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An improved synthesis of oligodeoxynucleotide N3′→P5′ phosphoramidates and their chimera using hindered phosphoramidite monomers and a novel handle for reverse phase purification

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ABSTRACT

Oligodeoxynucleotide N3′→P5′ phosphoramidates are promising candidates for antisense therapeutics, as well as for diagnostic applications. We recently reported a new method for the synthesis of these oligonucleotide analogs which makes use of a phosphoramidite amine-exchange reaction in the key coupling step. We report herein an improved set of monomers that utilize a more reactive, hindered phosphoramidite to produce optimal yields in a single coupling step followed by oxidation, thereby eliminating the need for the previously reported couple–oxidize–couple–oxidize approach. On the 10 µmol scale, the synthesis is performed using only 3.6 equivalents (equiv.) of monomer. An improved oxidation reagent consisting of hydrogen peroxide, water, pyridine and THF is also introduced. Reported here for the first time is the use of a reverse-phase purification methodology employing a ribonucleotide purification handle that is removed under non-acidic conditions, in contrast to the conventional dimethoxytrityl group. The synthesis and purification of uniformly modified N3′→P5′ phosphoramidate oligodeoxynucleotides, as well as their chimera containing phosphodiester and/or phosphorothioate linkages at predefined positions, using these new methodologies are included herein. The results of ^{31}P NMR studies that led to this improved amine-exchange methodology are also described.

INTRODUCTION

The use of ‘antisense’ oligodeoxynucleotides (ODNs) to inhibit protein expression has become a potentially valuable treatment for a variety of diseases, particularly viral infections and cancers (1). These ODNs, complementary to a portion of a target mRNA, suppress the expression of the encoded protein by either sterically blocking translation or processing of the RNA or by the irreversible cleavage of the target RNA by endogenous RNase H (1). An extensive number of chemically modified ODNs have been synthesized in an attempt to develop analogs that have improved stability to nucleases, the ability to hybridize to complementary RNA with high specificity and affinity, increased uptake in cells and the appropriate pharmacokinetic properties. Phosphorothioate ODNs, while showing considerable promise as first-generation antisense agents, unfortunately have decreased RNA-binding affinity compared with isosequential unmodified phosphodiester ODNs (2) and also exhibit sequence-independent binding to proteins (3). On the other hand, most fully modified second generation analogs do not confer RNase H-induced cleavage of bound target RNA (4). For this reason, the use of chimera containing an RNase H-active internal region (e.g. phosphorothioate) flanked by terminal regions that have improved sequence-specific binding characteristics and reduced non-specific binding to cellular proteins, has become increasingly popular (4,5).

Recently, uniformly modified oligonucleotide N3′→P5′ phosphoramidates, wherein a 3′-amino group was substituted for the 3′-hydroxyl group of the 2′-deoxyribose ring, were described (6–8). These analogs bind with high affinity and in a sequence-specific manner to RNA and have shown increased efficacy relative to phosphorothioate ODNs in both cell culture assays and *in vivo* therapeutic models, despite their inability to activate RNase H (9–12). Additionally, their tight binding to double-stranded DNA targets has made them potentially useful as ‘antigene’ agents via triplex formation (13,14).

We recently reported (15,16) a new method for the synthesis of oligonucleotide N3′→P5′ phosphoramidates, which relies on a phosphoramidite amine exchange reaction. In this method, the amino functionality of a 5′-*O*-(*N*,*N*-diisopropylamino)phosphoramidite of a 3′-(trityl)amino-nucleoside monomer, **1**, is exchanged

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for the 3′ amino group of a 5′ support-bound (oligo)nucleotide, and the resulting internucleotide phosphoramidite is then oxidized to a phosphoramidate. This research scale method was a significant improvement over the previous oxidative phosphorylation approach (6–8) and allowed the synthesis of sufficient quantities of phosphoramidate ODNs for pre-clinical investigations. However, the method suffered from several process inefficiencies that made commercial production impractical. Reported herein are further process improvements that have been applied to this amine-exchange methodology. This work has culminated in the successful development of both a practical research scale (1 µmol) method for the synthesis of the title compounds and a 10 µmol scale method using a vortexing-mode synthesizer, which uses significantly lower stoichiometric excesses of monomer.

MATERIALS AND METHODS

General procedures

^{1}H NMR (400 MHz; with tetramethylsilane as an internal standard) and ^{31}P NMR (162 MHz; with H_3PO_4 as an external standard) were recorded on a Bruker Avance DRX-400 spectrometer. Analytical anion exchange chromatography (IEC) was performed on a Dionex PA-100 NucleoPac column (4 × 250 mm) using a gradient of 0–50% Buffer B versus Buffer A over 40 min at 1 ml/min. Buffer A was 0.01 M NaOH, 0.01 M NaCl, H_2O; Buffer B was 0.01 M NaOH, 1.5 M NaCl, H_2O. Preparative IEC on the 1 µmol scale was performed as previously described (16). Preparative IEC on the 10 µmol scale was performed on a Pharmacia Source 15 Q column (2.6 × 11.3 cm) by pre-equilibrating the column in 5% Buffer D versus Buffer C, loading the crude ODN (5 ml/min), and then eluting it using a gradient of 15–35% Buffer D versus Buffer C over 80 min, followed by 35–100% Buffer D versus Buffer C at 10 ml/min. The eluant was held at 100% Buffer D for 5 min at 10 ml/min before re-equilibrating the column to 5% Buffer D versus Buffer C. Buffer C was 0.01 M NaOH in 5% aqueous EtOH; Buffer D was 0.01 M NaOH, 1.5 M NaCl in 5% aqueous EtOH. Analytical RP-HPLC (reverse phase high pressure liquid chromatography) was performed on a Polymer Labs PLRP-S column (0.46 × 15 cm) using a gradient of 5–40% Buffer F versus Buffer E over 40 min, followed by holding for 10 min in 40% Buffer F at 1 ml/min. Buffer E was 0.1 M triethylammonium bicarbonate (TEAB), 2% acetonitrile, pH 8, and Buffer F was acetonitrile. Preparative RP-HPLC at the 1 µmol scale was performed on a Polymer Labs PLRP-S column (0.8 × 30 cm) using a gradient of 5–40% Buffer F versus Buffer E over 40 min, followed by holding for 10 min in 40% Buffer F at 2 ml/min.

Reagents

3′-(Trityl)amino-2′,3′-dideoxynucleosides, 3′-(trityl)amino-2′,3′-dideoxynucleoside-5′-*O*-(*N*,*N*-diisopropylamino 2-cyanoethyl)-phosphoramidite monomers, **1** and CPG loaded with 3′-(trityl)-amino-2′,3′-dideoxynucleosides were synthesized as reported previously (16). 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxy-thymidine was synthesized as reported previously (7). N^2-isobutyryl-2′-deoxyguanosine, thymidine, N^4-benzoyl-2′-deoxy-cytidine, N^6-benzoyl-2′-deoxyadenosine and 4,4′-dimethoxytri-tylchloride were purchased from Raylo Chemicals. Tetrazole, iodine oxidation reagent and the acetic anhydride/*N*-methylimidazole capping reagents were obtained from Perkin Elmer/Applied Biosystems. 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethyl-silyl)uridine-3′-*O*-(*N*,*N*-diisopropylamino 2-cyanoethyl)phos-phoramidite was obtained from Glen Research. Isobutyric anhydride, dichloroacetic acid, 2,6-lutidine, hydrogen peroxide, *tert*-butyldimethylsilyl chloride, triethylamine trihydrofluoride, 1 M tetrabutyl-ammonium fluoride in THF, triethylamine, 1,8-diazabi-cyclo[5.4.0]undec-7-ene (DBU), *N*,*N*-diisopropylethylamine, anhydrous tetrahydrofuran, *cis*-2,6-dimethylpiperidine, 2,2,6,6-tetramethylpiperidine, *N*-*tert*-butyl-*N*-isopropylamine, 3-hydroxy-propionitrile, phosphorus trichloride, acetonitrile-d_3, and sodium fluoride were obtained from Aldrich. The DBU was distilled from CaH_2 prior to use. All solvents were obtained from Burdick and Jackson and used without further purification.

