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A Convenient Method for the Preparation of Oligonucleotide 5'-Phosphates

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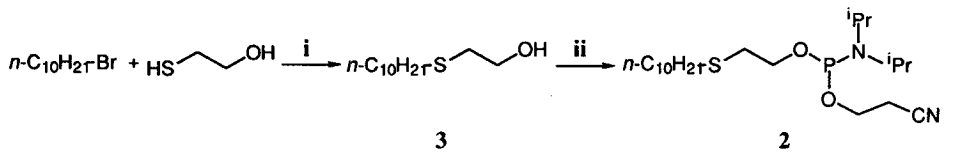
Abstract: Oligodeoxyribonucleotides (4-, 5-, and 11-mer) bearing a 5'-phosphate group have been synthesized using 2-*n*-decylthioethyl as protecting group of the 5'-terminal phosphate function. The lipophilic protecting group, which is easily available from 1-bromo-*n*-decane and 2-mercaptoethanol, allows a simple and efficient purification of the oligonucleotides. It is quantitatively removed by oxidation of the sulfide to the sulfoxide, followed by β-elimination in aqueous base media.

INTRODUCTION

In our ongoing program on oligodeoxynucleotide probes, we had to devise a convenient method for the synthesis of oligodeoxyribonucleotides bearing a phosphate group at the 5'-terminus.[1] On the basis of reported protection schemes,[2] we decided to use 2-*n*-decylthioethyl as protecting group for the 5'-terminal phosphate function. This group is orthogonal to the oligonucleotide synthetic protocols, and can be specifically and quantitatively removed under basic conditions after oxidation of the thioether without leading to side reactions. The feasibility of the strategy and the protecting group was first tested with two model sequences, 5'-TTTT (**1a**) and 5'-TACGT (**1b**), and the method was then applied to 5'-CTCGTCCACCA (**1c**).

RESULTS AND DISCUSSION

The synthesis of the phosphoramidite **2** is outline in Scheme 1. The intermediate 2-*n*-decylthioethanol (**3**) was prepared in high yield from 1-bromo-*n*-decane and 2-mercaptoethanol in the presence of dicyclohexylamine. O-β-cyanoethyl-O-[S-*n*-decyl-2-mercapto-1-ethyl]-N,N-diisopropyl phosphoramidite (**2**) was obtained by addition under an argon atmosphere of O-β-cyanoethyl-N,N,N',N'-tetraisopropyl phosphordiamidite and tetrazole to 2-*n*-decylthioethanol dissolved in dry MeCN. The product, a colourless oil, was obtained by chromatography on silica gel.



3 2

Scheme 1. *Reagents and conditions*: **i**: dicyclohexylamine (1.0 equiv.), DMF, 20 °C, 15 h; **ii**: O-β-cyanoethyl-N,N,N',N'-tetraisopropyl phosphordiamidite (1.6 equiv.)/tetrazole (1.3 equiv.), anhydrous MeCN, 20 °C, 15 h under an argon atmosphere.

Oligonucleotides **1a-c** were synthesized on an Applied Biosystems 394 DNA synthesizer using chemical protocols based on O-β-cyanoethyl-phosphoramidite chemistry. Incorporation of the phosphoramidite **2** was accomplished manually under standard conditions to give the phosphite triesters **3a-c**. Aqueous iodine oxidation to the phosphate triesters **4a-c** resulted in partial oxidation of the thioether and subsequent β-elimination of the protecting group during the post-synthetic cleavage of the oligonucleotide from the CPG solid support.[3] This side reaction was avoided by carrying out the oxidation with a freshly prepared 0.1 M solution of *tert*-butylhydroperoxide in dichloromethane.[4] After completion of the syntheses, the CPG-bound oligonucleotides were treated overnight with concentrated aqueous ammonia at 55 °C. The lipophilic nature of the linear aliphatic moiety allowed a simple and efficient purification, since the 5'-terminal O-protected phosphate oligonucleotides were well resolved over a C_{18} reversed-phase HPLC column from side products and unreacted material. The purity of **5a-c** was verified by reversed-phase analytical HPLC and capillary electrophoresis (see Figure 1A for **5c**), and the identity was assessed by correct MALDI-TOF mass spectral analyses (see Figure 1B for **5c**).[5]

