorporation of labeling reagents into an oligonucleotide as a racemic mixture results in 2^n diastereomers where n equals the number of incorporations. Diastereomeric mixtures of oligonucleotides are essentially inseparable by standard methods, and complicate the study of physical properties as well as biological activity. Therefore, a reagent that incorporates labels with diastereomeric definition has great benefit in areas such as antisense or diagnostic research. The reagents reported herein are designed to address this need.

In this paper, we report a system for the direct labeling and modification of oligonucleotides based on a unique, enantiomerically pure, 2-deoxyribosyl backbone in

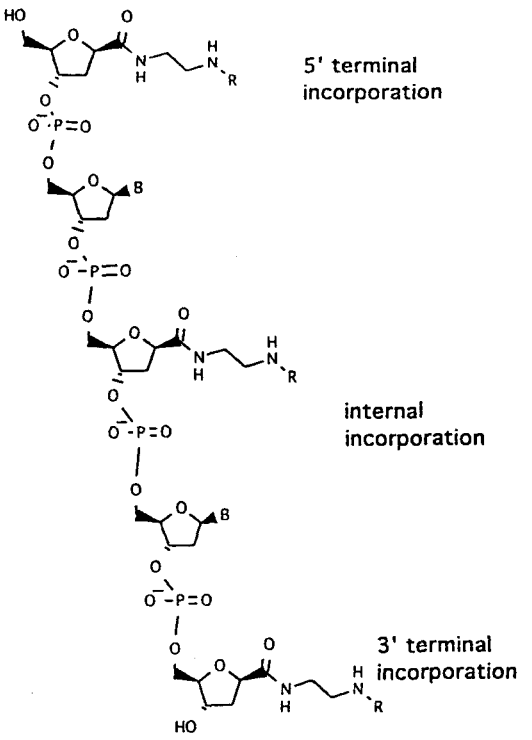

FIGURE 2. Structure of an oligonucleotide labeled with 2-dexoyribosyl backbone reagents.

order to address these issues. Introduction of labeling reagents having a 2-deoxyribosyl backbone (**FIGURE 1**) results in oligonucleotides (**FIGURE 2**) in which the enantiomeric, anomeric, and conformational definition as well as the internucleotide distance of the natural oligonucleotide is preserved. This gives rise to a chemically homogenous product in which any perturbations in the physical and biochemical properties of the sugar-phosphate backbone are eliminated. From the 2-deoxyribosyl backbone we have prepared a series of CE-phosphoramidites and solid supports. These reagents have been successfully employed in automated DNA synthesis to directly label/modify oligonucleotides with various combinations of biotin, primary amine, and cholesterol. Herein we describe the synthesis and use of these new reagents.

RESULTS

In our design of a labeling system incorporating a stereochemically defined backbone with the proper internucleotide distance between nucleoside residues 2-deoxy-D-