^{31}P NMR measurement of the the overall equilibrium constant K_1K_2 for a TT coupling using diisopropyl-aminophosphoramidite monomer with tetrazole activation

The equilibria represented by equations **1** and **2** were studied by dissolving 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine [11.9 mg, 21.9 µmol, 1 equivalent (equiv.)] and 3′-(trityl)-amino-3′-deoxythymidine-5′-*O*-(*N*,*N*-diisopropylamino 2-cyano-ethyl)phosphoramidite, **1t** (monomer amidite, 53.4 mg, 78.2 µmol, 3.6 equiv.), in dry deuterioacetonitrile (0.46 ml) under argon, and adding 0.4 ml of a 0.5 M solution of tetrazole (0.20 mmol, 9.1 equiv.) in dry acetonitrile. The solution was transferred under an argon atmosphere to an NMR tube and the acquisition of the ^{31}P NMR spectrum was initiated immediately. After 2 min the acquisition was stopped, the FID was Fourier transformed, and a new acquisition was initiated. This process was repeated several times. The earliest time point contains pulses accumulated before the reaction reached equilibrium. The late time points contain increasing amounts of resonances due to side-reaction at O^4 of T and H-phosphonate resonances due to (relatively slow) hydrolysis by adventitious water. The reported spectrum, from an intermediate time-point, was selected to minimize these factors; however, the presence of these side-reactions limits the precision of the equilibrium constant determinations to perhaps ±20%. These side reactions are more troublesome in the independent measurements of the activation equilibrium constant K_1 (see below) because, in the absence of a 3′-aminonucleoside, the primary species present 'at equilibrium' is the activated intermediate.

^{31}P NMR measurement of the tetrazole activation equilibrium constant K_1 for various phosphoramidite monomers

The same general procedure as described above was followed, except 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine was not included. In these experiments the initial monomer concentration was 0.1 M and the tetrazole concentration was 0.25 M.

2-Cyanoethylphosphorodichloridite

To a solution of phosphorus trichloride (500 ml, 5.73 mol) in 250 ml of acetonitrile was added dropwise at room temperature, with stirring and bubbling of argon, a solution of 3-hydroxypropionitrile (47 ml, 0.69 mol) in 250 ml of acetonitrile. The solution was stirred 15 min at room temperature, with absorption of evolving HCl into a solution of 10% aqueous KOH, then concentrated on the rotary evaporator and filtered into a distillation flask. The 2-cyanoethylphosphorodichloridite (88.5 g, 75.7%) distilled as a

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colorless liquid at 78–80°C at 1.0 mm Hg. ^{31}P NMR ($CDCl_3$): δ 180.3.

2-Cyanoethyl *cis*-2,6-dimethylpiperidinylchlorophosphoramidite

To a solution of 2-cyanoethylphosphorodichloridite (35.0 g, 203.6 mmol) in 300 ml toluene:hexane (1:4, v/v) was added *cis*-2,6-dimethylpiperidine (55 ml, 408.1 mmol) dropwise with stirring at 4°C. The reaction was stirred for 2 h at room temperature, then filtered and the solid washed with 40 ml of toluene:hexane (1:4, v/v). The filtrate was concentrated on the rotary evaporator. To the resultant oil was added 5 ml of CH_2Cl_2 and 300 ml of hexane which resulted in crystallization of the product. After the crystallization was complete (4°C, overnight), the 2-cyanoethyl *cis*-2,6-dimethylpiperidinylchlorophosphoramidite was filtered under argon, crushed with a spatula, washed with 100 ml of hexane:CH_2Cl_2 (100:3, v/v) and dried *in vacuo*. The mother liquor was concentrated and recrystallized to give a second crop of product. The combined products yielded 38.8 g (76.5%) of pale yellow crystals. ^{31}P NMR ($CDCl_3$): δ 172.7.

2-Cyanoethyl 2,2,6,6-tetramethylpiperidinylchlorophosphoramidite

To a solution of 2,2,6,6-tetramethylpiperidine (21.0 ml, 124 mmol) in 50 ml toluene was added dropwise with stirring at 4°C, a solution of 2-cyanoethylphosphorodichloridite (10.3 g, 60.0 mmol) in 20 ml of toluene. The mixture was stirred for 1 h at 4°C and 30 min at room temperature, then filtered and concentrated to an oil. The product was recrystallized from CH_2Cl_2 (3 ml) and hexane (200 ml), filtered and washed with 50 ml of hexane:CH_2Cl_2 (100:3, v/v) under argon to yield 10.5 g (63.0%) of 2-cyanoethyl 2,2,6,6-tetramethylpiperidinylchlorophosphoramidite as yellow crystals. ^{31}P NMR ($CDCl_3$): δ 190.4.

2-Cyanoethyl *N-tert*-butyl-*N*-isopropylaminochlorophosphoramidite

To a solution of 2-cyanoethylphosphorodichloridite (40.0 g, 232 mmol) in 350 ml toluene:hexane (1:4, v/v) was added *N-tert*-butyl-*N*-isopropylamine (56 g, 77 ml, 486 mmol) dropwise with stirring at 4°C. The reaction was stirred for 3 h at room temperature, then filtered and the solid washed with 30 ml of toluene:hexane (1:4, v/v). The filtrate was concentrated on the rotary evaporator and the resulting oil was distilled at 112–114°C at 0.1 mm Hg to yield 46.6 g (79.9%) of 2-cyanoethyl *N-tert*-butyl-*N*-isopropylaminochlorophosphoramidite as a colorless oil. ^{31}P NMR ($CDCl_3$): δ 194.6.

General procedure for the preparation of 5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxynucleosides

Base-protected 2′-deoxynucleosides (dA^{Bz}, dC^{Bz}, dG^{iBu}, T) were azeotroped twice from pyridine and suspended in pyridine (10 ml/g). To this stirring mixture was added *N,N*-dimethylaminopyridine (0.1 equiv.), triethylamine (1.2 equiv.) and then *tert*-butyldimethylsilyl chloride (1.05–1.2 equiv.; 5 ml/g). After stirring for 8–24 h at room temperature, the pyridine was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 and extracted with water (2×) and saturated aqueous NaCl. The solution was dried over Na_2SO_4, filtered and concentrated to a solid that was used in the next reaction without further purification.

General procedure for the preparation of 5′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleosides

To the stirring solution of 5′-(*tert*-butyldimethylsilyl)-2′-deoxynucleoside (dA^{Bz}, dC^{Bz}, dG^{iBu}, T) in pyridine (10 ml/g), previously azeotroped twice with pyridine, was added 4,4′-dimethoxytrityl chloride (1.2–1.3 equiv.). The solution was stirred for 16–24 h at room temperature and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and extracted sequentially with water and saturated aqueous solutions of $NaHCO_3$ and NaCl. The solution was dried over Na_2SO_4, filtered and concentrated to a foam that was either used directly in the next reaction (T) or purified on silica (dA^{Bz}, dC^{Bz} and dG^{iBu}) using a gradient of 1–5% MeOH in CH_2Cl_2.

General procedure for the preparation of 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleosides

The 5′-TBDMS protecting group was cleanly removed by dissolving the 5′-(*tert*-butyldimethylsilyl)-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleoside (dA^{Bz}, dC^{Bz} or T, but not $dG^{iBu,Dpc}$) in THF (3 ml/g) and reacting it with tetrabutylammonium fluoride (1 M in THF; 2.0 equiv.) for 16–24 h. The solution was concentrated *in vacuo*, redissolved in CH_2Cl_2 and extracted with water (2×) and saturated aqueous NaCl. The organic layer was dried over Na_2SO_4, filtered and evaporated *in vacuo*. The crude 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleosides were purified on silica using a gradient of 1–5% MeOH in CH_2Cl_2.

N^6-benzoyl-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine

The overall yield from dA^{Bz} was 55.4 g (56.7%). ^1H NMR ($CDCl_3$/TMS): δ 9.14 (1H, br s, exchanges with D_2O), 8.72 (1H, s), 8.06 (1H, s), 8.02 (2H, d, J = 7.43 Hz), 7.62 (1H, t, J = 7.33 Hz), 7.53 (2H, d, J = 7.74 Hz), 7.50 (2H, d, J = 7.54 Hz), 7.39 (4H, d, J = 8.81 Hz), 7.34 (2H, t, J = 7.54 Hz), 7.26 (2H, t, J = 7.95 Hz), 6.87 (4H, dd, J = 8.91, 2.43 Hz), 6.37 (1H, dd, J = 9.95, 5.26 Hz), 5.79 (1H, br d, J = 10.38 Hz, exchanges with D_2O), 4.66 (1H, d, J = 5.32 Hz), 4.08 (1H, s), 3.81 (6H, s), 3.76 (1H, d, J = 12.78 Hz), 3.35 (1H, t, J = 11.86 Hz), 2.73 (1H, ddd, J = 13.21, 10.10, 7.99 Hz), 1.76 (1H, dd, J = 13.31, 5.30 Hz). HRMS (FAB^+): calculated for $[M + H]^+$, 658.2666; found, 658.2666.

