Supporting Material for

3'-Nitrophenylpropyloxycarbonyl (NPPOC) Protecting Groups for High-fidelity Automated 5'→3' Photochemical DNA Synthesis

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5'-TBDPS-3'-NPPOC-thymidine.

A solution of 2-(2-nitrophenyl)propanol (1.7 g, 0.938 mmol, MW = 181.17) in 30 mL anhydrous THF was added slowly to a solution of 20% phosgene in toluene (20 mL, 56.7 mmol, d = 0.935, MW = 98.92) in 10 mL THF at ice bath temperature. The mixture was stirred at 0 °C for 30 min, then room temperature for another 2 h. TLC indicated a complete conversion of the starting material (R_f = 0.3 in 2:1 / hexane : ethyl acetate) into its chloroformate product (R_f = 0.7). The solvent was removed carefully in vacuo. The oily product was dissolved in 25 mL anhydrous THF and slowly added to a solution of 5'-TBDPS thymidine (azeotroped twice from pyridine, 3.0 g, 6.24 mmol, MW = 480.6) in 30 mL anhydrous pyridine at 0 °C. The mixture was stirred at 0 °C for 2 h followed by another 12 h at room temperature. To this was added 3.0 mL methanol and the solvent was concentrated in vacuo after 30 min stirring. The oily residue was taken up in ethyl acetate and washed with saturated $NaHCO_3$ and saturated NaCl, dried with Na_2SO_4 and concentrated. The crude product was subjected to flash chromatography on a silica gel column with CH_2Cl_2/CH_3OH (0.5% to 3.3%) to give 3.8 g desired product (yield = 88%). ^{1}H-NMR ($CDCl_3$): δ 8.60 (s, 1H, -NH), 7.36-7.80 (15H, Ar-H, -H6), 6.37 (dd, 1H, H1'), 5.30 (m, 1H, H3'), 4.35 (m, 2H, -CH_2), 4.12 (q, 1H, H4'), 3.88 (m, 2H, H5', -CH), 3.74 (m, 1H, H5''), 2.48 (m, 1H, H2'), 2.20 (m, 1H, H2''), 2.154, (d, 3H, T-CH_3), 1.38 (dd, 3H, -CH_3) 1.09 (s, 9H, 3 X-CH_3). MS (FAB+, 3-NBA): m/z 688.4 (calculated M + 1 = 688.26).

Also isolated from the column was 0.7g 5'-TBDPS-3', O^4-di-NPPOC-thymidine (yield = 10%). ^{1}H-NMR ($CDCl_3$): 7.36-7.80 (18H, Ar-H, -H6), 6.31 (dd, 1H, H1'), 5.28 (m, 1H, H3'), 4.61 (m, 2H, -CH_2), 4.35 (m, 2H, -CH_2), 4.12 (m, 1H, H4'), 3.92 (m, 2H, H5', -CH), 3.76 (m, 1H, H5''), 2.49 (m, 1H, H2'), 2.21 (m, 1H, H2''), 1.451 (m, 9H, T-CH_3, 2X-CH_3), 1.09 (s, 9H, 3 X-CH_3). MS (FAB+, 3-NBA): m/z 895.5 (calculated M + 1 = 895.31).

(Ref: A Hasan, K-P. Stengele, H. Gegrich, P Cornwell, K Isham, R. Sachleben, W. Pfleiderer and R Foote, *Tetrahedron*, **1997**, 53, 4247-4264)

5'-TBDPS-3'-NPPOC-N^4-isobutyryldeoxycytidine.