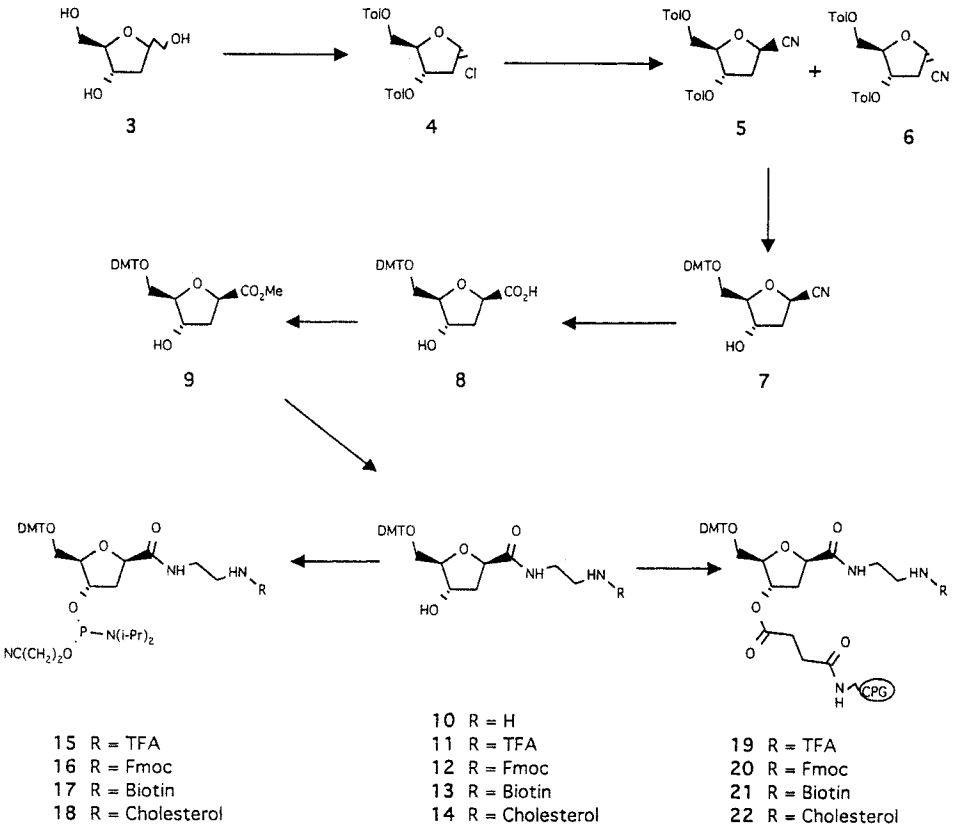

FIGURE 3. Synthetic scheme for preparation of 2-deoxyribosyl labeling reagents.

ribose appeared to be an ideal starting material. It is available commercially in optically pure form, and can be readily transformed to a protected synthon [10] which has been successfully incorporated into a wide variety of structures. An examination of the literature revealed that a 2-deoxyribosyl backbone had previously been proposed, but apparently never utilized, for a nucleic acid labeling system.[11] Other approaches [8] applicable for the introduction of multiple aminoalkyl functionalities into oligonucleotides have been reviewed. The ready availability of chirally pure starting material, maintenance of the natural sugar-phosphate backbone, and the expected non-discriminatory impact on hybridization properties of this non-nucleosidic system are advantages which lead us to explore the 2-deoxyribosyl backbone.

After considerable experimentation a synthetic approach to the ultimately successful labeling system was developed and is shown in **FIGURE 3**. This divergent synthetic

strategy features the primary amine **10** as a common intermediate which, in principle, can be elaborated to any CE-phosphoramidite or solid support desired. 2-Deoxy-D-ribose (**3**) is converted to the ditoluoyl chloride **4** via a literature procedure.[10] The chloride is converted to a mixture of anomeric ditoluoyl nitriles **5** and **6** which were readily separable by silica gel chromatography.[11,12] While a variety of methods have been used to effect this transformation, in our hands the best results were obtained via $SnCl_4$ catalyzed reaction of **4** with TMSCN which afforded a 5:1 mixture of **5** and **6**. The toluoyl groups of the β–anomer **5** were removed via treatment with NaOMe followed by neutralization with Dowex 50 H^+ resin,[13] and the 5-OH was tritylated via the DMAP catalyzed reaction with DMT Cl in DMF [14] to afford **7**. Base hydrolysis of the nitrile afforded the carboxylic acid **8**. This was not isolated, but reacted directly with excess MeOH in the presence of dicyclohexylcarbodiimide to provide the methyl ester **9**. It was now necessary to introduce a nucleophilic function to permit eventual attachment of the labels. This was best achieved via reaction of **9** with excess ethylenediamine to provide **10** which contained the required primary amine functionality. This was not purified, but either protected with the TFA or Fmoc protecting groups to afford **11** or **12** respectively, or labeled with biotin NHS ester or cholesterol chloroformate to provide **13** or **14**.

For this labeling approach to be successful it is imperative that the labeling moieties be stable to standard oligonucleotide deprotection conditions. Conversely, amine protecting groups must be quantitatively cleaved under these same conditions to provide a free primary amine functionality for subsequent labeling. To assess the suitability of our linkage chemistry, precurors **11-14**, were subjected to the standard conditions of oligonucleotide deprotection (conc NH_4OH, EtOH (for solubilization), 55°, 16 hr). The labeled precursors **13** and **14** were inert to these conditions. The amine protected precursors, **11** and **12**, on the other hand were quantitatively converted to the primary amine **10**.