N^4-benzoyl-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine

The overall yield from dC^{Bz} was 70.0 g (74.7%) including additional mixed fractions that were purified further by precipitation from CH_2Cl_2 into a 20× volume of 3:1 hexane:ether over 1.5 h. ^1H NMR ($CDCl_3$/TMS): δ 8.66 (1H, br s, exchanges with D_2O), 8.09 (1H, d, J = 7.35 Hz), 7.87 (2H, d, J = 7.43 Hz), 7.62 (1H, t, J = 7.36 Hz), 7.53 (2H, d, J = 7.80 Hz), 7.48 (2H, d, J = 7.63 Hz), 7.37 (4H, d, J = 8.86 Hz), 7.32 (2H, t, J = 7.53 Hz), 7.25 (1H, t, J = 7.17 Hz), 6.85 (4H, d, J = 8.76 Hz), 6.25 (1H, dd, J = 7.63, 6.12 Hz), 4.36–4.43 (1H, br m), 3.94 (1H, d, J = 2.19 Hz), 3.81 (6H, s), 3.66 (1H, br d, J = 11.86 Hz), 3.26 (1H, br d, J = 11.90 Hz), 2.48 (1H, br s, exchanges with D_2O), 2.22 (1H, dd, J = 13.13, 5.20 Hz), 2.08 (1H, quintet, J = 6.94 Hz). HRMS (FAB^+): calculated for $[M + Na]^+$, 656.2373; found, 656.2383.

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3′-O-(4,4′-dimethoxytrityl)-thymidine

The overall yield from T was 45.2 g (81.8%). ^{1}H NMR ($CDCl_3$/TMS): δ 8.61 (1H, br s, exchanges with D_2O), 7.46 (2H, d, J = 7.47 Hz), 7.36 (4H, d, J = 8.83 Hz), 7.32 (2H, t, J = 7.94 Hz), 7.25 (1H, t, J = 7.43 Hz), 6.86 (4H, d, J = 7.39 Hz), 6.15 (1H, dd, J = 8.87, 5.76 Hz), 4.38 (1H, d, J = 6.20 Hz), 3.99 (1H, d, J = 2.13 Hz), 3.81 (6H, s), 3.68 (1H, br d, J = 11.79 Hz), 3.30–3.37 (1H, br m), 2.47–2.55 (1H, br m, exchanges with D_2O), 1.95 (1H, ddd, J = 13.98, 8.42, 6.00 Hz), 1.87 (3H, s), 1.67–1.74 (1H, m). HRMS (FAB$^+$): calculated for $[M + Na]^+$, 567.2107; found, 567.2111.

3′-O-(4,4′-dimethoxytrityl)-O^6-(N,N-diphenylcarbamoyl)-N^2-isobutyryl-2′-deoxyguanosine

To a stirring solution of 5′-O-(tert-butyldimethylsilyl)-3′-O-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine (101.9 g, 135.2 mmol) in pyridine (300 ml) was added N,N-diisopropylethylamine (26.6 g, 206.1 mmol) and N,N-diphenylcarbamoyl chloride (41.4 g, 178.7 mmol). The dark solution was stirred for 2 h, and then concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2, extracted with water (2×) and saturated aqueous NaCl, dried over Na_2SO_4, filtered, and concentrated to a purple foam. The crude nucleoside was dissolved in 9:1 CH_2Cl_2:pyridine (800 ml), and then triethylamine trihydrofluoride (155.0 g, 961.5 mmol) was added and reacted for 16 h at room temperature. The solvents were removed *in vacuo* and the residue was dissolved in CH_2Cl_2 and washed with water (2×) and saturated aqueous NaCl. The solution was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to a dark red foam. The crude product was purified on silica (50–70% EtOAc/hexane) to afford 43.3 g (35.6% overall yield from dG^{iBu}) of triply protected 2′-deoxyguanosine. ^{1}H NMR ($CDCl_3$/TMS): δ 8.00 (1H, s), 7.91 (1H, br s, exchanges with D_2O), 7.49 (2H, d, J = 7.75 Hz), 7.20–7.45 (19H, mm with 4H, d, J = 8.93 Hz at 7.38), 6.86 (4H, dd, J = 8.82, 2.09 Hz), 6.26 (1H, dd, J = 9.80, 5.12 Hz), 4.65 (1H, d, J = 5.25 Hz), 4.35 (1H, dd, J = 10.35, 3.26 Hz, exchanges with D_2O), 4.04 (1H, s), 3.80 (6H, s), 3.73 (1H, br d, J = 11.48 Hz), 3.39 (1H, br t, J = 11.54 Hz), 2.64–2.80 (2H, m), 1.68 (1H, dd, J = 13.18, 5.16 Hz), 1.24 (6H, d, J = 6.93 Hz). HRMS (FAB$^+$): calculated for $[M + Na]^+$, 857.3275; found, 857.3270.

3′-(Trityl)amino-3′-deoxythymidine-5′-O-(2,2,6,6-tetramethylpiperidinyl 2-cyanoethyl) phosphoramidite, 2t

To 4.1 g (8.4 mmol) of 3′-(trityl)amino-3′-deoxythymidine (previously azeotroped twice from CH_3CN) in 25 ml of CH_2Cl_2 cooled to 4°C under argon, was added 1.9 ml (12.6 mmol) of DBU and 5.2 ml (8.4 mmol) of a solution of 2-cyanoethyl 2,2,6,6-tetramethylpiperidinechlorophosphoramidite in CH_2Cl_2 (1.63 mmol/ml). The ice bath was removed and the solution was stirred for 30 min. To avoid decomposition, the crude reaction mixture was loaded directly onto a silica column (3% MeOH, 5% Et_3N, toluene) for purification. Compound **2t**, was purified further on silica using 5% MeOH, 5% Et_3N, toluene and afforded 3.33 g (54.8%) pure phosphoramidite. ^{31}P NMR ($CDCl_3$): δ 165.3, 166.1.

3′-(Trityl)amino-3′-deoxythymidine-5′-O-(N-tert-butyl-N-isopropylamino 2-cyanoethyl) phosphoramidite, 4t

To 1.0 g (2.1 mmol) of 3′-(trityl)amino-3′-deoxythymidine (previously azeotroped twice from CH_3CN) in 7 ml of CH_2Cl_2 was added N,N-diisopropylethylamine (0.54 ml, 3.11 mmol) and 2-cyanoethyl N-tert-butyl-N-isopropylaminochlorophosphoramidite. The mixture was stirred for 15 min at room temperature and then loaded directly onto a silica column in 3% methanol, 5% Et_3N, toluene. The off-white product, **4t**, was isolated in a 67.6% yield (0.97 g). ^{31}P NMR ($CDCl_3$): δ 158.55, 159.50.

General method for the synthesis of 5′-O-(cis-2,6-dimethylpiperidinyl 2-cyanoethyl) phosphoramidite monomers

To 10.0 mmol of 3′-(trityl)amino-2′,3′-dideoxynucleoside or 3′-O-(4,4′-dimethoxytrityl)-2′-deoxynucleoside (previously azeotroped twice from CH_3CN) and 3.0 ml (20.0 mmol) of DBU in 30 ml of CH_2Cl_2 was added, with stirring, a solution of 2-cyanoethyl cis-2,6-dimethylpiperidinylchlorophosphoramidite (3.0 g, 12.0 mmol) in 8 ml of CH_2Cl_2, under an argon atmosphere. The reaction mixture was stirred for 15 min at ambient temperature. To avoid decomposition, the crude reaction was desalted by loading the mixture directly onto a silica column pre-equilibrated in 5% Et_3N/CH_2Cl_2 and quickly eluting it in the same solvent system. In all cases, further purification was necessary as indicated for each product.

N^6-benzoyl-3′-(trityl)amino-2′,3′-dideoxyadenosine-5′-O-(cis-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, 3a

Compound **3a** was purified on silica using 60–70% EtOAc/hexane containing 3% Et_3N. Yield 6.72 g (83.1%). ^{31}P NMR (CD_3CN): δ 148.82, 149.16.

N^4-benzoyl-3′-(trityl)amino-2′,3′-dideoxycytidine-5′-O-(cis-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, 3c

Compound **3c** was purified on silica using 70% EtOAc/hexane containing 3% Et_3N. Yield 6.50 g (82.7%). ^{31}P NMR (CD_3CN): δ 149.31, 149.68.

O^6-(N,N-diphenylcarbamoyl)-N^2-isobutyryl-3′-(trityl)amino-2′,3′-dideoxyguanosine-5′-O-(cis-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, 3g

Compound **3g** was purified on silica using 60% EtOAc/hexane containing 3% Et_3N. Yield 7.58 g (76.9%). ^{31}P NMR (CD_3CN): δ 148.93, 149.50.

3′-(Trityl)amino-3′-deoxythymidine-5′-O-(cis-2,6-dimethylpiperidinyl 2-cyanoethyl) phosphoramidite, 3t

Compound **3t** was purified on silica using 50% EtOAc/hexane containing 3% Et_3N. Yield 5.52 g (79.4%). ^{31}P NMR (CD_3CN): δ 149.13, 149.49.

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N[6]-benzoyl-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine-5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)-phosphoramidite, 5a

Compound **5a** was purified on silica using 60–70% EtOAc/hexane containing 3% Et_3N. Yield 6.67 g (76.7%). ^{31}P NMR (CD_3CN): δ 149.26, 149.39.

