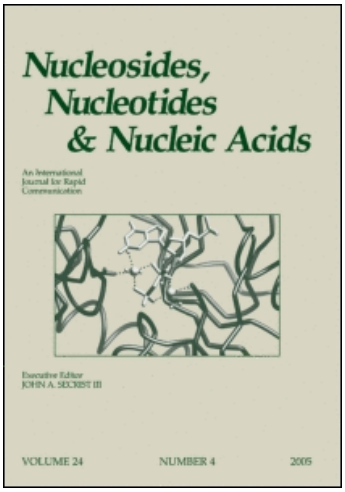

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STUDIES WITH 2-(BUTYLDIPHENYLSILOXYMETHYL)-BENZOYL PROTECTED OLIGODEOXYRIBONUCLEOTIDES

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ABSTRACT: Using 2-(tert-butyldiphenylsiloxymethyl)-benzoyl as the base protecting group, fully protected oligodeoxynucleotides can be obtained after ammonia-saturated methanol cleavage. A wide-pore reverse-phase C-8 chromatography column was suitable for the purification of the desired product.

Oligonucleotide synthesizers can be used for preparing regular oligodeoxynucleotides, as well as certain types of modified oligonucleotides. The commonly used base protecting groups, benzoyl and isobutyryl, have some disadvantages including (1) a long deprotection time of more than 6 hours (2) harsh cleavage conditions which are not suitable for oligonucleotide modification and conjugation, and (3) lack of selectivity where the deprotection and cleavage are done at the same time. Van Boom and co-workers[1,2] have recently reported the use of the fluoride labile 2-(tert-butyldiphenylsiloxymethyl)-benzoyl (SiOMB) N-protecting group (Figure 1), which might overcome the disadvantages of the benzoyl and isobutyryl groups. These investigators have used this N-protecting group to prepare a 4-mer having the group at two bases.

Fully base-protected oligodeoxyribonucleotides could be useful intermediates in the synthesis of various types of oligonucleotide conjugates and backbone modified oligonucleotides. Oligonucleotide conjugates with fluorescent groups, intercalators, crosslinking agents, and peptides have been reported,[3] and different approaches have been taken. For example, conjugation to the 5' terminus is generally carried out while the oligomer is attached to the solid sup-

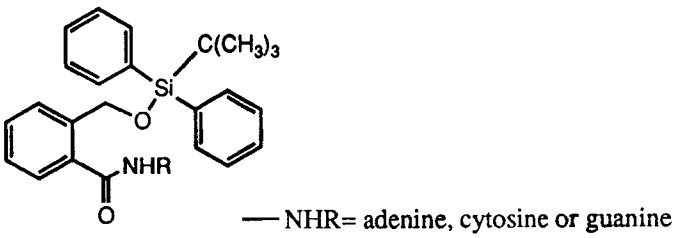

Figure 1. The 2-(t-Butyldiphenylsiloxymethyl)-benzoyl (SiOMB) protection group for exocyclic amino groups.

port.[3] In contrast, conjugation at the 3' terminus generally involves post synthetic modification after cleavage of the oligomer from the solid support using phosphotriester chemistry,[4] or the use of derivatized solid supports.[5] A fully protected oligodeoxyribonucleotide with a DMT blocking the 5' terminus could be used to prepare conjugates at the 3' terminus without the need of derivatized solid supports. One way to prepare such oligonucleotides is to use labile supports,[6,7] whereas the other way is to choose suitable protecting groups. Protecting groups that are removable under mild conditions can permit the use of base-sensitive ester linkages to the 3' and 5' termini of oligonucleotides already cleaved from the solid support. Such an easily removable protecting group would be useful, for example, in the preparation of base-sensitive nucleoproteins[8] or methylphosphate oligodeoxyribonucleotides. Studies on the synthesis and purification of SiOMB base-protected, tritylated oligodeoxynucleotides are now presented.