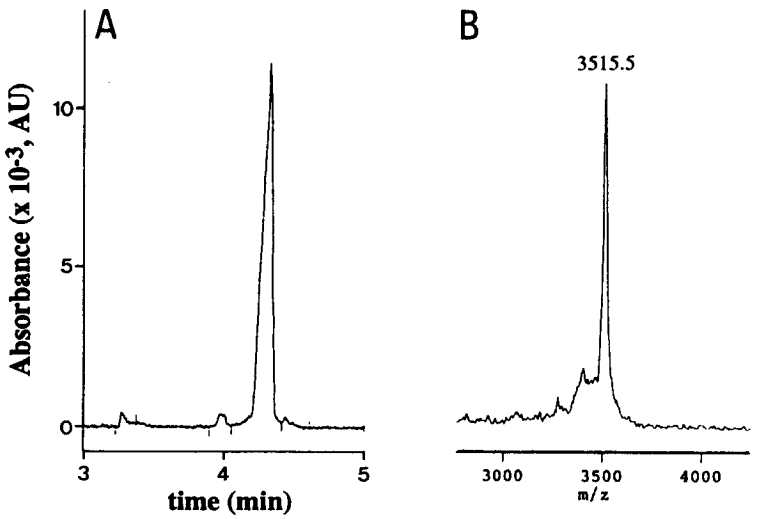
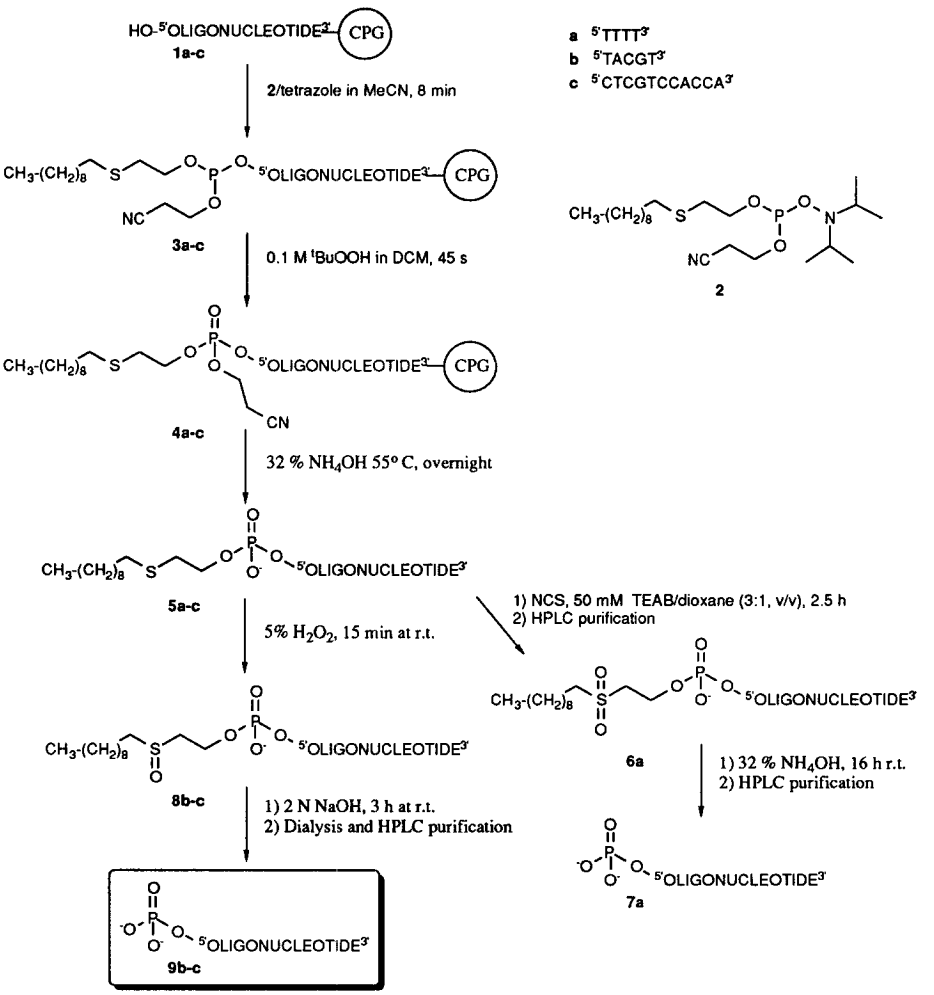


Figure 1.- A) Capillary electrophoretic scan run (λ= 260 nm) of the 5'-terminal O-protected oligonucleotide phosphate **5c**. B) MALDI-TOF mass spectra of **5c** (negative-ion mode): calcd. 3516.6 [M-H], found 3515.5.