N[4]-benzoyl-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, 5c

Compound **5c** was purified on silica using 60–75% EtOAc/hexane containing 3% Et_3N. Yield 6.29 g (74.4%). ^{31}P NMR (CD_3CN): δ 149.37, 149.76.

3′-*O*-(4,4′-dimethoxytrityl)-*O*[6]-(*N,N*-diphenylcarbamoyl)-*N*[2]-isobutyryl-2′-deoxyguanosine-5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, 5g

Compound **5g** was purified on silica using 50% EtOAc/hexane containing 3% Et_3N. Yield 7.43 g (71.0%). ^{31}P NMR (CD_3CN): δ 149.32, 149.51.

3′-*O*-(4,4′-dimethoxytrityl)-thymidine-5′-*O*-(*cis*-2,6-dimethyl-piperidinyl 2-cyanoethyl) phosphoramidite, 5t

Compound **5t** was purified on silica using 60% EtOAc/hexane containing 3% Et_3N. Yield 5.61 g (74.1%). ^{31}P NMR (CD_3CN): δ 149.24, 149.65.

Optimized 1 µmol scale synthesis of oligonucleotide N3′→P5′ phosphoramidates using 5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite monomers and RP-HPLC purification

Oligonucleotide N3′→P5′ phosphoramidates or chimera containing various representative numbers of phosphodiester, phosphorothioate or phosphoramidate linkages were prepared on a Perkin Elmer/ Applied Biosystems 392 or 394 DNA synthesizer using 1 µmol of 3′-(trityl)amino nucleoside bound to aminopropyl-CPG via a 5′-succinyl linker. The synthesis proceeded in the 5′→3′ direction using the method shown in Table 2 for the chain assembly. Because of the change in direction of the synthesis (most commercially available synthesizers are programmed for 3′→5′ synthesis), the desired sequence must be entered into the synthesizer in reverse order. For synthesis of PN/PS/PN chimera, be certain to program each flank to end with the first residue of the subsequent flank. If this is overlooked, the attaching residues at the PN/PS and PS/PN junctions will contain a thioamidate and a phosphodiester linkage, respectively. For instance, if the desired sequence is 5′-d(GGACCsCsTsCsCsTsCsCsGGAGCC), where ‘s’ is a phosphorothioate linkage and the other linkages are phosphoramidate linkages, then the synthesizer should be programmed and run three times as follows: (i) 5′-cCAGG-3′, with oxidation; (ii) 5′-Gcctcctcc-3′, with sulfurization; and (iii) 5′-cCGAGG-3′, with oxidation, where lower case letters represent 3′-*O*-DMT monomers and upper case letters represent 3′-(trityl)amino monomers. Using this procedure ~15 equiv. (compared with initial loading of support-bound nucleoside) of monomer was used for each coupling step. When ion exchange purification was desired, the ODN was synthesized in the ‘trityl off’ mode, deprotected and purified by IEC as reported previously (15,16). For RP-HPLC purification, the 3′-terminal base of the desired sequence must be a 3′-hydroxyl generated by using a 3′-*O*-DMT-nucleoside monomer, **5**. The 5′-*O*-DMT-2′-*O*-TBDMS-uridine-3′-(*N,N*-diisopropylamino 2-cyanoethyl)phosphoramidite was coupled to the deprotected 3′-hydroxyl group using the method shown in Table 2, except that 0.5 M tetrazole in acetonitrile was substituted for the lower concentration tetrazole solution in the terminal coupling reaction and a 10 min coupling time was used. The ‘trityl-on’ ODN was then cleaved from the CPG and deprotected with 3:1 concentrated aqueous ammonia:ethanol at 58°C for 8–12 h. The mixture was filtered, and the CPG was washed with 2 × 200 µl of cold 3:1 concentrated ammonia:ethanol. The combined ammonia washes were concentrated, buffered to final concentration of 0.1 M TEAB pH 8, and purified by RP-HPLC. The two product peaks (due to some premature desilylation during the deprotection and work-up; Fig. 5b) were collected, combined, concentrated to dryness and then treated with a mixture of 1 M aqueous NaF (0.2 ml) and concentrated aqueous ammonia (0.2 ml) for 16 h at 58°C to remove the uridine group. After removal of the ammonia, the ODN was desalted on a Sephadex G-25 column (Pharmacia NAP-5, which also removes the uridine byproducts) and lyophilized.

Large-scale (10 µmol) synthesis of ODN N3′→P5′ phosphoramidates

Phosphoramidate ODNs were synthesized on a Perkin Elmer/ Applied Biosystems 390Z equipped with flow restrictors using the method described in Table 2. Either 3.6 equiv. (single couple–oxidize) or 7.2 equiv. (couple–oxidize–couple–oxidize; 2 × 3.6 equiv.) of the *cis*-2,6-dimethylpiperidinylphosphoramidite monomers were used relative to the initial loading of support-bound nucleoside. The 3′-terminal trityl or DMT was removed at the end of the synthesis. The ODN was deprotected with concentrated aqueous ammonia (30 ml) at 58°C for 12 h, filtered, concentrated on the rotary evaporator to ~5 ml and then IEC purified. The product peak was fractionated and the samples that were >85% pure were combined, concentrated to ~8 ml and precipitated with 16 ml of absolute ethanol. This procedure was repeated twice more (3 ml of H_2O, 6 ml of EtOH), after which the product was dissolved in water, desalted on Sephadex G-25 and lyophilized.

RESULTS AND DISCUSSION

Solid-phase synthesis using diisopropylamino phosphoramidite monomers

Our initial explorations of the amine-exchange methodology for phosphoramidate oligonucleotide synthesis led to the development of the previously reported ‘first-generation’ research scale method (15,16). These solid-phase syntheses are performed in the 5′→3′ direction using 5′-*O*-(*N,N*-diisopropylamino 2-cyanoethyl)-phosphoramidite-3′-(trityl)amino-2′,3′-dideoxynucleoside monomers, **1**, (A^{Bz}, C^{Bz}, G^{iBu}, T). The CPG-supported 3′-(trityl)amino-nucleoside (1 µmol) is loaded in a reaction column and the following cycle is used for chain extension: (i) detritylation with dichloroacetic acid, (ii) coupling using 1*H*-tetrazole as the activator, (iii) oxidation with iodine and water, (iv) repetition of steps ii and iii (couple–oxidize–couple–oxidize), (v) capping with isobutyric anhydride and *N*-methylimidazole (NMI), (vi) repetition of steps i–v until the chain is fully assembled, (vii) detritylation, and (viii) deprotection with concentrated aqueous ammonia.

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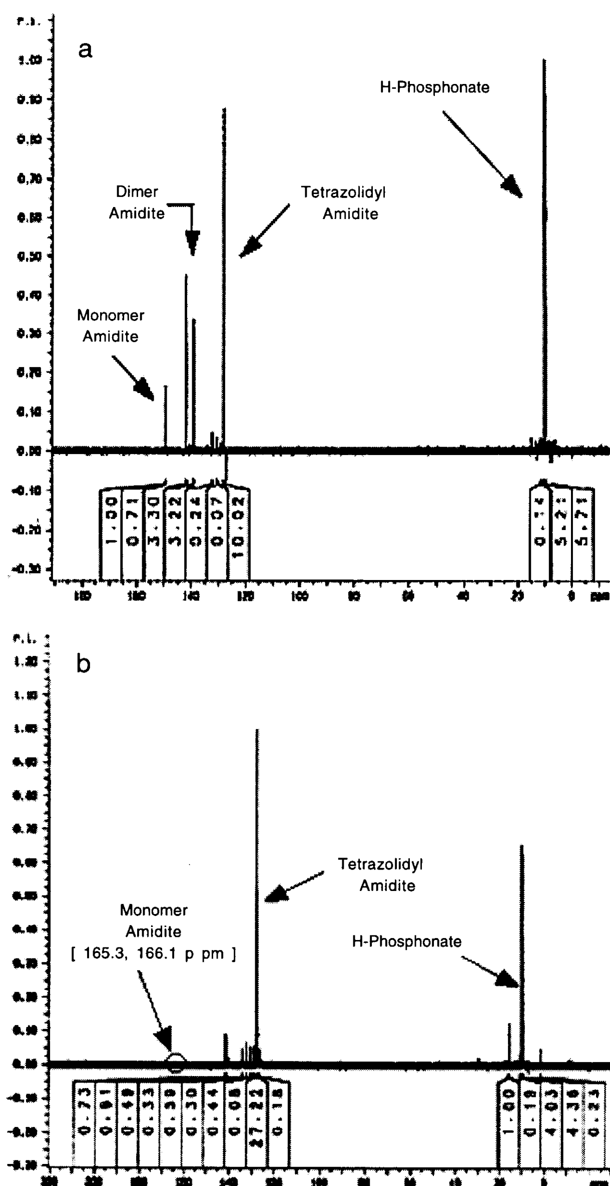
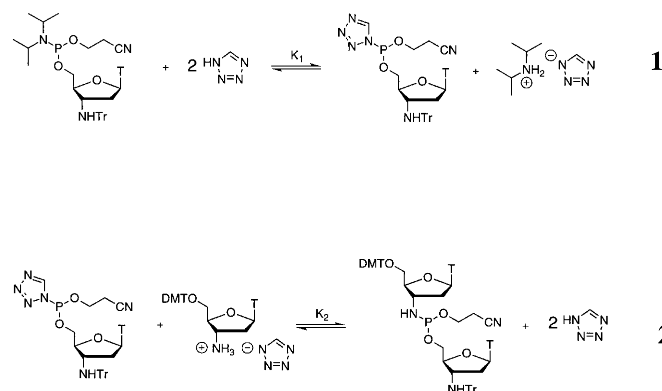


Figure 1. (**a**) ^{31}P NMR spectrum of the amine-exchange reaction at equilibrium using diisopropylamino phosphoramidite, **1t**, and 5′-DMT-3′-NH_2-T. (**b**) ^{31}P NMR spectrum of the activation with tetrazole of 5′-*O*-(2,2,6,6-tetramethylpiperidinyl 2-cyanoethyl)phosphoramidite T monomer, **2t**.