Precursors **11-14** were converted to the phosphoramidites **15-18** via reaction with bis(diisopropylamino)-2-cyanoethoxyphosphine and tetrazole. Solid supports **19-22** were prepared via conversion of **11-14** to the corresponding succinates via DMAP catalyzed reaction with succinic anhydride in dichloroethane at 50°.[15] The succinates were then reacted in the presence of BOP/HBT [16] with LCAA-CPG to provide **19-22.** In the case of the "amine" phosphoramidites **15** and **16**, Fmoc N-protection was preferred over TFA protection due to the limited solubility of **15** in acetonitrile. However, in the case of the solid supports TFA protection was found to be preferable. We observed considerable loss of the Fmoc protecting group when we capped the unreacted amino groups on solid support **20**. We observed no loss of the Fmoc moiety during the instrument capping cycle on residues introduced with phosphoramidite **16**.

Phosphoramidites **15-18** and solid supports **19, 21,** and **22** were successfully employed in automated DNA synthesis using a commercial instrument. Several experiments were performed to verify that the labels survived the synthesis process. Purified biotinylated oligos prepared with **17** and/or **21** were spotted on a nylon membrance and detected with a streptavidin/alkaline phosphatase conjugate.[17] Oligomers functionalized with a primary amine prepared with **16** and/or **19** were labeled successfully with biotin using standard methodology. These oligos behaved identically to those prepared from **17** and **21** on PAGE analysis and in the above described membrane spot test. Cholesterol labeled oligonucleotides prepared with **18** or **22** migrated approximately two bases slower than an unlabeled oligonucleotide during PAGE analysis. Similar electrophoretic behavior has been previously reported for cholesterol modified oligonucleotides.[18]

DISCUSSION

An efficient synthesis of diastereomerically homogenous reagents capable of incorporating various reporter or modifying functionalities into oligonucleotides at any position (5', internal, 3') via automated synthesis has been developed. Multiple incorporations are easily achieved by repetitive couplings in the same fashion as normal nucleoside phosphoramidite reagents. The flexibility of the synthetic approach should make it amenable to the development of reagents incorporating any moiety which is capable of surviving the conditions of automated nucleic acid synthesis in either its native or some protected form. The labels are covalently attached through a 5 atom spacer arm connected to the anomeric position of the 2-deoxyribosyl phosphate backbone. Due to the use of the 2-deoxyribosyl moiety as the spacer unit, the enantiomeric, anomeric, and conformational definition, as well as the internucleotide distance of a natural oligonucleotide, is preserved in oligonucleotides incorporating these reagents.

Experiments with oligonucleotides incorporating 2-deoxyribosyl backbone reagents have shown that the reporter moiety is incorporated intact with high efficiency and is readily detectable via standard assay procedures. Oligonucleotides incorporating 2-deoxyribosyl based reagents at the 3'-position have been shown to be resistant to exonuclease digestion (Snake Venom Phosphodiesterase) (**FIGURE 4**), a finding which could have implications for the use of these reagents in antisense research.[19] Hybridization experiments (**TABLE 1**) indicate that incorporation of 2-deoxyribosyl backbone reagents into the central position of an oligonucleotide tends to destabilize the duplex with the complementary oligonucleotide. This occurs to an extent similar to that observed with an oligonucleotide having a mismatched standard nucleoside residue at the same position. This destabilization seems to be less than is observed with other abasic residues in similar

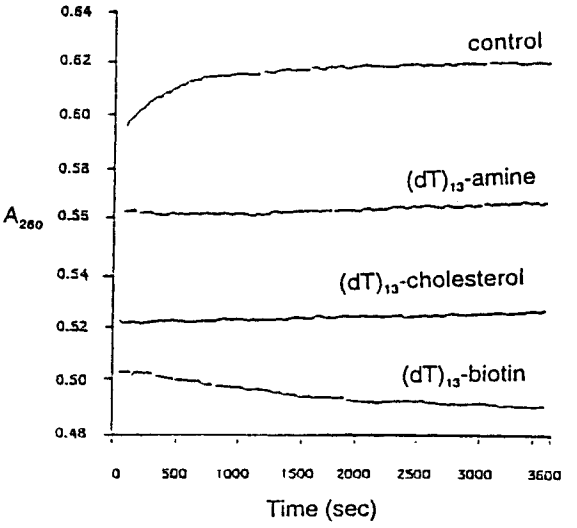

FIGURE 4. Snake Venom Phosphodiesterase digestion of $(T)_{13}$ modified on the 3'-terminus with 2-deoxyribosyl solid supports.

TABLE 1. Melting temperatures (T_m) for 13-mer duplexes of $(dA)_{13}$ and complementary oligomers containing functionalized 2-deoxyribosyl residues and regular nucleoside mismatches.