5'-TBDPS-N^4-isobutyryldeoxycytidine (4.0 g, 7.47 mmol, MW = 535.7) was treated with 2-(2-nitrophenyl)propanol (1.7 g, 9.34 mmol, MW = 181.2) and phosgene (50 mL) following the procedure described above to give 5.1 g 5'-TBDPS-3'-NPPOC-N^4-isobutyryldeoxycytidine (yield = 92%). ^{1}H-NMR ($CDCl_3$): 8.89 (dd, 1H, H5), 7.20-7.80 (m, 15H, ArH, H6), 6.27 (m, 1H, H1'), 5.21 (m, 1H, H3'), 4.33 (m, 2H, -OCH2-), 4.21 (m, 1H, H4'), 3.97 (dd, 1H, -CHAr), 3.90 (m, 1H, H5'), 3.73 (m, 1H, H5''), 2.27(m, 1H, -CH=), 2.67 (m, 2H, H2'), 2.17 (m, 1H, H2''), 1.39 (dd, 3H, -CH_3), 1.24 (d, 6H, 2X-CH_3), 1.06 (s, 9H, 3X-CH_3). MS (FAB+, 3-NBA): m / z 743.2 (calculated M + 1 = 743.30).

3'-NPPOC-thymidine.

To a solution of 5'-TBDPS-3'-NPPOC-thymidine (3.7 g, 5.38 mmol, MW = 687.8) in 120 mL anhydrous THF was added 10 mL triethylamine trihydrofluride (61.4 mmol, MW = 161.2, d = 0.989) (Ref: M. Pirrung, L Fallon, D. Lever, S. Shuey, *J. Org. Chem.* **1996**, *61*, 2129-2136). The mixture was stirred at room temperature overnight and concentrated in vacuo. The residue was diluted with 200 mL methylene chloride and washed with saturated $NaHCO_3$ and saturated NaCl. The organic phase was dried with Na_2SO_4 and concentrated. The crude product was chromatographed on a silica gel flash column (2% to 3.5% methanol in methylene chloride) to give 2.2 g pure 3'-NPPOC-thymidine (yield = 91%). ^{1}H-NMR ($CDCl_3$): δ 8.65 (s, 1H, -NH), 7.36-7.81 (m, 4H, Ar-H), 6.15 (m, 1H, H1'), 5.25 (m, 1H, H3'), 4.28 (m, 2H, -CH_2), 4.14 (m, 1H, H4'), 3.85 (m, 3H, H5', -CH, H5''), 2.48 (m, 2H, H2', H2''), 1.92 (d, 3H, T-CH_3), 1.39 (dd, 3H, -CH_3). HRMS (FAB+, 3-NBA): m / z 450.15 (calculated M+1 = 450.14).

3'-NPPOC-N^4-isobutyryldeoxycytidine.

3'-NPPOC-N^4-isobutyryldeoxycytidine (5.0 g, 6.73 mmol, MW = 742.9) was treated with excess triethylamine trihydrofluride following the procedure described above to give 3.1 g pure 3'-NPPOC-N^4-isobutyryldeoxycytidine (yield = 91.3%): ^{1}H-NMR ($CDCl_3$): δ8.28 (b, 1H, H5), 7.3-7.8 (m, 5H, ArH, H6), 6.15 (t, 1H, H1'), 5.26 (m, H3'), 4.2-4.4 (m, 3H, -OCH_2-, H4'), 3.7-4.0 (m, 3H, -CHAr, H5', H5''), 2.65 (m, 2H, -CH=, H2'), 2.46 (m, 1H, H2''), 1.38 (dd, 3H, -CH_3), 1.22 (d, 6H, 2X-CH_3). HRMS (FAB+, 3-NBA): m / z 505.20 (calculated M+1 = 505.19).

3'-NPPOC-thymidine 5'-cyanoethyl(diisopropyl)phosphoramidite.