While this synthesis cycle is strikingly similar to the phosphodiester chain assembly cycle based on phosphoramidite chemistry, there are several notable differences. Because the amine-exchange coupling step in phosphoramidate synthesis is a reversible equilibration, the couple–oxidize–couple–oxidize approach is more efficient than a single coupling, even when using double the equivalents of monomer for the single coupling (16). Also, the 3′-(trityl)amino group is sufficiently nucleophilic to react slowly with the widely used acetic anhydride/NMI capping reagent, therefore the unreacted 3′-amines are capped with a bulky electrophile, isobutyric anhydride/NMI, which does not exhibit this problem. The synthesis cycle is repeated until the desired sequence is fully assembled, then the resulting ODN is detritylated, cleaved from the support, and deprotected in concentrated aqueous ammonia at 58°C for 8–12 h. The N3′→P5′ phosphoramidate ODNs are generally synthesized 'trityl-off' by this first-generation amine-exchange method because once the cyanoethyl group is removed during ammonialysis, the phosphoramidate is no longer stable to the acidic detritylation conditions. This fact unfortunately makes purification by RP-HPLC, using the trityl group as a hydrophobic handle, impractical and therefore necessitates purification by IEC. This first-generation amine-exchange method allowed for the production of sufficient quantities of phosphoramidate ODNs for initial pre-clinical studies, but the use of a total of 30 equiv. of monomer per cycle precluded the use of this method for either more extensive pre-clinical experiments or economical commercial production of phosphoramidate ODNs.

Equilibrium studies with diisopropylamino phosphoramidite monomers

To better understand the key coupling step, the tetrazole-mediated phosphoramidite amine-exchange equilibrium was studied in solution by ^{31}P NMR, utilizing 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine (5′-*O*-DMT-3′-amino T) as a model for the solid-supported 3′-amino(oligo)nucleotide. The equilibria, represented by equations **1** and **2**, were studied by dissolving 5′-*O*-DMT-3′-amino T and 3′-(trityl)amino-3′-deoxythymidine-5′-*O*-(*N*,*N*-diisopropylamino 2-cyanoethyl)phosphoramidite, **1t** (monomer amidite), in dry deuterioacetonitrile under argon and adding a solution of tetrazole in dry acetonitrile. The solution was transferred under an argon atmosphere to an NMR tube and the ^{31}P NMR spectrum that was recorded appears in Figure 1a.



The spectrum consists of resonances corresponding to the phosphoramidite monomer, the tetrazolidyl-amidite intermediate, the product dimer phosphoramidite, the hydrogen phosphonate resulting from hydrolysis of some of the monomer (through the intermediacy of the tetrazolidyl amidite), and minor side-reactions. The total of the integrations of these species was assumed to be equal to the initial phosphoramidite concentration of 0.090 M, and the concentrations at equilibrium were calculated from the relative integrations of the individual resonances. The concentrations of the species that contain no phosphorus, and therefore do not appear in the spectrum, were calculated mathematically using the known initial and equilibrium values for the phosphorus-containing species. The values for the equilibrium constants obtained from this ^{31}P NMR spectrum were $K_1 = 42/\mathrm{M}$, $K_2 = 1.5$ M and $K_1K_2 = 62$.

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It is immediately clear that the two amines being exchanged are not equivalent. If they were, then the overall equilibrium constant K_1K_2 would be unity. The favorable equilibrium that is observed is in agreement with suggestions in the literature (17) that, under acidic conditions, amine-exchange reactions of phosphoramidites are driven in the direction in which the more basic amine leaves, forming its salt with the acidic component. In this case diisopropylamine, with a reported pK_a of 11.1 (18), is favored to leave to form its tetrazolide salt. The surprisingly low pK_a value of 9.1–9.2 has been reported for 3′-amino-3′-deoxythymidine (19). The log of the measured overall equilibrium constant (K_1K_2 = 62) is 1.8. This value is consistent with the suggestion that the exchange equilibrium is dominated by the pK_a difference (1.9–2.0 in this case) between the amines being exchanged.

The small value of 1.5 M for K_2 means that in order to achieve a high equilibrium concentration of product, monomer will also be expended to produce a relatively high equilibrium concentration of the tetrazolidyl intermediate, unless the tetrazole concentration is kept very low. In addition, the production of each equivalent of tetrazolidyl intermediate also creates an equivalent of diisopropylammonium tetrazolide salt, which drives the overall equilibrium from product back to starting material. Neither of these effects are operative in normal phosphodiester synthesis, in which the formation of the phosphite triester product is irreversible. Adventitious hydrolysis of monomer is also more detrimental to the coupling in the amine-exchange method because each equivalent of hydrogen phosphonate formed consumes another equivalent of monomer, as in conventional phosphodiester synthesis, and releases another equivalent of diisopropylammonium tetrazolide which, unlike in conventional diester synthesis, drives the equilibrium back further from product to starting material. All of these factors increase the number of equivalents of monomer necessary to achieve optimal coupling efficiencies.

Equilibrium studies with hindered-amine phosphoramidites

One way to affect the equilibrium constants K_1 and K_1K_2, and potentially achieve lower stoichiometric requirements of monomer to produce a desired yield of product, is to raise the relative energy level of the phosphoramidite monomer. We reasoned that this might be possible by making the amino leaving group of the phosphoramidite very hindered, such that the reverse reaction of this amine with the tetrazolidyl intermediate would be difficult or impossible. Therefore a 5′-phosphoramidite-3′-(trityl)amino T monomer, **2t**, was synthesized in which the diisopropylamino group was substituted by the very hindered-amine 2,2,6,6-tetramethylpiperidine. Its activation with tetrazole was studied by ^{31}P NMR by dissolving the amidite in dry deuterioacetonitrile and adding a solution of tetrazole in dry acetonitrile. The ^{31}P NMR spectrum which was recorded appears in Figure 1b.

Under the conditions of the experiment, none of the tetramethylpiperidinyl phosphoramidite was detectable at equilibrium. It was estimated that the monomer would have been observable above the noise level of the spectrum if its concentration were at least 0.19 mM. From this information it was calculated that the K_1 for this equilibrium must be at least 5260/M, or 125 times greater than that of the diisopropylaminophosphoramidite monomer. Presumably it is the large amount of steric hindrance of the tetramethylpiperidine that prevents this group from participating in the reverse reaction with the tetrazolidyl amidite. The factor of 125 would imply a pK_a difference of 2.1 (log 125) between tetramethylpiperidine and diisopropylamine, if the pK_a were the dominating factor, yet the reported pK_as of the two amines are both 11.1 (18).

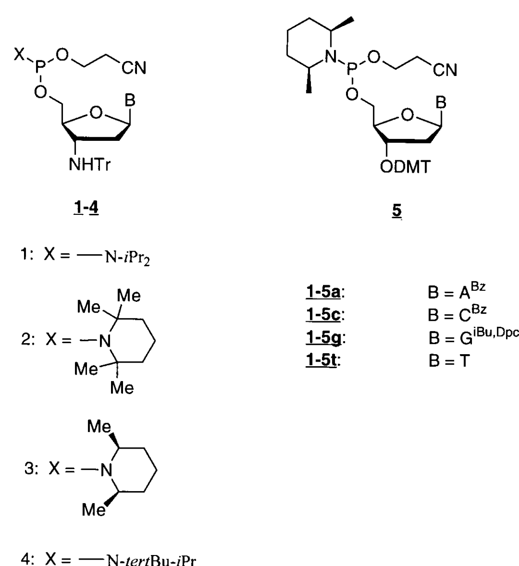

Figure 2. Structures of the 5′-phosphoramidite monomers.