$(dA)_6$-dA-$(dA)_6$
$(T)_6$-X-$(T)_6$

X	T_m (°C)
T	29.8
dR-Biotin	13.6
dR-Amine	13.8
dA	13
dC	12.8
dG	14

experiments.[20] Duplexes with complementary oligonucleotides are not destabilized when 2-deoxyribosyl reagents are incorporated into either, or both, of the end positions of an olignucleotide (Data not shown). The labeling system described herein represents a significant advance in the technology for labeling oligonucleotides with utility in a variety of biological applications.

MATERIALS AND METHODS

General methods

Oligonucleotide Purification Elution Columns (OPEC) and Biotin-NHS ester were provided by CLONTECH Laboratories, Inc. All other chemicals were purchased from either Chem Impex International Inc. or Aldrich Chemical Company. All reactions were carried out under Ar atmosphere. Solvent extracts of aqueous solutions were dried over anhydrous Na_2SO_4. Solutions were concentrated under reduced pressure using a rotary evaporator. Thin layer chromatography (TLC) was done on Analtech Silica Gel GF (0.25 mm) plates. Chromatograms were visualized with either UV light or sulfuric acid. All compounds reported were homogeneous by TLC unless otherwise noted. HPLC analyses were performed on a Rainin Rabbit HPX system using a C18 Microsorb (4.6 x 150 mm, 5 μm) reverse phase column. A Perkin-Elmer 1310 Infrared Spectrometer was used for infrared data. UV measurements were obtained with a Perkin Elmer Lambda 2 spectrophotometer equipped with a Peltier temperature controller and a six cell transport device. ^1H and ^{31}P NMR spectra were obtained by the NMR Laboratory at the University of California, Berkeley, on a Bruker AM-400 instrument in $CDCl_3$ using tetramethylsilane as an internal standard (^1H) or trimethylphosphate (^{31}P) as an external standard. Mass spectra were obtained from the Mass Spectrometry Lab, University of California, Berkeley. Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA/RNA Synthesizer according to manufacturer's protocol.

3,5-Di-O-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl nitrile (5). To a solution of 3,5-di-*p*-toluoyl-2-deoxy-D-ribofuranosyl chloride (**4**)[10] (171 g, 0.44 mol) in dichloromethane (1.4 L) was added trimethylsilyl cyanide (48.4 g, 0.49 mol) in one portion with mechanical stirring. The mixture was cooled to -78° C and $SnCl_4$ (0.2 mL) was added dropwise with stirring. The mixture was stirred cold for 25 min followed by 20 min at ambient. 5% $NaHCO_3$ (1.4 L) was added, and the mixture was stirred for 30 min. The mixture was extracted with ethyl acetate (2.4 L). The organic layer was washed with 5% $NaHCO_3$ (3 x 1.5 L), dried, and evaporated. The residue was chromatographed (silica

gel, 83:17-0:100 petroleum ether / ethyl acetate) to afford 107 g (64%) of **5**[12] and 24 g (14%) of **6** [12] as white solids.

2-Deoxy-5-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl nitrile (7). 5 (36.3 g, 96 mmol) was dissolved in MeOH (1 L) and the solution cooled in an ice bath. NaOMe (10.85 g, 200 mmol) was added with stirring. The reaction was stirred in ice for 1.25 h and at ambient for 3.75 h. Dowex 50X8-100 ion-exchange resin (190 g) was added to the reaction mixture and stirred for 20 min. The resin was collected by filtration, and washed with MeOH (500 mL). The filtrates were combined and evaporated. The residue was dissolved in DMF (200 mL) and cooled in an ice bath. Pyridine (7.8 g, 100 mmol) and 4-dimethylaminopyridine (DMAP) (1.16 g, 9.6 mmol) were added with stirring, followed by dimethoxytrityl chloride (32.4 g, 96 mmol) in 4 portions over 1.5 h. The reaction was stirred at ambient for 2.75 h. MeOH (300 mL) was added and stirring was continued for 15 min. 5% $NaHCO_3$ (800 mL) was added and stirring was continued for an additional 15 min. The mixture was extracted with ethyl acetate (1.5 L). The organic layer was separated and washed with 5% $NaHCO_3$ (2 x 800 mL) and brine (800 mL), dried, and evaporated. The residue was chromatographed (silica gel, 90:10:5 - 55:40:5 hexane-ethyl acetate-triethylamine) to afford 29 g (68%) of **7** as a viscous oil. TLC R_f = 0.37 (55:40:5 hexane / ethyl acetate / triethylamine). ^1H NMR 2.19 (m, 2H), 3.15 (m, 2H), 3.72 (s, 6H), 3.94, (m, 1H), 4.32 (m, 1H), 4.73 (m, 1H), 6.78 (dd, 4H), 7.20 (m, 9H). IR 3480, 3000, 2930, 2050, 1610, 1510, 1250 cm^{-1}.