RESULTS AND DISCUSSION

The 5'-O-(4,4'-Dimethoxytrityl)-4-N-2-(tert-butyldiphenylsiloxymethyl)-benzoyl deoxycytidine 3'-O-2-(cyanoethyl)-N,N-(diisopropylamino)-phosphoramidite monomer was prepared using the procedure of Van Boom and coworkers[1,2] with modifications based on the work of Jones.[9] Commercially available thymidine phosphoramidite monomer was used in the synthesis of the oligomer. The fully blocked oligomer with the sequence 5'-TCTCTCTCTCTCTCT-3' was prepared using an automatic DNA synthesizer and standard protocols. The coupling yield of each synthesis cycle was deter-

mined by measuring the absorbance at 498 nm of the released DMT-groups, and averaged 96% per cycle.

The oligomer was cleaved from the CPG support by treatment with 29% ammonium hydroxide for 1 hour at room temperature. Reverse-phase C-18 column chromatography, as typically used for oligonucleotides, cannot be used for the purification of Trityl-on, SiOMB-on oligonucleotides because of the strong hydrophobicity of these multiple protecting groups. However, on a wide-pore reverse-phase C-8 column, the crude oligomer revealed a broad jagged peak between 35 and 55 minutes (Figure 2 A). Collecting the material between 35 and 55 minutes, treating with tetrabutyl ammonium fluoride for 1 hour and reinjecting this onto the reverse-phase column resulted in a major peak (eluting with 21% buffer B) corresponding to the deprotected trityl-on oligomer at 26 minutes (Figure 2 B). Collecting this peak, treating the material with 2% dichloroacetic acid in dichloromethane, and reinjecting the material onto the reverse-phase column resulted in a single peak at 14 minutes, corresponding to the purified, deblocked oligomer (Figure 2 C).

HPLC analysis of the crude 17-mer (i. e. after deprotection and deblocking, but without reverse-phase purification) on an ion-exchange column gave a major peak at 42 minutes, corresponding to the complete 17-mer. Smaller peaks at 41 minutes and at lower retention times are failure sequences (Figure 3 A). A calibation marker, 15-mer, was found to elute at 40 minutes on the ion-exchange column. Lack of peaks at retention times greater than 42 minutes, corresponding to oligomers having more than 16 charges along the phosphate backbone, was taken as evidence for the lack of formation of branched oligomers. This is significant, since the branching would occur at the N-4 of cytosine that is blocked by the SiOMB group. This lack of branching implies that the protecting group is stable to the synthesis conditions. The reverse-phase chromatography purified trityl-off, SiOMB-off product gave a major peak at 42 minutes, which corresponded to the 17 mer, and a second peak at 41 minutes which could have been uncapped n-1 failure sequences (Figure 3 B).

Gryaznov and Letsinger[10] have recently shown that coupling of a T nucleotide to an unblocked C nucleotide results in a significant amount of double coupling products observed by HPLC using a reverse-phase column. If the N-blocking group was falling off before or during the synthesis, we would have expected to see a significant amount of branching by HPLC analysis. Ion-ex-