Several other phosphoramidite monomers were synthesized and studied using this ^{31}P NMR method. The K_1s for 3′-(trityl)amino-3′-deoxythymidine-5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite, **3t**, and the 3′-(trityl)amino-3′-deoxythymidine-5′-*O*-(*N*-*tert*-butyl-*N*-isopropylamino 2-cyanoethyl)phosphoramidite, **4t**, were determined to be 936/M and >5000/M, respectively. The structures of these hindered phosphoramidites are shown in Figure 2.

Solid-phase synthesis using hindered-amine phosphoramidite monomers

Solid-phase synthesis using hindered-amine phosphoramidite monomers was initially studied by synthesizing TT dimers on the 10 µmol scale using a 'vortexing-vessel' (batch-reactor) synthesizer. The results of these syntheses, which appear in Table 1, clearly demonstrate the advantage of the use of the hindered-amine phosphoramidites compared with the diisopropylaminophosphoramidite monomers. In these experiments, 3 equiv. or less of the hindered phosphoramidite monomers were sufficient to produce the maximum yield, whereas 10 equiv. of the diisopropylaminophosphoramidite monomers were required to achieve a comparable result. In the couplings involving the more reactive monomers, it was necessary to use less tetrazole than for the diisopropylamino monomers in order to prevent the monomer from being trapped as the tetrazolidyl intermediate. In these cases, 2.5 equiv. of tetrazole relative to monomer was routinely used.

A variety of N3′→P5′ phosphoramidate ODNs were then synthesized on the 10 µmol scale using these hindered-amine monomers. Successful syntheses were accomplished using only 3.6 equiv. of the *cis*-2,6-dimethylpiperidinylphosphoramidite monomers, **3**, (with 9 equiv. of tetrazole) in a single coupling mode and were furthermore shown to be equivalent to those syntheses employing the couple–oxidize–couple–oxidize (2 × 3.6 equiv.)

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protocol. On the 1 µmol scale, using a 'flow-through mode' synthesizer, 12–15 equiv. of monomer (single couple–oxidize protocol) are generally required, not due to limitations in the chemistry, but because of the proportionally higher dead-volumes and loss of reagent from the top of the column in this type of synthesizer. The phosphoramidite of choice for the monomers is the 5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite because of its unique properties that combine the appropriate reactivity and the necessary stability. These monomers are free-flowing solids and are sufficiently stable in acetonitrile solution for ~1 week.

Table 1. Comparison of 10 µmol $T_{np}T_{NH2}$ dimer syntheses using different phosphoramidites and amounts of monomer and tetrazole

Phosphoramidite monomer	Monomer equiv.	Tetrazole equiv.	HPLC yield (%)
Diisopropylamino	10	50	93.8
	5	50	89.8
	2.8	28	87.1
	2.8	7	82.9
Tetramethylpiperidinyl	2.8	7	94.1
cis-Dimethylpiperidinyl	5	12.5	92.4
	2 × 5	2 × 12.5 (COCO)	92.4
	3	7.5	93.2
	2 × 3	2 × 7.5 (COCO)	92.5

In order to allow for straightforward syntheses of chimeric phosphorothioate– or phosphodiester–phosphoramidate ODNs, the 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleoside-5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidites, **5**, were synthesized. While the more reactive phosphoramidite is not necessary for the internal phosphodiester or phosphorothioate linkages, it is necessary for all couplings to a 3′-amino group using the single couple–oxidize format. Also, because the tetrazole requirements are different for the (*N,N*-diisopropylamino)phosphoramidites and the (*cis*-2,6-dimethylpiperidinyl)phosphoramidites, and since there is typically only one position for tetrazole on conventional synthesizers, it is more convenient to use the hindered phosphoramidites for all types of monomers along with the lower concentration (0.167 M) of tetrazole.

Identification and reduction of other process inefficiencies

Oxidation. Careful study of the types of 'failure' impurities in the synthetic phosphoramidate ODNs, seen by IEC, led to the belief that a small amount of chain scission was occurring during the synthesis cycle. A mechanism for this cleavage was proposed based on first, a small amount of an Arbuzov side-reaction (20) occurring during the iodine oxidation, and second, a cleavage of the now susceptible decyanoethylated phosphoramidate linkage during the acidic detritylation step.

The oxidation reaction was studied by ^{31}P NMR in solution using the phosphoramidite monomer, 3′-(trityl)amino-3′-deoxythymidine-5′-*O*-(*N,N*-diisopropylamino 2-cyanoethyl) phosphoramidite **1t**, as a model for the internucleotide linkage, because attempts to isolate a pure TT dimer phosphoramidite were unsuccessful. When this model phosphoramidite was mixed with an excess of the conventional iodine oxidation reagent, none of the desired fully protected phosphoramidate was formed (Fig. 3a). A variety of other oxidizing agents were studied by ^{31}P NMR and a reagent consisting of 1.5% H_2O_2 in 3.5% H_2O, 20% pyridine, 75% THF was found to be best. The ^{31}P NMR spectrum of a solution of the above model phosphoramidite in this reagent indicated the formation of only the desired, fully protected diastereomeric phosphoramidate products (Fig. 3b). This reagent worked very well in phosphoramidate ODN synthesis as well, however it was necessary to wash and neutralize the solid support with 20% pyridine in acetonitrile immediately after draining the coupling solution and before the oxidation step, in order to obtain the best yields.

O^6*-Guanosine protection*. A recognized problem in ODN synthesis is the inadvertent phosphitylation of *O*^{6} of guanosine by phosphoramidite monomers. In normal phosphodiester synthesis

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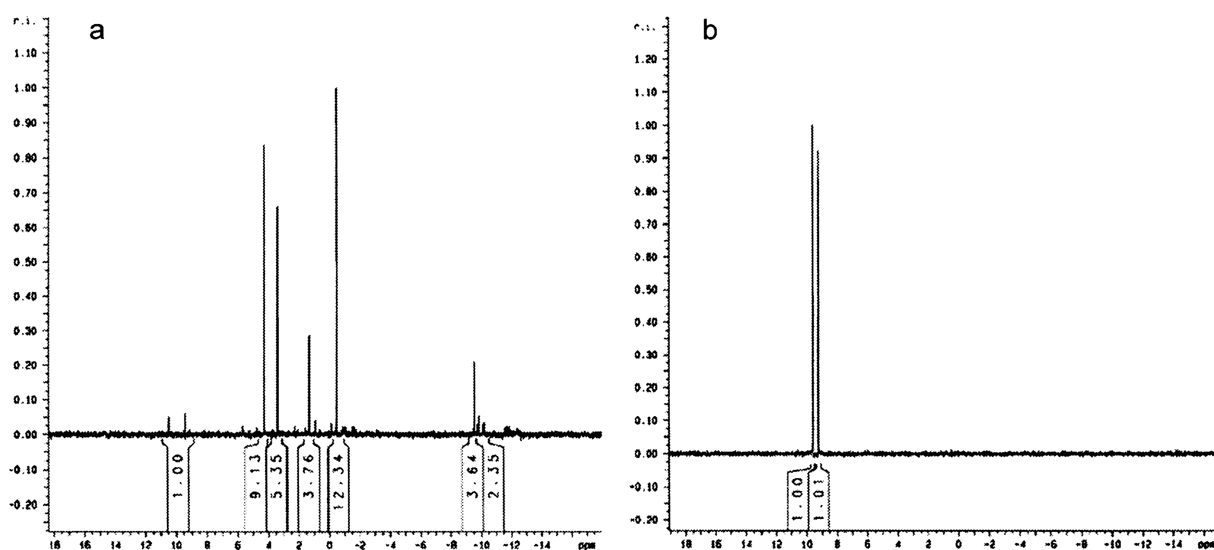

Figure 3. ^{31}P NMR spectra of the reaction of a model phosphoramidite, **1t**, with (**a**) I_2/H_2O/pyridine/THF or (**b**) H_2O_2/H_2O/pyridine/THF.