3-Deoxy-6-O-(4,4'-dimethoxytrityl)-allonic acid, methyl ester (9). A solution of 2-deoxy-5-O-DMT-D-ribofuranosyl nitrile (**7**) (35 g, 79 mmol), EtOH (350 mL), and 15% NaOH (63 mL) was stirred at ambient 1.5 h , and then at 60° C for 1 h. The reaction mixture was cooled to ambient in an ice bath and 10% citric acid solution (1.5 L) was added. The reaction mixture was extracted with ethyl acetate (6 x 500 mL). The combined organic layers were washed with water (3 x 1.5 L) and brine (1.2 L), dried and evaporated. The residue was dissolved in DMF (420 mL). MeOH (50.6 g) and dicyclohexylcarbodiimide (16.2 g, 79 mmol) were added and the reaction mixture was stirred at ambient for 16 h. The reaction mixture was chilled, filtered, and the filtrate was partitioned between ethyl acetate (2 L) and water (1 L). The organic layer was separated, washed with 10% Na_2CO_3 (2 x 1 L) and brine (1 L), dried, and evaporated. The residue was chromatographed (silica gel, 3:2 - 1:1 hexanes / ethyl acetate) to afford 23 g (61%) of **9** as an off-white foam. TLC R_f = 0.32 (3:2 hexanes / ethyl acetate). ^1H NMR 2.22 (m, 2H), 3.18 (m, 1H), 3.31 (m, 1H), 3.72 (s, 3H), 3.78 (s, 6H), 3.99 (m, 1H), 4.35 (m, 1H), 4.68 (t, 1H), 6.82 (d, 4H), 7.32 (m, 9H). HRMS (FAB) for $C_{28}H_{30}O_7$ [M]: Calcd, 478.1991; Found, 478.2003.

N-Aminoethyl-(N'-TFA)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (11). To a solution of ethylenediamine (21 g, 348 mmol) in acetonitrile (33 mL) at 0° was added a solution of 3-deoxy-6-O-DMT-allonic acid, methyl ester (**9**) (4.2 g, 8.8 mmol) in acetonitrile (33 mL) over 1 h with thorough stirring. After the addition was complete the solution was stirred at 0° for 15 min and at reflux for 16 h. The reaction mixture was cooled to ambient, diluted with ethyl acetate (300 mL), washed with 5% $NaHCO_3$ (2 x 150 mL) and brine (150 mL), dried, and evaporated. The residue was dissolved in ethyl acetate (15 mL). Triethylamine (1.35 g, 13.3 mmol) and ethyl trifluoroacetate (1.5 g, 10.6 mmol) were added, and the mixture stirred at ambient for 1 h and evaporated. The residue was taken up in ethyl acetate (150 mL), washed with 10% Na_2CO_3 (2 x 90 mL), and brine (90 mL), dried, and evaporated. The residue was chromatographed (silica gel, 1:2 hexane / ethyl acetate) to afford 1.8 g (36%) of **11** as an off-white foam. TLC R_f = 0.30 (2:1 ethyl acetate / hexanes). ^1H NMR 2.15 (m, 1H), 2.27 (m, 1H), 3.19 (m, 4H), 3.23 (dd, 1H), 3.35 (dd, 1H), 3.73 (s, 6H), 3.91 (m, 1H), 4.28 (m, 1H), 4.53 (t, 1H), 6.75 (m, 4H), 7.11-7.35 (m, 9H). HRMS (FAB) for $C_{31}H_{33}F_3N_2O_7$ [M]: Calcd, 602.2240; Found, 602.2245.