3'-NPPOC thymidine (1.0 g, 2.2 mmol , MW = 449) was dried by co-evaporation with 50% methylene chloride in benzene twice followed by P_2O_5 / vacuum dessicator overnight. The dried foam was dissolved in 90 mL anhydrous methylene chloride and N,N-diisopropylethylamine (0.767 ml, 4.4 mmol, MW = 129.25, d = 0.742). To this was slowly added 2-cyanoethyl diisopropyl chlorophosphoramidite (0.59 ml, 2.64 mmol, MW = 236.68, d = 1.061) at 0 °C under the protection of argon. The mixture was stirred at 0 °C for 20 min followed by another 3 h at room temperature. The mixture was quickly extracted with 30 mL 10% Na_2CO_3 (degassed) followed by 30 mL saturated NaCl (degassed) under the protection of argon. The organic phase was dried with Na_2SO_4 for 20 min and concentrated. Purification of the product was accomplished by hexane (60 ml) precipitation twice from methylene chloride (10 ml) to give 1.1 g desired product (yield = 77%). ^{1}H-NMR ($CDCl_3$): δ 8.40 (s, 1H, -NH), 7.26-7.81 (m, 4H, Ar-H), 6.31 (m, 1H, H1'), 5.20 (m, 1H, H3'), 4.31 (m, 4H, -CH_2, H5', H5''), 3.91(m, 2H, H4', -CH), 3.80 (m, 2H, -OCH_2), 3.60 (m, 2H, 2X-CHN), 2.64 (t, 2H, -CH_2CN), 2.48 (m, 1H, H2'), 2.17 (m, 1H, H2''), 1.89 (d, 3H, T-CH_3), 1.11-1.41(m, 15, 5X-CH_3). ^{31}P-NMR ($CDCl_3$): δ 149.60, 148.97, 148.92. HRMS (FAB+, 3-NBA): m / z 650.26 (calculated M+1 = 650.25).

3'-NPPOC-N^4-isobutylrydeoxycytidine 5'-cyanoethyl(diisopropyl) phosphoramidite.

3'-NPPOC-N^4-isobutylrydeoxycytidine (2.0g, 4.0 mmol, MW = 504.5) was treated with 2-cyanoethyl diisopropyl chlorophosphoramidite (1.2 ml, 5.36 mmol) in the presence of DIPEA (1.76 ml) following the procedure described above to give 2.0 g pure 3'-NPPOC-N^4-isobutylrydeoxycytidine 5'-cyanoethyl(diisopropyl)phosphoramidite (yield = 70%). ^{1}H-NMR ($CDCl_3$): δ 8.73 (s, 1H, -NH), 8.22 (m, 1H, NH), 7.27-7.81 (m, 5H, Ar-H), 6.29 (m, 1H, H1'), 5.16 (m, 1H, H3'), 4.33 (m, 4H, -CH_2, H5', H5''), 3.93(m, H, H4', -CH), 3.82 (m, 2H, -OCH_2), 3.63 (m, 2H, 2X-CHN), 2.77 (m, 1H, H2), 2.66 (m, 2H, -CH_2CN), 2.12 (m, 1H, H2''), 1.11-1.45 (m, 15H, 5X-CH_3); ^{31}P-NMR ($CDCl_3$): δ 149.63, 149.65, 149.34. HRMS (FAB+, 3-NBA): m/z 7.5.30 (calculated M+1 = 7.5.29).

Photochemical synthesis of oligonucleotides

All synthesis was carried out on a modified Applied Biosystems 392 DNA synthesizer, which interfaced with an Oriel 71445 shutter controller. Reagent delivery from the synthesizer was controlled using OligoNet software, with appropriate adjustment of the standard protocols to accommodate the particular needs of the photo-deprotection step in each cycle. Phosphoramidites were used at a concentration of 0.1 M in anhydrous acetonitrile. All reactions were conducted in 0.2 μmole scale on controlled pore glass beads in custom-built plastic columns as described below.

1) Plastic column preparation

The column cartridge was assembled using a high clarity plastic tube (Chemfluor Fluoropolymer PFA plastic tube 5/12 I.D. × 1/4 O.D., VWR catalog #63014-879, made by Norton Performance Plastic #D84204202) and two polymer filters (4.1 mm in diameter × 2.2 mm thick, Glen Research, #20-0021-0F, replacement filter for Expedite column). The plastic tube was cut to 2.4 cm in length. Into this was pushed one polymer

filter to about 1 cm from the end. The column was loaded with about 3.3 mg of 60 μg/g dT-5'-CPG (Glen Research, #20-0302), followed by installation of the second polymer filter from the other end. The trityl group of the thymidine on the glass beads was manually removed using 10 mL 3% TCA/CH_2Cl_2 by syringe injection. The inner diameter of this home-made column is similar to that of the standard reaction column, so it can be directly mounted vertically to the column adapter on the DNA synthesizer.