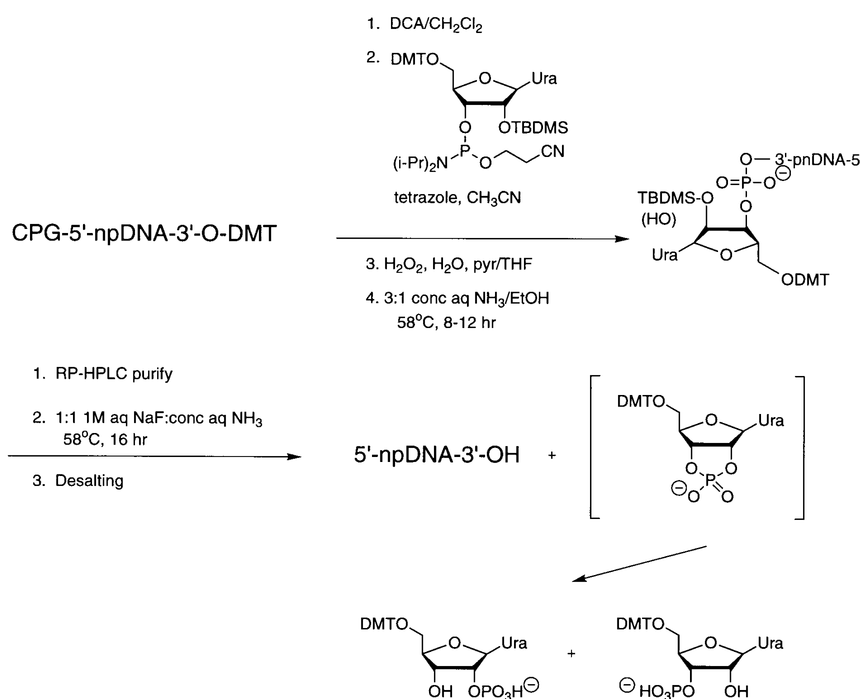

Figure 4. Scheme showing the introduction and removal of the hydrophobic handle for RP-HPLC of oligonucleotide phosphoramidates.

this problem is circumvented by capping the unreacted 5′-hydroxyls before oxidation of the phosphite linkages. Under the conditions of the acetic anhydride/NMI capping step, the guanosine O^6 phosphites are cleaved selectively in the presence of the internucleotide phosphite linkage (21). After oxidation, the guanosine O^6 phosphates are refractory to such cleavage. Unfortunately, because internucleotide phosphoramidite linkages are also somewhat unstable to the capping conditions, the capping step is performed after the oxidation. Several known methods of protecting the O^6 of guanosine were studied, and the *N*,*N*-diphenylcarbamoyl group (22) was found best. This protecting group is removed under the normal deprotection conditions (concentrated ammonium hydroxide, 58°C, 8–12 h). The O^6-diphenylcarbamoyl protection of guanosine also serves two other useful purposes. Firstly, hydrogen peroxide can oxidize guanosine residues to a certain extent and the O^6 protection eliminates this problem. Secondly, the O^6 protection makes the phosphoramidite easier to purify and markedly improves the stability of the (*cis*-2,6-dimethylpiperidinyl) phosphoramidite monomer in solution.

New hydrophobic handle for RP-HPLC purification. Phosphodiester and phosphorothioate ODNs are generally purified by RP-HPLC, which easily separates the DMT-containing product from capped failure sequences. The RP-HPLC chromatography is readily scaleable and affords highly pure ODNs as long as the homogeneity of the DMT-containing product resulting from the synthesis is good. The DMT group is typically removed by treatment with 80% aqueous acetic acid.

A limitation in the purification of phosphoramidate ODNs to date has been the need for IEC purification of the 'trityl-off' crude reaction mixtures, as ammonialytic removal of the cyanoethyl protecting groups renders the phosphoramidate linkages unstable to the acidic, post-RP-HPLC detritylation conditions. The IEC purification is time consuming, due to the necessary fractionation and analysis of the product peak, and also suffers from losses in resolution when scaled-up. To circumvent the need for acid to remove the trityl group from the product, a method that uses a hydrophobic handle that is stable to the chain assembly conditions and the ammonialytic deprotection, yet can be cleaved under non-acidic conditions post-RP-HPLC, was developed.

The method reported here is applicable to fully modified phosphoramidate ODNs, as well as chimeric ODNs containing any combination of phosphoramidate, phosphorothioate, and/or phosphodiester linkages, as long as the ODN contains a 3′-terminal hydroxyl group. The method relies on the 3′-addition of a commercially available RNA monomer, such as 5′-*O*-DMT-2′-*O*-TBDMS-uridine-3′-*O*-(*N*,*N*-diisopropylamino 2-cyanoethyl)phosphoramidite, to the terminal 3′-OH via tetrazole activation, followed by oxidation to a 3′→3′ phosphodiester linkage. The 5′-terminal DMT group of the RNA moiety is retained following synthesis, and the ODN is cleaved from the support and deprotected. After RP-HPLC purification, the uridine group with the attached DMT hydrophobic handle is readily cleaved from the desired product using fluoride and base, as outlined in Figure 4. The removal of the RNA moiety to produce a terminal hydroxyl group is analogous to the method used to produce a terminal hydroxyl group when using Rainbow™ Universal CPG (23).

Optimized synthesis and reverse-phase purification of phosphoramidate ODNs and chimeric ODNs containing any combination of phosphoramidate, phosphorothioate, and/or phosphodiester linkages

All of the improvements to the chain assembly method for phosphoramidate ODNs and chimera discussed above are incorporated into the optimized method described in Table 2. Additionally, it is best to use new or freshly distilled dichloroacetic acid for the detritylation because degradation of the phosphoramidate ODNs has been observed, on occasion, with older bottles of acid. The phosphoramidate ODNs and chimera used for

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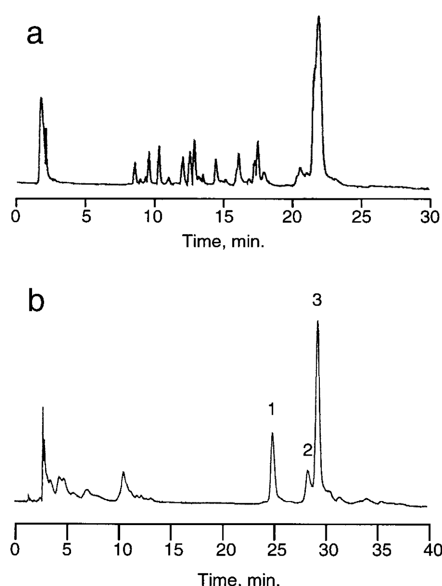

Figure 5. (**a**) Analytical IEC (40.1% pure) and (**b**) RP-HPLC (42.9% pure) of the phosphoramidate ODN, d(CCCTCCTCCGGAGCCpU^{DMT}) (where p is a 3′,3′-phosphodiester linkage). Two product peaks are seen in the RP-HPLC because some of the TBDMS group on the uridine purification handle is removed prematurely by the ammonia treatment and/or subsequent work-up. Peak 1, product containing uridine with DMT but not TBDMS; peak 2, byproduct from the Dpc protection of G; peak 3, product containing uridine with DMT and TBDMS.

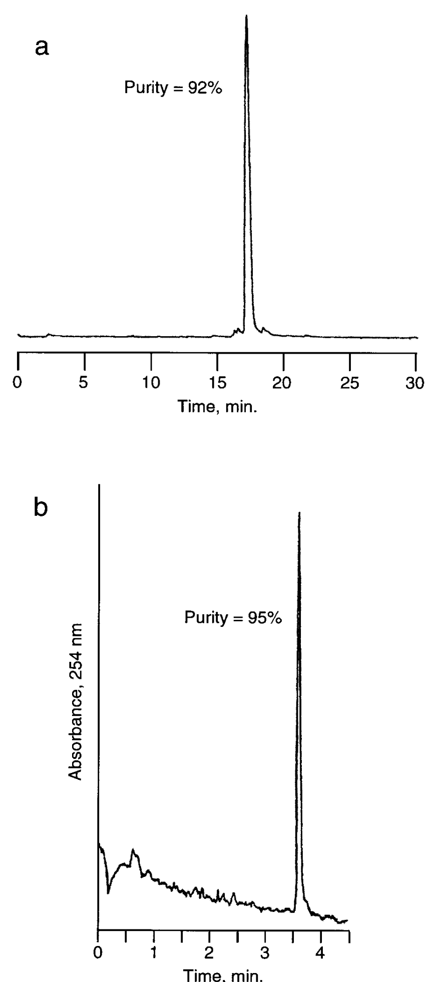

Figure 6. (**a**) Analytical IEC and (**b**) CGE of the RP-HPLC purified and deprotected phosphoramidate ODN, d(CCCTCCTCCGGAGCC).

therapeutic, diagnostic and research applications are usually synthesized with a terminal 3′-hydroxyl group that is incorporated using the 5′-*O*-phosphoramidite-3′-*O*-DMT-protected nucleosides, **5**. After detritylation, the protected uridine phosphoramidite monomer is coupled to the 3′-terminal hydroxyl group using the method described in Table 2, except that the coupling time is extended to 10 min and the tetrazole/acetonitrile concentration used is 0.5 M instead of 0.167 M because the uridine monomer is a (*N*,*N*-diisopropylamino)phosphoramidite. The 3′→3′ uridine coupling efficiency was measured on a model ODN by trityl cation analysis and found to be 98–99%. The DMT group is retained on the ODN at the end of the synthesis and is used as the hydrophobic handle for RP-HPLC. At the completion of the synthesis, the ODN is cleaved and deprotected with a 3:1 solution of concentrated aqueous ammonia:ethanol at 58°C for 8–12 h. After removal of the CPG by filtration, the ODN is concentrated and buffered to a final concentration of 0.1 M TEAB pH 8, and purified by RP-HPLC.