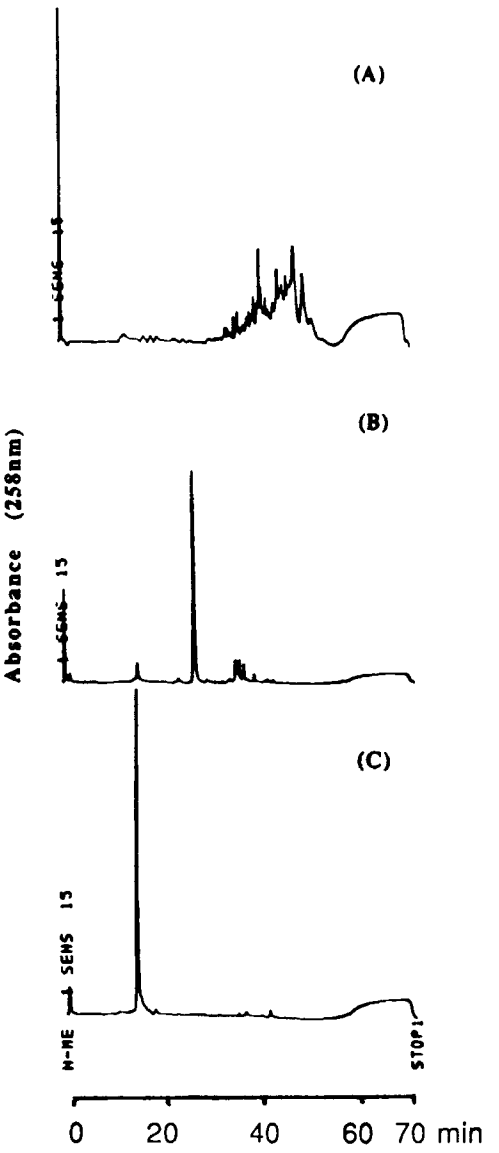

Figure 2. Reverse-phase C-8 HPLC chromatogram: (A) DMT-on/SiOMB-on oligomer which was cleaved from CPG with 29% ammonium hydroxide for 1 hr; (B) DMT-on/SiOMB-off oligomer; (C) DMT-off/SiOMB-off oligomer.

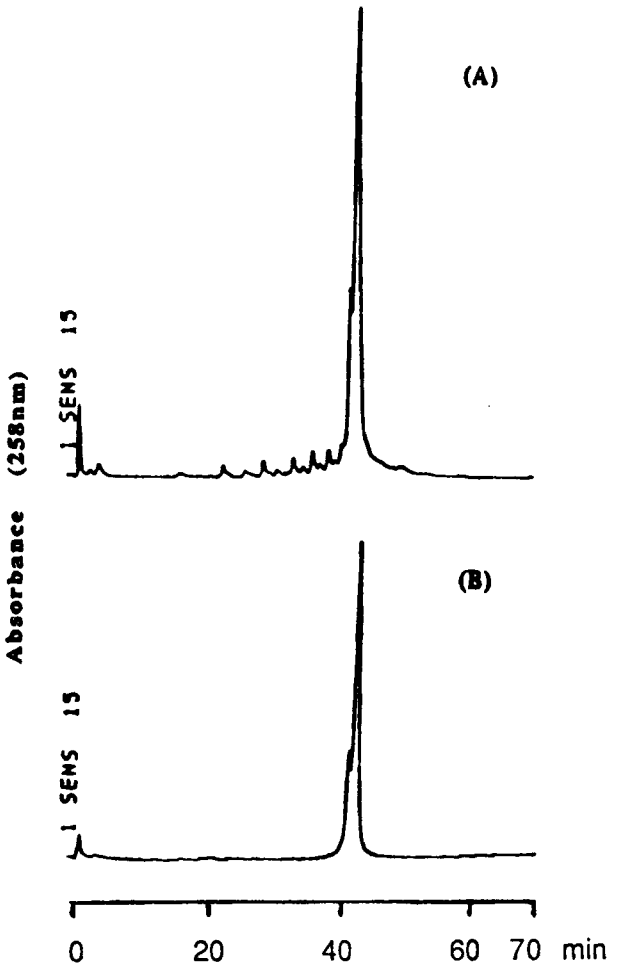

Figure 3. Ion-exchange HPLC chromatogram: (A) Crude DMT-off/SiOMB-off oligomer (B) DMT-off/SiOMB-off oligomer which was pre-purified by a reverse-phase trityl-on, trityl-off two-step procedure.

change results exclude branching, since the major peak was at the expected retention time and there were no observable peaks of longer retention time. There would also have been an increase in the amount of released DMT groups as the synthesis of the oligomer proceeded, if there were branching. This was not observed.

Studies were conducted to determine whether the multiple peaks of the SiOMB-on product were due to synthesis conditions. A 7-mer having the se-

quence 5'-TCTCTCT-3' was synthesized on a 1 μmol scale. The oligomer was subjected to one dummy synthesis cycle, substituting anhydrous acetonitrile for phosphoramidite monomer. Then, one half of the CPG resin was removed from the cartridge and the second half was subjected to 20 additional dummy cycles on the DNA synthesizer. Cleavage of each batch of oligomer by ammonium hydroxide from the CPG, followed by analysis of the crude 7-mer by reverse-phase HPLC, showed no difference in the peak patterns (Figure 4).