2) Synthesizer Program

The cycles for 5'→3' photo-synthesis were created based on the standard ABI "1.0 μM CE" protocol. The coupling time was increased to 120s from 25s. The detritylation step was replaced by a photo-deprotection step using a 20 min, 365 nm UV-light illumination in the presence of 50 mM piperidine or 50 mM DBU in anhydrous acetonitrile, which was delivered from bottle 10. The UV irradiation was controlled by the synthesizer through a relay connected to the light shutter. During UV irradiation, the synthesizer was programmed to refresh the solution every 2 min by flushing and filling steps to wash away the photo-cleaved byproduct, and more importantly to move the CPG resin around in the column to assure all the glass beads receive UV.

3) UV light supply

The UV light was supplied by ORIEL 1000W mercury-xenon arc lamp. The light is passed successively through a liquid filter (Oriel #6123, filled with de-ionized water) and a 364 nm interference filter (Oriel #56536), such that UV light with only a 10 nm bandwidth centered at 365 nm was used. The light was delivered to the reaction column on the DNA synthesizer through a 1 meter long light guide (Oriel 71260). The light illuminated the column perpendicularly from about 1 cm away from its center. The output from the light guide is about 4.0 mW/cm^2 at the optimal distance (about 1.0 cm away from its end) as measured with a power meter. Only about 1.0 mW/cm^2 of the

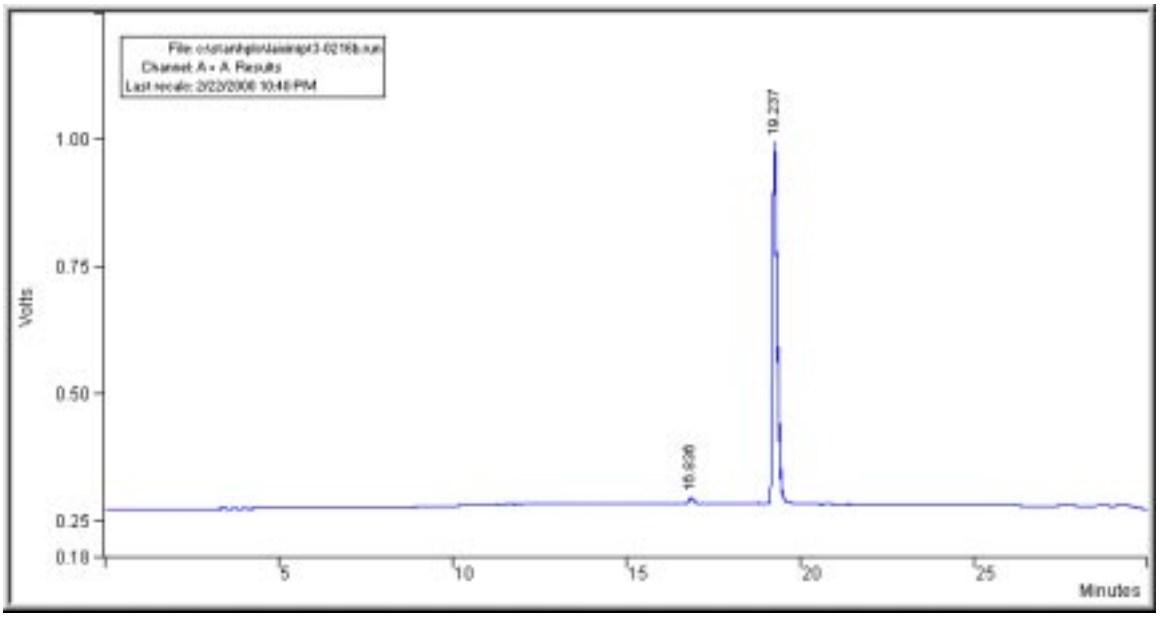
light passes the plastic column at the same distance. A reflector was placed behind the column towards the light source.