Representative analytical IEC and RP-HPLC chromatograms are shown for the phosphoramidate sequence 5′-d(CCCTCCTCC-GGAGCCpU^{DMT}) (where p is a 3′→3′ phosphodiester linkage) in Figure 5. There are two 'product' peaks seen by RP-HPLC because some of the TBDMS group is removed prematurely by the ammonia treatment and/or subsequent work-up. The RP-HPLC purification is performed on a polystyrene column using an increasing gradient of acetonitrile in TEAB buffer. It is important to recognize that TEAB is used because, unlike the more commonly used triethylammonium acetate (TEAA), the TEAB buffer remains basic during concentration and enables the isolation of pure phosphoramidate ODN without acid-mediated degradation. The two product peaks are combined, concentrated to dryness, and the uridine group is cleaved by a 16 h treatment at 58°C with a 1:1 mixture of 1 M aqueous NaF and concentrated aqueous ammonia. No chain length degradation of the ODNs has been observed from this treatment. When the cleavage of the uridine group is complete, the ammonia is removed *in vacuo* and the uridine byproducts and NaF are easily removed by desalting on a Sephadex G-25 column. The size exclusion chromatography is necessary because ethanol precipitation removes excess NaF but surprisingly does not remove the uridine byproducts. The purified, desalted ODN is then lyophilized. Figure 6 shows the IEC and capillary electropherogram (CGE) of the isolated phosphoramidate ODN, 5′-d(CCCTCCTCCGGAGCC). For this sequence on the 1 µmol scale, 20.1 OD at 260 nm were isolated with a purity of 92%, as measured by IEC, and 95%, as determined by CGE. The molecular weight found by electrospray ionization mass spectrometry was 4437 Da, which correlated well with the calculated value of 4436 Da (data not shown).

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Table 2. Optimized synthesis cycle for N3′→P5′ phosphoramidate ODNs and their chimera

i	3% Cl_2CHCO_2H in CH_2Cl_2 [60 s for (trityl)amino; 90 s for *O*-DMT at 1 µmol; 100 s for (trityl)amino; 130 s for *O*-DMT at 10 µmol], then CH_3CN wash (6×)
ii	Phosphoramidite monomer, **3** or **5** (0.1 M; 15 equiv. at 1 µmol; 3.6 equiv. at 10 µmol) + 1*H*-tetrazole (0.167 M; 65 equiv. at 1 µmol; 9 equiv. at 10 µmol) in CH_3CN (5 min)
iii	Neutralize and wash with a solution of 20% pyridine/CH_3CN (6×)
iv	1.5% H_2O_2 in 3.5% H_2O, 20% pyridine, 75% THF (0.8 ml at 1 µmol; 1.3 ml at 10 µmol; 2 min) or, for a phosphorothioate linkage, S-Tetra (25) in pyridine (0.2 M; 50 equiv. at 1 µmol; 15 equiv. at 10 µmol; 220 s), then CH_3CN wash (6×)
v	1:1:8 isobutyric anhydride:2,6-lutidine:THF (0.65 ml at 1 or 10 µmol) + 16.5% (v/v) NMI:THF (0.65 ml at 1 or 10 µmol; 2 min), then CH_3CN wash (6×); repetition of steps i–v until the oligonucleotide is fully assembled

Step i, for the couple–oxidize–couple–oxidize cycle, repeat steps ii–iv before proceeding to step v.

Table 3. Representative data for N3′→P5′ phosphoramidate ODN syntheses

ODN[a] (scale, method, equiv.)	Purification	Final OD	Purity (CGE) (%)
d(CCCTCCTCCGGAGCC) (1 µmol; couple–ox; 1 × 15 equiv.)	RP-HPLC	20	95
d(AGAGATTTTTACACC) (1 µmol; couple–ox; 1 × 15 equiv.)	IEC, Mono Q	29	98
d(GGACCsCsTsCsCsTsCsCsGGAGCC) (1 µmol; couple–ox; 1 × 15 equiv.)	RP-HPLC	26	97
d(CAGATCGTCCATGGTC) (10 µmol; couple–ox, 1 × 3.6 equiv.)	IEC, Source 15 Q	100.2	99
d(CAGATpCpGpTpCpCpApTGGTC) (10 µmol; couple–ox, 1 × 3.6 equiv.)	IEC, Source 15 Q	173.6	99
d(CAGATpCpGpTpCpCpApTGGTC) (10 µmol; couple–ox–couple–ox, 2 × 3.6 equiv.)	IEC, Source 15 Q	163.0	99

[a]The sequences are reported in the 5′→3′ direction. The linkages are phosphoramidate unless noted by ‘s’ or ’p’, which are phosphorothioate or phosphodiester linkages, respectively.

This RP-HPLC method is particularly useful for phosphorothioate–phosphoramidate chimera because the phosphorothioate portion of the molecule greatly reduces the resolution of the IEC. A ^{31}P NMR of a phosphoramidate–phosphorothioate chimera with the sequence 5′-d(GGACCsCsTsCsCsTsCsCsGGAGCC) is shown in Figure 7 and demonstrates the incorporation of nine phosphoramidate linkages and eight phosphorothioate linkages. Table 3 shows the yields and purities of a variety of representative phosphoramidate ODNs and chimera synthesized by this optimized method and purified by RP-HPLC or IEC.

The RP-HPLC method described herein should be useful for any ODN analog that is stable to treatment with base and fluoride and also eliminates problems with post-RP-HPLC acid-catalyzed depurination of adenosines (24). The method is reliable as long as the quality of the chain assembly and the resulting homogeneity of the ‘trityl-on’ peak is good. Currently for phosphoramidate ODNs and chimera, most sequences are >85% pure when the RP-HPLC method is used, however some sequences occasionally require IEC to obtain purities >85%.

CONCLUSION

The amine-exchange method for the synthesis of N3′→P5′ phosphoramidate ODNs has been improved such that only 3.6 equiv. of phosphoramidite monomer are necessary for optimal coupling on the 10 µmol scale. The key improvements are the use of the 5′-*O*-(*cis*-2,6-dimethylpiperidinyl 2-cyanoethyl)phosphoramidite monomers, which transform the crucial coupling step from a reversible equilibrium to an essentially irreversible reaction, and the use of a new hydrophobic handle that is cleaved after RP-HPLC under non-acidic conditions. Other process improvements, which increased the yield and purity of the products, include a hydrogen peroxide-based oxidation reagent, a neutralization step after coupling and additional protection of the O^6 position of the guanosine monomer with the *N*,*N*-diphenylcarbamoyl group.

An important advantage of this amine-exchange method is the ease with which chimeric ODNs possessing any combination of phosphoramidate, phosphorothioate and/or phosphodiester linkages in predefined positions are synthesized without any instrument modifications. The new ribonucleotide-based hydrophobic handle is used to purify N3′→P5′ phosphoramidate ODNs and their chimera containing a 3′-hydroxyl terminus, and is particularly necessary for phosphorothioate–phosphoramidate chimera. Because the ODNs are synthesized using phosphoramidite chemistry, commercially available phosphoramidite-based labeling reagents (e.g. polyethylene glycol spacers, fluorescein, etc.) are easily used with the method.

We have not yet determined if even lower monomer stoichiometry can be used with careful control of the moisture content of the monomers and solvent. Lower stoichiometric requirements may also be possible using an activator other than tetrazole. Optimization of this method at larger scales is under development.

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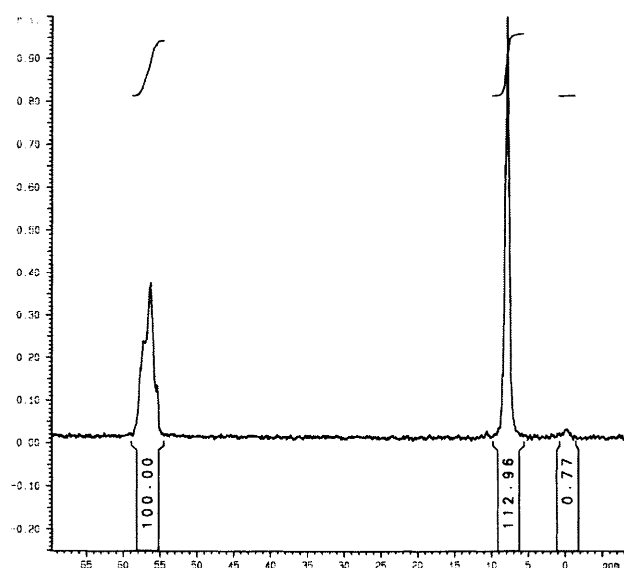

Figure 7. ^{31}P NMR of the phosphoramidate–phosphorothioate chimera with the sequence d(GGACCsCsTsCsCsTsCsCsGGAGCC), which demonstrates the incorporation of eight phosphorothioate linkages (δ 56.34 p.p.m.) and nine phosphoramidate linkages (δ 7.77 p.p.m.). Slightly more phosphodiester (δ –0.9 p.p.m.; 0.77% relative to phosphorothioate) is present than is usual for sulfurization of phosphite triesters with S-Tetra (25).

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