Having ruled out any deleterious effect occuring during the synthesis cycles, the next most likely explanation for the multiple peaks is that they represent DNA 17-mer with varying numbers of N-4 deprotected amine groups (i. e. where SiOMB groups were lost during the cleavage step). When the cleavage was done with ammonia-saturated methanol, a better chromatogram was obtained (c.f. Figure 2 A and Figure 5 B). Presumably, the fully-protected 17-mer would be the most hydrophobic product and would have the longest retention time on the reverse-phase column. From a time course cleavage experiment carried out with ammonium-saturated methanol, a limiting peak (at 54 min), corresponding to the most hydrophobic, fully protected product, could be discerned (Figure 5). Longer treatment with ammonia-saturated methanol caused a decrease in the amount of this fully protected 17-mer peak (Figure 5). Although the SiOMB group was sensitive to ammonia-saturated methanol solution, the wide-pore reverse-phase C-8 column was suitable for the separation of the fully-protected oligonucleotide from partially unprotected oligonucleotides. When the most hydrophobic peak, after storage at 4°C for 6 months, was rechromatographed, a single peak was obtained at the same retention time (data not shown), indicating stability of the base-protected product.

The possible effect of the solid supports was considered. Since van Boom et. al. carried out synthesis on monobeads,[2] we compared CPG and polystyrene solid supports. In this instance, an oligomer with the sequence 5' AAAAAT 3' was prepared using the SiOMB protected deoxyadenosine phosphoramidite (further details are not presented). Essentially, the same pattern was observed on reverse-phase HPLC for the crude product after ammonia-saturated methanol cleavage from each support (data not shown).

In conclusion, we have demonstrated the solid phase synthesis and HPLC purification of an oligodeoxyribonucleotide of useful length incorporating

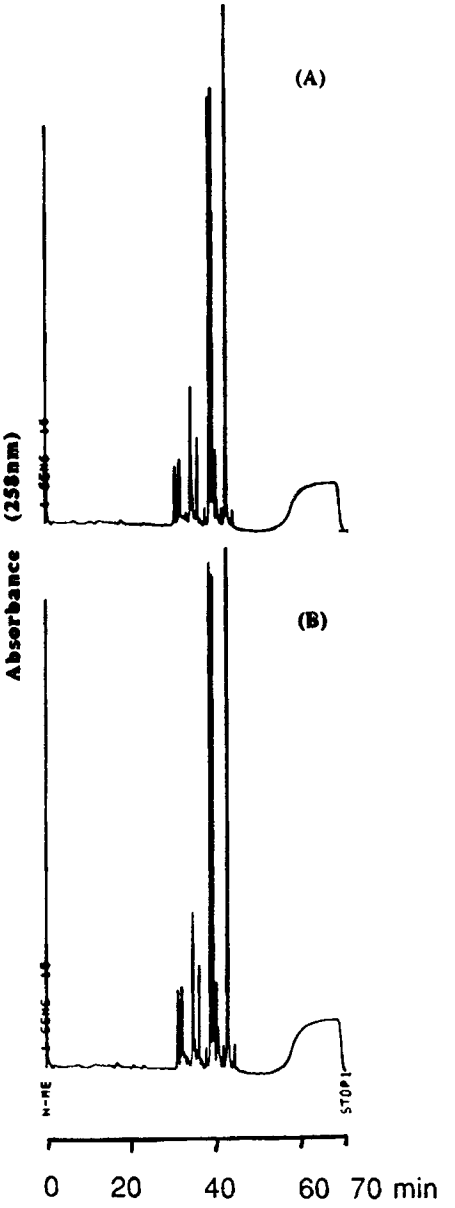

Figure 4. Effect of dummy cycles on the reverse-phase HPLC chromatogram of DNA oligomers: (A) DMT-off/SiOMB-on 7-mer exposed to 1 dummy cycle; (B) DMT-off/SiOMB-on 7-mer exposed to 21 dummy cycles.