The protecting group was removed by oxidation of the sulfide, followed by β-elimination in base media (Scheme 2). The deprotection conditions were optimized with the model compounds **5a** and **5b**. Complete and clean conversion of the thioether **5a** to the sulfone **6a** was obtained upon oxidation with an excess of *N*-chlorosuccinimide (≈ 20-fold excess) in 50 mM TEAB (pH = 7.5)/dioxane (3:1, v/v) for 2.5 h at room temperature. Under identical experimental conditions, the oxidation of **5b** resulted in multiple unidentified by-products, which, in our hands, renders the NCS-mediated oxidation futile for mixed sequences.[6] As an alternative, we decided to oxidize the thioether to the sulfoxide. This reaction was effectively accomplished

with a 5 % aqueous solution of H_2O_2. After 15 min at room temperature, no trace of the starting material was observed by analytical C_{18} reversed-phase HPLC. The MALDI-TOF mass spectra of the products showed the expected increase of 16 amu in molecular weight. The sulfoxide oligonucleotide derivatives **8b-c** were deprotected by treatment with 2 N NaOH for 3 h at room temperature.[7] Dialysis and reversed-phase HPLC purification gave the oligonucleotides bearing the 5'-terminal phosphate group. The purified oligonucleotides were characterized by capillary electrophoresis (see Figure 2A for **9c**) and MALDI-TOF mass analyses (see Figure 2B for **9c**).



Scheme 2. Procedure for the preparation of oligodeoxyribonucleotide 5'-phosphates.

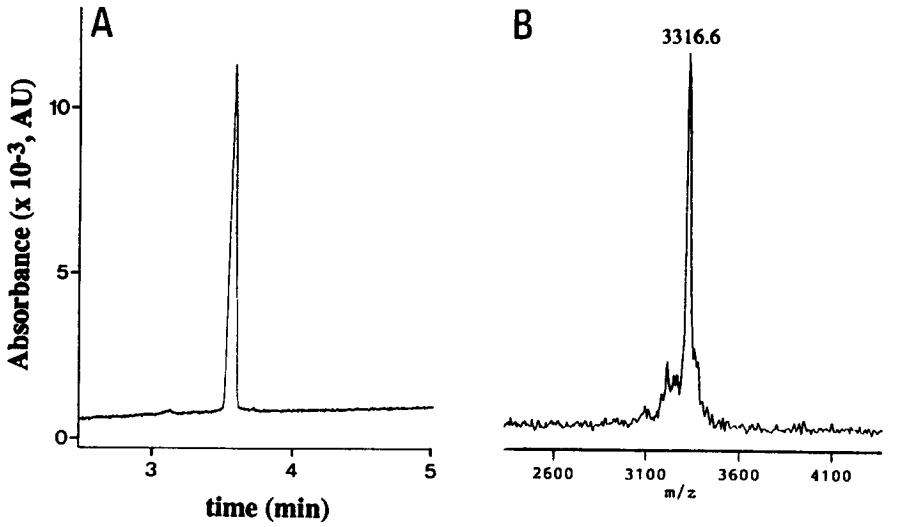


Figure 2.- A) Capillary electrophoretic scan run (λ= 260 nm) of the oligonucleotide 5'-phosphate **9c**. B) MALDI-TOF mass spectra of **9c** (negative-ion mode): calcd. 3316.2 [M-H], found 3316.6.

From the above, it can be seen that the phosphoramidite **2** described in this paper allows the efficient preparation of oligodeoxyribonucleotides containing a 5'-phosphate group. The protecting group, which is easily available from 1-bromo-*n*-decane and 2-mercaptoethanol, is orthogonal to the oligonucleotide synthetic protocols and can be quantitatively removed by hydrogen-peroxide-mediated oxidation of the sulfide to the sulfoxide, followed by β-elimination in 2 N NaOH.

EXPERIMENTAL

General.- Anhydrous solvents were purchased from Fluka. Reagents for oligonucleotide synthesis were from Cruachem. O-β-cyanoethyl-N,N,N',N'-tetraisopropyl phosphordiamidite was prepared according to ref. 8. Short column chromatography: silica gel 60 (70-230 mesh, Merck). TLC: Silica gel plates ('Kieselgel' *60 F_{254}*; Merck); visualization by dipping in a stainning solution (2.5 g of phosphormolybdic acid and 0.6 g of cerium (IV) sulfate in 1 l of 1 M H_2SO_4) following by heating the plates with an air gun. NMR spectra were recorded on a Bruker BZH spectrometer operating at 250 and 101 MHz for ^1H and ^{31}P, respectively; chemical shifts δ in ppm *versus* $SiMe_4$ (= 0 ppm, ^1H; internal standard) or H_3PO_4 (^{31}P; external standard), and coupling constants J in Hz. HPLC analyses were carried out with RPC_{18} ODS Hypersil columns on a Merck HITACHI liquid chromatography system formed by a L-3000 Photo Diode Array Detector, a L-6200A Intelligent-Pump system, and a D-2500 Chromato-Integrator. Mass spectra were run on an LDI 1700 instrument (Linear Scientific Inc.). Oligodeoxyribonucleotides were synthesized on an automated DNA synthesizer ABI 394B (Applied Biosystems). Capillary electrophoretic scans were performed on a Beckman P/ACE System 2100.