N-Aminoethyl-(N'-Fmoc)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (12). To a solution of ethylenediamine (57 g, 945 mmol) in acetonitrile (90 mL) at 0° was added a solution of 3-deoxy-6-O-DMT-allonic acid, methyl ester (**9**) (11.3 g, 24 mmol) in acetonitrile (90 mL) over 1 h with thorough stirring. After the addition was complete the solution was stirred at 0° for 15 min and at reflux for 16 h. The reaction mixture was cooled to ambient, diluted with ethyl acetate (700 mL), washed with 5% $NaHCO_3$ (2 x 350 mL) and brine (350 mL), dried, and evaporated. The residue was dissolved in DMF (60 mL). Diisopropylethylamine (5.18 g, 3.8 mL, 39.6 mmol) and Fmoc-chloride (5.73 g, 22 mmol) were added, and the mixture stirred at ambient for 15 min. The reaction was diluted with ethyl acetate (600 mL), washed with water (300 mL), 10% Na_2CO_3 (2 x 300 mL), and brine (300 mL), dried, and evaporated. The residue was chromatographed (silica gel, 25:75 - 0:100 hexane / ethyl acetate) to afford 11 g (65%) of **12** as an off-white foam. TLC R_f = 0.25 (3:1 ethyl acetate / hexanes). ^1H NMR 1.47 (m, 4H), 2.19 (m, 2H), 3.25 (m, 2H), 3.68 (s, 6H), 3.91 (m, 1H), 4.07 (m, 2H), 4.24 (m, 1H), 4.52 (t, 1H), 5.18 (t, 1H), 6.75 (d, 4H), 7.05 (m, 2H), 7.33 (m, 13H), 7.48 (t, 2H), 7.67 (d, 2H). IR 3400, 3050, 2930, 1710, 1670, 1505, 1250 cm^{-1}. HRMS (FAB) for $C_{44}H_{44}N_2O_8$ [M]: Calcd, 728.3098; Found, 728.3095.

N-Aminoethyl-(N'-biotinoyl)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (13). To a solution of ethylenediamine (57 g, 945 mmol) in acetonitrile (90 mL) at 0° was added a solution of 3-deoxy-6-O-DMT-allonic acid, methyl ester (**9**) (11.3

g, 24 mmol) in acetonitrile (90 mL) over 1 h with thorough stirring. After the addition was complete the solution was stirred at 0° for 15 min and at reflux for 16 h.. The reaction mixture was cooled to ambient, diluted with ethyl acetate (700 mL), washed with 5% $NaHCO_3$ (2 x 350 mL) and brine (350 mL), dried, and evaporated. The residue was dissolved in DMF (60 mL). Diisopropylethylamine (5.18 g, 3.8 mL, 39.6 mmol) and Biotin-NHS (7.64 g, 22 .0 mmol) were added, and the mixture stirred at ambient for 1 h. The reaction was diluted with ethyl acetate (700 mL), washed with water (2 x 200 mL), 10% Na_2CO_3 (200 mL), and brine (200 mL), dried, and evaporated. The residue was chromatographed (silica gel, 95:5 - 90:10 dichloromethane / MeOH) to afford 16 g (94%) of **13** as a pale yellow foam. TLC R_f = 0.45 (9:1 dichloromethane / MeOH). ^1H NMR 1.52 (m, 6H), 2.05 (m, 3H), 2.28 (m, 1H), 2.62 (d, 1H), 2.78 (m, 1H), 3.20 (M, 4H), 3.71 (S, 6H), 3.97, (m, 1H), 4.21 (m, 2H), 4.36 (m, 1H), 4.52 (t, 1H), 5.71 (s, 1H), 6.66 (s, 1H), 6.79 (m, 4H), 7.25 (m, 9H). IR 3340, 3020, 2950, 1700, 1690, 1680 cm^{-1}. HRMS (FAB) for $C_{39}H_{48}N_4NaO_8S$ [M + Na]: Calcd, 755.3091; Found, 755.3103.