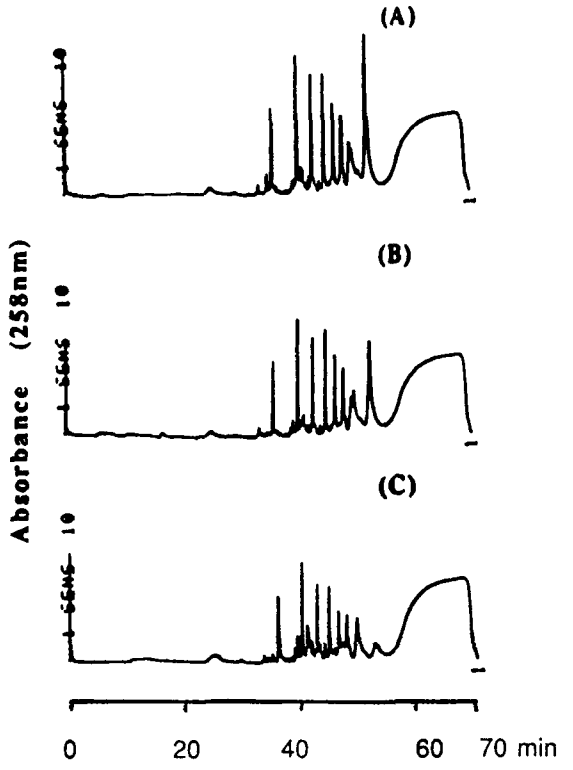

Figure 5. 17-mer on Reverse-phase C-8 HPLC chromatogram: Cleavage from CPG with ammonia-saturated methanol (A) 30 min; (B) 1 hr; (C) 3 hr.

a fluoride-labile N-protecting group at eight positions. The SiOMB protecting group is stable to synthesis conditions, but is sensitive to cleavage conditions. Ammonia-saturated methanol, rather than aqueous ammonia, is a better reagent for the cleavage step. The fully protected, as well as the deprotected, oligonucleotide can be purified using a wide-pore reverse-phase C-8 column under the same HPLC conditions.

EXPERIMENTAL

Pyridine, toluene, N,N-diisopropylamine, N,N-diisopropylethylamine, and triethylamine were dried by refluxing over calcium hydride and distilled immediately before use. Tetrahydrofuran was dried by refluxing over sodium benzophenone ketyl and distilled immediately before use. Dioxane was dried by refluxing over lithium aluminium hydride and distilled immediately before use. 2-Bromobenzylalcohol, tert-butyldiphenylsilyl chloride, oxalyl chloride, magne-

sium, trimethylsilylchloride, 2-cyanoethyl N,N-diisopropylaminochlorophosphine, deoxycytidine monohydrate, and 1.0 M tetrabutylammonium fluoride in tetrahydrofuran were purchased from Aldrich, Milwaukee, WI. Dichloroacetic acid (2%) in dichloromethane was purchased from American Bionetics, Emeryville, CA. Column chromatography was performed on 60Å, 75-150 μm particle size silica gel (Analtech, Newark, DE). Thin layer chromatography was performed on Silica Gel GHLF 250 micron thickness glass plates (Analtech). ^{1}H, ^{13}C, and ^{31}P NMR spectra were measured at a Varian VXR-200 spectrometer. HPLC analysis was carried out on a Hitachi L-6200 Intelligent Pump with a L-4200 UV-VIS Detector and a D-2000 Chromato-Integrator. Reverse-phase chromatography was carried out using an LC-308 (300Å) 2 cm x 4.6 mm i. d. cartridge (Supelco, Bellefonte, PA). Gradient elution was performed with a 1 ml/minute flow rate starting with 5 minutes isocratic buffer A (5% acetonitrile/95-% 0.1 M triethylammonium acetate, pH 7) then adding buffer B (95% acetonitrile/5% 0.1 M triethylammonium acetate, pH 7) at a 1 percent/minute increase for 25 minutes, changing to a 3 percent/minute increase for 25 minutes, then holding at isocratic buffer B for 10 minutes. Ion-exchange chromatography was carried out using a DEAE 60-7 Column 4 x 125 mm (Nucleogen, Duren, W. Germany). Gradient elution was performed with a 1 ml/minute flow rate starting with 10 minutes isocratic buffer A (60% 20 mM sodium acetate, pH 6.5/40% acetonitrile) then adding buffer B (buffer A containing 0.7 M lithium chloride) at 1 percent/minute for 60 minutes. The detector output at 258 nm was recorded in all cases. Solid phase synthesis was performed on an Applied Biosystems Model 380 A updated instrument. The synthesis was conducted on a 1 μmol scale using CPG support (Applied Biosystems, Foster City, CA).