4) Product analysis

All oligonucleotides were synthesized using the above described program and a no-deprotection ending procedure. The oligonucleotide products were cleaved from the CPG column by 2 to 3 h treatment using 1.0 mL concentrated ammonia at room temperature, followed by a 5 h incubation at 50 °C to make sure all protecting groups were completely removed. The solution was concentrated to dryness in a speed-vac at room temperature. The pellet was dissolved in 0.5 mL of 25 mM TEAA buffer (pH = 6.5). About 20 μl of the solution was used for HPLC analysis. HPLC was performed on a HP1100 series Liquid Chromatograph System. The UV detector was set at 260 nm. The data were collected and processed using Varian Star Chromatograph Workstation software on a PC. Column: Supelcosil LC-18S 4.6 × 250 mm, 5 μm. Solvent A: 25 mM pH=6.5 TEAA buffer; solvent B: 40% acetonitrile in water. Solvent flow rate: 1.0 mL/min. A linear gradient, 20% to 40%B in the first 30 min, was used for all analyses except for decathymidine.

Figure 1. HPLC chromatograms of the fully deprotected product mixtures of TTT (A) and TTTT (B) photochemical synthesis

(A)



(B)

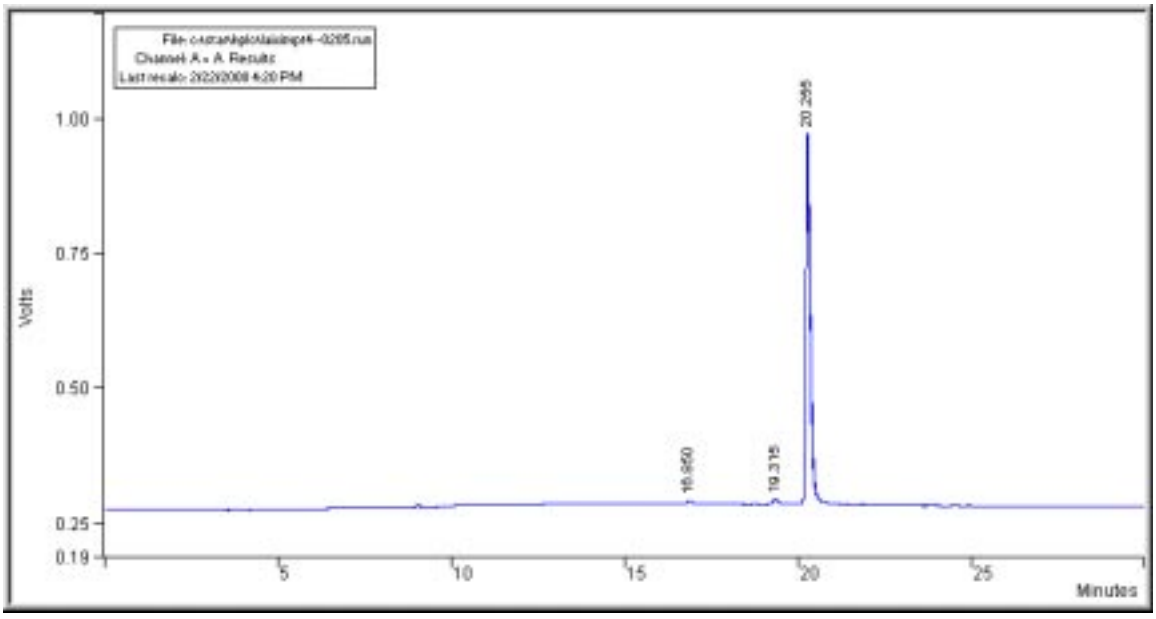


Figure 2. MALDI-TOF MS of TTTT prepared by photochemical synthesis.