N-Aminoethyl-(N'-cholesteryl)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (14). To a solution of ethylenediamine (16.8 g, 280 mmol) in acetonitrile (50 mL) at 0° was added a solution of 3-deoxy-6-O-DMT-allonic acid, methyl ester (**9**) (6.75 g, 14 mmol) in acetonitrile (55 mL) over 1 h with thorough stirring. After the addition was complete the solution was stirred at 0° for 15 min and at reflux for 16 h. The reaction mixture was cooled to ambient, diluted with ethyl acetate (375 mL), washed with 5% $NaHCO_3$ (2 x 200 mL) and brine (200 mL), dried, and evaporated. The residue was dissolved in dichloromethane (110 mL) containing pyridine (11.3 mL, 140 mmol) and cooled to 0°. Cholesteryl chloroformate (5.93 g,13.2 mmol) in dichloromethane (60 mL) were added over 25 min. Stirring was continued at 0° for 1 hr after the addition. The reaction mixture was evaporated. The residue was coevaporated with toluene (75 mL) and partitioned between ethyl acetate (200 mL) and water (75 mL). The organic phase was separated, washed with brine (100 mL), dried, and evaporated. The residue was chromatographed (silica gel, 25:75 - 0:100 hexane / ethyl acetate) to afford 7.36 g (58%) of **14** as an off-white foam. TLC R_f = 0.3 (ethyl acetate / hexanes, 3:2). ^1H NMR 1.50 (m, 43H), 2.93 (m, 4H), 3.24 (m, 2H), 3.70 (s, 6H), 3.90 (m 1H), 4.25 (m, 1H), 4.38 (m, 1H), 4.50 (t, 1H), 5.00 (m, 1H), 5.25 (d, 1H), 6.74 (m, 4H), 7.04 (m, 1H), 7.22 (m, 9H). IR 3400, 2930, 2860, 1740, 1610, 1510 cm^{-1}. HRMS (FAB) for $C_{57}H_{78}N_2NaO_8$ [M + Na]: Calcd, 941.5656; Found, 941.5666.

General procedure for preparing CE-phosphoramidites 16-18 from precursors 12-14. Precursors (10 mmol) were dissolved in dichloromethane (75 mL).

Tetrazole (770 mg, 11 mmol) and bis(diisopropylamino)-2-cyanoethoxyphosphine (3.3 g, 11 mmol) were added and the mixtures was stirred for 30 min at ambient. The reaction mixtures were filtered and partitioned between ethyl acetate (500 mL) and 10% Na_2CO_3 (400 mL). The organic phases were separated and washed with 10% Na_2CO_3 (3 x 400 mL) and brine (400 mL), dried, and evaporated. The residues were chromatographed (silica gel) to afford the phosphoramidites as diastereomeric mixtures in 60-80% yields.

N-Aminoethyl-(N'-Fmoc)-4-O(P-β-cyanoethyl-N,N-diisopropylaminophosphinyl)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (16). TLC R_f = 0.33 and 0.23 diastereomers (50:45:5 hexanes / ethyl acetate / triethylamine). ^1H NMR 1.19 (dd,12H), 2.34 (m, 2H), 2.63 m, 2H), 3.25 (m,4H), 3.35 (m, 2H), 3.43 (m, 2H), 3.66 (m, 2H), 3.82 (s, 6H), 4.23 (m, 1H), 4.37 (m, 2H), 4.56 (m, 1H), 4.70 (t, 1H), 5.55 (m, 1H), 6.91 (m, 4H), 7.37 (m, 13H), 7.63 (t, 2H), 7.81 (d, 2H). ^{31}P NMR 145.23, 145.61. IR 3060, 2960, 2930, 2840, 2250, 1720, 1680, 1610, 1510 cm^{-1}. HRMS (FAB) for $C_{53}H_{61}N_4NaO_9P$ [M + Na]: Calcd, 951.4074; Found, 951.4065.

N-Aminoethyl-(N'-biotinoyl)-4-O(P-β-cyanoethyl-N,N-diisopropylaminophosphinyl)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (17). TLC R_f = 0.55 (9:1 dichloromethane / MeOH). ^1H NMR 1.06 (d, 12H), 1.41 (m, 7H), 2.11 (t, 2H), 2.20 (m, 2H), 2.44 (t, 1H), 2.61 (t, 1H), 2.69 (d, 1H), 2.84 (m, 1H), 3.12 (m, 2H), 3.27 (m, 4H), 3.55 (m, 2H), 3.62 (m, 2H), 3.79 (s, 6H), 4.14 (m, 1H), 4.26 (m, 2H), 4.43 (m, 1H), 4.56 (t, 1H), 6.32 (s, 1H), 6.84 (m, 4H), 7.05 (s, 1H), 7.27 (m, 9H). ^{31}P NMR 145.79, 146.24. IR 3065, 2960, 2930, 2840, 2260, 1705, 1685, 1505, 1365 cm^{-1}. HRMS (FAB) for $C_{48}H_{65}N_6NaO_9PS$ [M + Na]: Calcd, 955.4169; Found, 955.4169.