5'-O-(4,4'-Dimethoxytrityl)-4-N-2-(tert-butyldiphenylsiloxymethyl)-benzoyl deoxycytidine

Deoxycytidine (1 g, 4.4 mmol) was co-distilled twice with 50 ml of pyridine, suspended in 60 ml of pyridine, and the volume concentrated to 50 ml under dry nitrogen. The reaction mixture was cooled on ice and 3 ml (23 mmol) of trimethylchlorosilane dissolved in 5 ml of pyridine was added over 15 minutes. The reaction mixture was stirred for 30 minutes on ice and 18 ml of a 0.5 M solution of 2-(tert-butyldiphenylsiloxymethyl)-benzoyl chloride[1] in 1,4-dioxane (9 mmol) was added over 10 minutes. The reaction mixture was then stirred for 16 hours at room temperature, cooled on ice and 8 ml of cold water was added. After stirring for 15 minutes, 8 ml of 29% aqueous ammonia was added. After stirring for 30 minutes on ice, the reaction mixture was concentrated to an oil under vacuum. The oil was dissolved in 20 ml of water and extracted with 20 ml of diethyl ether. The separated organic layer was evaporated to a solid foam. The foam was dissolved in 30 ml acetone and the unprotected nucleoside was removed by gravity filtration. The acetone solution was concentrated and then co-evaporated twice with 50 ml of pyridine, suspended in 30 ml of pyridine, and the volume concentrated to 20 ml. In a second flask, 4,4'-dimethoxytrityl chloride (1.8 g, 5.3 mmol) and 4-N,N-dimethylaminopyridine (0.027 g, 0.2 mmol) were co-evaporated twice with 30 ml of pyridine, suspend-

ed in 20 ml of pyridine, and the volume concentrated to 10 ml. The mixture in the second flask was added to the reaction mixture. Then, 0.9 ml (6.2 mmol) of triethylamine was added and the reaction mixture was stirred for 16 hours at room temperature. To the mixture was added 50 ml of water. The reaction mixture was extracted with two 200 ml portions of diethyl ether. The ether layers were concentrated under vacuum. Column chromatography (80 g of silica gel, dichloromethane/methanol, 100/0 to 95/5, v/v) and precipitation of an ethyl acetate solution of the product from hexane at -30 to -40°C. gave 1.4 g (1.5 mmol,37% yield) of the solid. Rf: 0.78 (9/1 CH_2Cl_2/MeOH). ^1H NMR ($CDCl_3$): δ 1.08 (s, 9H, $C(CH_3)_3$), 2.24 (m, 1H, H-2'), 2.74 (m, 1H, H-2"), 3.46 (m, 2H, H-5' and H-5"), 3.73 (s, 6H, OCH_3), 4.16 (m, 1H, H-4'), 4.51 (m, 1H, H-3'), 5.01 (s, 2H, CH_2OSi), 6.29 (m, 1H, H-1'), 6.84 (d, 4H, H-2 or 3 of $C_6H_4OCH_3$, $J_{2,3}$=8.8 Hz), 7.15-7.48 (m, 18H, aromatic H), 7.58 (d, 1H, H-6, $J_{5,6}$=7.4 Hz), 7.67 (d, 4H, H-2 or 3 of $C_6H_4OCH_3$, $J_{2,3}$=8.8 Hz), 8.25 (d, 1H, H-5, $J_{5,6}$=7.4 Hz). ^{13}C NMR($CDCl_3$): d 19.3 ($C(\underline{C}H_3)_3$), 26.9 ($\underline{C}(CH_3)_3$), 42.1 (C-2'), 55.2 (OCH_3), 62.7 (C-5'), 63.8 (CH_2OSi), 70.8 (C-3'), 86.4 (C-4'), 86.9 (C-1'), 87.2 (Cq, DMT), 113.3-135.6 (CH, aromatic), 132.0 and 133.1 (2xCSi, phenyl), 140.7 ($\underline{C}CH_2$), 144.3 (Cq, phenyl), 158.7 ($2x\underline{C}OCH_3$), 162.3 (C=O).