2-n-Decylthioethanol (3).- To a solution of 1-bromo-*n*-decane (106 ml, 500 mmol) in DMF (1 l) was added dicyclohexylamine (100 ml, 500 mmol) and 2-mercaptoethanol (37 ml, 500 mmol). The solution was stirred overnight at room temperature under an argon atmosphere. After completion of the reaction, the suspension was filtered and the precipitate was washed extensively with DMF. The DMF filtrate was evaporated to dryness and the residue was dissolved in AcOEt. The solution was washed with brine, dried over anhydrous

Na_2SO_4, and evaporated to dryness. The compound was purified by distillation, bp: 120-122 °C, 0.1 Torr;[9] Yield: 101 g (462.5 mmol, 93%). TLC: R_f 0.69 ($CHCl_3$:AcOEt, 1:1, v/v). ^1H NMR ($CDCl_3$) δ 3.70 (br t, *J* = 7 Hz, 2H), 2.72 (t, *J* = 7 Hz, 2H), 2.52 (t, *J* = 7 Hz, 2H), 1.57 (m, 2H), 1.3 (br s, 14 H), 0.90 (br t, *J* = 7 Hz, 3H).

O-β-Cyanoethyl-O-[S-n-decyl-2-mercapto-1-ethyl]-N,N-diisopropyl phosphoramidite (2).-To a solution of 2-*n*-decylthioethanol (98 g, 449 mmol) in anhydrous MeCN (1 l) was added O-β-cyanoethyl-N,N,N',N'-tetraisopropyl phosphordiamidite (255 ml, 710 mmol) and tetrazole (39.2 g, 560 mmol). The mixture was stirred overnight under an argon atmosphere. The suspension was filtered to remove a white-yellow precipitate, which was washed with MeCN. To the MeCN filtrate was added triethylamine (20 ml) and the solution was evaporated to dryness. The residue was dissolved in AcOEt (1 l) and after washing the solution with 1 N $NaHCO_3$ (2 x 300 ml, cold), and brine (2 x 250 ml), it was dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue (238 g) was then subjected to chromatography on silica gel using CH_2Cl_2:AcOEt (8:2, v/v; 1% NMM) as eluent. Yield: 98.3 g (235 mmol, 52%), colourless oil. ^{31}P NMR ($CDCl_3$): δ 147.74 (H_3PO_4 as external standard).

Oligodeoxyribonucleotide synthesis.- Oligonucleotides **1a-c** were synthesized on an Applied Biosystems 394B DNA synthesizer using standard chemical protocols based on the O-β-cyanoethyl-phosphoramidite chemistry. Incorporation of the phosphoramidite **2** to the free 5'-hydroxyl group was accomplished manually according to the following protocol: i) washes with anhydrous MeCN (6 x 15 s); ii) coupling of the phosphoramidite **2** (1 x 8 min); single coupling. For a 1 μmol scale synthesis, the phosphoramidite **2** (≈ 40 mg) was dissolved under anhydrous condition in acetonitrile/tetrazole (400 μl; Applied Biosystems) and incorporated directly to the CPG bound oligonucleotide to give the phosphite triesters **3a-c**; iii) washes with anhydrous MeCN (6 x 15 s). Once incorporation of the phosphoramidite was completed, the phosphite triester were oxidized to the phosphate triester **4a-c** with a freshly prepared 0.1 M solution of *tert*-butylhydroperoxide in dichloromethane (1 x 45 s). After completion of the oxidation step, the solid supports were washed with anhydrous acetonitrile (6 x 15 s) and dried. The oligonucleotides were liberated from the solid support and the side chains deblocked by overnight treatment of the CPG bound oligonucleotides with concentrated aqueous ammonia at 55 °C. The oligonucleotides were purified by reversed-phase high-pressure liquid chromatography on a Hypersil C_{18} column (250 x 8 mm, 5 μm) eluted with a MeCN/50 mM TEAA (7:3, v/v)-50 mM TEAA (pH= 7.0) gradient. The identity of the purified 5'-terminal O-protected diphosphate oligonucleotides **5a-c** was assessed by correct MALDI-TOF mass spectral analyses. MALDI-TOF mass spectra (negative-ion mode): calcd. 1434.2 [M-H], found 1434.3, **5a**; calcd. 1757.4 [M-H], found. 1757.9, **5b**; calcd. 3516.6 [M-H], found 3515.5, **5c**. The purity of the purified compounds was verified by analytical HPLC on a Hypersil-C_{18} column (125 x 4.6 mm, 5 μm), flow rate 1.0 mL/min, detection at 260 nm: linear gradient over 70 min of MeCN/50 mM TEAA (7:3, v/v) and 50 mM TEAA (pH= 7.0) from 15:85 to 85:15, single peak at t_R= 31.3 min (**5a**); linear gradient over 50 min from 15:85 to 65:35, single peak at t_R= 30.3 min (**5b**); linear gradient over 37 min from 15:85 to 52:48, single peak at t_R= 23.4 min (**5c**); see Figure 1A for the capillary electrophoretic scan run of **5c**.