N-Aminoethyl-(N'-cholesteryl)-4-O(P-β-cyanoethyl-N,N-diisopropylaminophosphinyl)-3-deoxy-6-O-(4,4'-dimethoxytrityl)-allonamide (18). TLC R_f = 0.58 and 0.44 diastereomers (1:1 hexanes / ethyl acetate). ^1H NMR 1.43 (m, 43H), 2.35 (t, 2H), 2.52 (t, 2H), 3.48 (m, 10H), 3.72 (s, 6H), 4.04 (m, 1H), 4.42 (m, 1H), 4.51 (t, 1H), 4.91 (m, 1H), 5.27 (m, 1H), 6.76 (m, 4H), 6.99 (m, 1H), 7.25 (m, 9H). ^{31}P NMR 146.33, 146.44. IR 2960, 2930, 2860, 2250, 1730, 1605, 1505 cm^{-1}. HRMS (FAB) for $C_{66}H_{95}N_4NaO_9P$ [M + Na]: Calcd, 1141.6734; Found, 1141.6720.

General procedure for preparing solid supports 19, 20, 21 from precursors 11, 13, 14. Precursors (1.5 mmol) were dissolved in dichloroethane (6.3 mL). Succinic anhydride (300 mg, 3 mmol), DMAP (91.5 mg, 0.75 mmol), and triethylamine (152 mg, 1.5 mmol) were added and the solution stirred at 50° C for 45 min. The reaction mixture was cooled to ambient, diluted with dichloromethane (60 mL), washed with cold 10% citric acid (2 x 30 mL), water (30 mL), and brine (30 mL), dried, and evaporated. The residue was dissolved in dichloromethane (37 mL). Long chain alkylamine CPG (25 g), triethylamine (1.9 mL), HBT (220 mg, 1.63 mmol), and BOP (720 mg, 1.63 mmol) were added in order, and the reaction agitated for 2 hours. The support was collected by filtration, washed with dichloromethane (2 x 200 mL) and dried *in vacuo*. The support was suspended in pyridine (105 mL) / acetic anhydride (13 mL) / N-methylimidazole (13 mL) solution, and agitated for 15 min. The support was collected by filtration, washed with pyridine (2 x 200 mL), DMF (2 x 200 mL), MeOH (5 x 200 mL), and ether (200 mL), and dried *in vacuo* to afford 25 g of support. The loading capacity was measured according to standard procedures and determined to be between 35-45 μmole/g.

Oligonucleotide synthesis

CE-Phosphoramidites **16-18** and solid supports **19, 21, 22** were used in automated DNA oligonucleotide synthesis using an Applied Biosystems 394 DNA/RNA synthesizer according to manufacturer's suggested DNA synthesis protocols on a 1.0 μmol scale. Coupling efficiencies were measured by using the dimethoxytrityl cation concentration. Cholesterol labeled phosphoramidite **18** was used as a 0.1 M solution in dichloromethane. The remaining phosphoramidites reported were used as 0.1 M solutions in acetonitrile. Deprotection was accomplished with concentrated ammonium hydroxide at 55°C for 16 h. Purification was accomplished using OPEC columns (Clontech Laboratories, Inc.) according to recommended procedures. Oligonucleotides were analyzed by gel electrophoresis (20% PAGE-denaturing) and in every case demonstrated single band purity.

Melting experiments

Melting transitions were measured at 260 nm in 1.0 M NaCl, 0.001 M phosphate, pH 7.5 buffer, at an oligomer strand concentration of approximately 2 μM. Absorbance vs temperature for each duplex was obtained at a heating and cooling rate of 0.5°C/min and melting temperatures (T_m) were determined as the maxima of the differential curves, with an error of ±1°C.

Enzymatic digestion of oligonucleotides

0.5 OD units of the oligonucleotide in 1.0 mL buffer (50 mM Tris.HCl pH 8.6; 50 mM NaCl; 7 mM $MgCl_2$) was digested with 0.05 U snake venom phosphodiesterase (Pharmacia) [=1 μL of a solution of the enzyme in the following buffer: 5 mM Tris.HCl pH 7.5; 50% glycerol (v/v)] at 37°C. During the digestion the increase in absorbance at 260 nm was followed for 8 h. The increase in absorbance vs time was plotted for each oligonucleotide.

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Received March 12, 1996
Accepted June 29, 1996