5'-O-(4,4'-Dimethoxytrityl)-4-N-2-(tert-butyldiphenylsiloxymethyl)-benzoyl deoxycytidine 3'-O-2-(cyanoethyl)-N,N-(diisopropylamino)-phosphoramidite

5'-O-(4,4'-Dimethoxytrityl)-4-N-2-(tert-butyldiphenylsiloxymethyl)-benzoyl deoxycytidine (200 mg, 0.22 mmol) was co-distilled twice with 10 ml of 9/1 CH_2Cl_2/Pyridine (v/v) and then allowed to remain under vacuum overnight. The flask was vented under dry nitrogen and 5 ml of anhydrous CH_2Cl_2 and 0.10 ml (0.55 mmol) of diisopropylethylamine were added. 2-Cyanoethyl N,N-diisopropylaminochlorophosphine (0.03 ml, 0.28 mmol) was added dropwise over 5 minutes. After stirring the reaction mixture at room temperature for 1 hour, the mixture was diluted with 50 ml of CH_2Cl_2 and transfered to a separatory funnel. The organic layer was washed with 20 ml of 10% $NaHCO_3$ and 20 ml of brine, dried over anhydrous $MgSO_4$ and concentrated under vacuum. The resulting oil was dissolved in ethyl acetate and the nucleotide was precipitated from hexane cooled to -30 to -40°C to give a 5'-O-(4,4'-dimethoxytrityl)-4-N-2-(tert-butyldiphenylsiloxymethyl)-benzoyl deoxycytidine 3'-O-2-(cyanoethyl)-N,N-(diisopropylamino)-phosphoramidite as a solid 225 mg (93% yield). TLC Rf: 0.65 (9/9/2 Ethyl Acetate/Dichloromethane/Triethylamine). ^{31}P NMR ($CDCl_3$): δ 150.9 and 151.4.

Cleavage, deprotection and purification of the DNA oligomer

The CPG support was removed from the DNA synthesizer cartridge, placed in a 4.5 ml plastic tube and treated with 1 ml of 29% NH_4OH for 1 hour at

room temperature. The solution was removed and the resin washed twice with 500 µl of 1:1 (95% 0.1 M triethylammonium acetate, pH 7, 5% acetonitrile)/methanol (v/v). The crude oligomer was dried under vacuum, dissolved in methanol and a portion (0.5 Unit, A_{258}) was analyzed by reverse-phase HPLC (Figure 2 A). A second portion of the oligomer (0.8 Unit A_{258}) collected from the reverse-phase column, dried and redissolved in 10 µl of methanol was treated with 1 µl of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran for 1 hour and analyzed by reverse-phase HPLC (Figure 2 B). The peak at 26 minutes was collected and the SiOMB-off/Trityl-on oligomer was dried under vacuum, and treated with 20 µl of dichloroacetic acid (2%) in dichloromethane. After stirring for 2 minutes by vortex, 200 µl of diethyl ether and 100 µl of water were added, and the diethyl ether layer was decanted. The aqueous layer was analyzed by reverse-phase HPLC (Figure 2 C). The peak at 14 minutes was collected, the SiOMB-off/Trityl-off oligomer was dried under vacuum, dissolved in 50 µl of water, and analyzed by an ion-exchange HPLC (Figure 3 B). In a separate experiment, a portion of the oligomer (0.8 Unit A_{258}) in 8 µl of methanol was treated with 1 µl of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran for 1 hour at room temperature. Then 20 µl of dichloroacetic acid (2%) in dichloromethane was added and stirred for 2 minutes by vortex. To this solution was added 200 µl of diethyl ether and 100 µl of water. The diethyl ether layer was decanted, the aqueous layer was analyzed by ion-exchange HPLC (Figure 3 A).

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