Deprotection experiments. The deprotection conditions were optimized with **5a** and **5b**.

Oxidation and deprotection of 5a. **5a** (71 nmol) was dissolved in 100 μl of 50 mM TEAB (pH= 7.5)/dioxane (3:1, v/v) and treated with an excess of *N*-chlorosuccinimide (1.5 μmol) for 2.5 h at room temperature. The solution was evaporated to dryness and the crude **6a** was dissolved in water and purified by HPLC: linear gradient over 50 min of MeCN/50 mM TEAA (7:3, v/v) and 50 mM TEAA (pH= 7.0) from 15:85 to 65:35 at flow rate 1.0 ml/min. MALDI-TOF mass spectra (negative-ion mode): calcd. 1466.2 [M-H], found 1466.8.

6a was deprotected by treatment with 200 μl of concentrated aqueous ammonia, 16 h at room temperature. After concentration and purification, an aliquot of **7a** was analyzed by MALDI-TOF (negative-ion mode): calcd. 1233.8 [M-H], found 1233.2.

Oxidation and deprotection of 5b. Under identical experimental conditions as above, the oxidation of **5b** with *N*-chlorosuccinimide resulted in multiple unidentifed by-products. As an alternative, **5b** was oxidized to the sulfoxide **8b**. **5b** (23 nmol) was dissolved in 200 μl of a 5 % solution of H_2O_2. After 15 min at room temperature, the analytical HPLC chromatogram - linear gradient over 50 min of MeCN/50 mM TEAA (7:3, v/v) and 50 mM TEAA (pH= 7.0) from 15:85 to 65:35 - of an aliquot of the solution confirmed the

disappearance of the starting material and the MALDI-TOF analysis showed the expected increase in molecular weight: calcd. 1773.4 [M-H], found 1771.9. The solution was evaporated to dryness and **8b** was treated with 60 µl of a 2 N solution of NaOH for 3 h at room temperature. The solution was dyalized and, after concentration and purification, an aliquot of **9b** was analyzed by MALDI-TOF (negative-ion mode): calcd. 1557.0 [M-H], found 1557.3.

Oxidation and deprotection of **5c**. **5c** (19 nmol) was dissolved in 100 µl of a 5 % solution of H_2O_2. After 15 min at room temperature, the analytical HPLC chromatogram - linear gradient over 37 min of MeCN/50 mM TEAA (7:3, v/v) and 50 mM TEAA (pH= 7.0) from 15:85 to 52:48 - of an aliquot of the solution confirmed the disappearance of the starting material and the MALDI-TOF analysis showed the expected increase in molecular weight: calcd. 3532.6 [M-H], found 3532.1. The solution was evaporated to dryness and **8c** was treated with 80 µl of a 2 N solution of NaOH for 3 h at room temperature. The solution was dyalized and, after concentration, the crude compound **9c** was purified by HPLC: linear gradient over 60 min of MeCN/50 mM TEAA (7:3, v/v) and 50 mM TEAA (pH= 7.0) from 5:95 to 55:45 at flow rate 1.0 ml/min. The purity of **9c** was verified by capillary electrophoresis (see Figure 2A), and the identity was assessed by correct mass spectral analysis, MALDI-TOF (negative-ion mode): calcd. 3316.2 [M-H], found 3316.6.

Abbreviations: AcOEt, ethyl acetate; CPG, controlled-pore glass support; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DNA, deoxyribonucleic acid; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption time-of-flight mass spectrometry; MeCN, acetonitrile; NCS, *N*-chlorosuccinimide; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; RNA, ribonucleic acid; TEAA, triethylammonium acetate; TEAB, triethylammonium hydrogencarbonate; TLC, thin layer chromatography; t_R, retention time.

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