Volume 17 Number 9 1989 Nucleic Acids Research

Prevention of chain cleavage in the chemical synthesis of 2'-silylated oligoribonucleotides

Taifeng Wu, Kelvin K.Ogilvie[1]* and Richard T.Pon[2]

Department of Chemistry, McGill University, 801 Sherbrooke St. W., Montreal, Quebec H3A 2K6, [1]Acadia University, Wolfville, Nova Scotia B0P 1H0 and [2]Department of Medical Biochemistry, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Received March 2, 1989; Accepted April 12, 1989

ABSTRACT

Strong aqueous ammonium hydroxide used to remove N-acyl protecting groups from synthetic oligoribonucleotides causes removal of some alkylsilyl protecting groups from 2'-hydroxyls and leads to chain cleavage. This problem is most severe when 30% ammonium hydroxide is used and substantially reduced but still detectable when 3:1 ammonium hydroxide-ethanol is used. We have virtually eliminated this unwanted cleavage by incorporating the labile phenoxyacetyl amino protecting group on adenosine and guanosine. The N-benzoyl protecting group remains adequate for cytidine nucleosides. Synthetic oligoribonucleotides containing these N-acylated nucleosides and 2'-t-butyldimethylsilyl or 2'-triisopropylsilyl protecting groups can be deacylated by room temperature treatment in saturated anhydrous methanolic ammonia (8-12 h) without causing any detectable chain cleavage.

INTRODUCTION

The chemical synthesis of long sequences of RNA has recently become a practical reality (1, 2). The scope of this technique has been established by the total chemical synthesis of a 77-unit RNA sequence corresponding to the initiator tRNA of Escherichia coli (2). This chemical synthesis represented the end result of nearly twenty years of development in which a key element leading to the successful synthesis of long RNA sequences was the use of alkylsilyl (principally t-butyldimethylsilyl and triisopropylsilyl) protecting groups on the 2'-hydroxyl positions (3-5). We have used these protecting groups in combination with phosphotriester, chlorophosphite and phosphoramidite coupling procedures (1-7). Others have recently reported successful RNA syntheses using the same alkylsilyl groups and the H-phosphonate coupling procedure (8-9).

As a set of compatible protecting groups and effective nucleotide coupling procedures were developed, the major obstacles to RNA synthesis were overcome. We have since focused our attention on improving the efficiency of the total process (10). In particular, we sought to maximize the amount of material available for biological characterization by optimizing the deprotec-

tion steps since certain preparations, despite rigorous exclusion of ribonucleases, contained less full-length product than expected from the coupling results.

In this manuscript we wish to describe how the harsh aqueous-ethanolic ammonia treatment previously used in the removal of N-acyl protecting groups (1-2, 7-9) can cause chain cleavage by removal of 2'-silyl protecting groups. We will also describe how this chain cleavage can be circumvented by using more labile N-phenoxyacetyl-protected nucleosides and by using anhydrous methanolic ammonia for the N-deacylation reaction. A preliminary communication of our observations has already appeared (11). Since the completion of our study, Stawinski and co-workers have reported similar observations using the H-phosphonate method (9).

MATERIALS AND METHODS

The N-benzoylated ribonucleosides, phosphoramidites, and derivatized controlled pore glass (CPG) supports were prepared as previously described (1-2, 7). Phenoxyacetyl chloride and 1M tetrabutylammonium fluoride/THF solution were obtained from Aldrich (Milwaukee, WI). Phenoxyacetic anhydride was prepared as previously described (11, 18). TLC was performed on fluorescent Merck F254 silica gel plates and flash chromatography was performed on Merck 60 silica gel. Ammonium hydroxide-ethanol solutions (3:1 v/v) were prepared from 28-30% concentrated NH_4OH and 95% ethanol and were stored refrigerated. Anhydrous methanolic ammonia solutions were prepared by bubbling ammonia through a stirred methanol solution at 0° for approximately 15-20 min. The ammonia solution was then tightly sealed and used immediately.

Solid-phase syntheses were performed on an Applied Biosystems 380B DNA synthesizer programmed with our previously used synthesis cycle (1-2). Coupling yields were determined by colorimetric trityl assay and average coupling yields for entire syntheses are reported. Conditions for the removal of the product from the solid support and elimination of methyl protecting groups were as previously described (1-2).

Alkylsilyl groups were removed by dissolving the lyophilized 2'-silylated material in 1M TBAF/THF (0.5 ml). The solutions were left at room temperature (16 h) and then desalted on a sterile Sephadex G-25 column using sterile water.

Deprotected oligoribonucleotides were handled using sterilized and diethylpyrocarbonate treated equipment to avoid ribonuclease contamination. Polyacrylamide gel electrophoresis was performed using 20% polyacrylamide/8M

urea denaturing gels. Typically 1 A_{260} unit of sample was loaded per lane and bands were visualized by UV shadowing. HPLC analyses were performed on either a Whatman C-8 (4.6 x 250 mm) or Aquapore RP-300 (4.6 x 100 mm) reversed-phase columns using a Spectra Physics SP8000 HPLC system equipped with a single wavelength UV detector (254 nm).

^1H Nuclear magnetic resonance spectra were recorded on a Varian XL-200. Signals were referenced to the internal $CDCl_3$ signal (7.25 ppm) unless otherwise specified. ^{31}P NMR spectra were obtained as downfield shifts relative to 85% phosphoric acid on a Varian XL-300. UV spectra were recorded on either an HP8452 or Philips PU8745 UV/Vis spectrometers.

Studies on the pentadecameric homopolymer of uridine (U_{15}).

After chain assembly (97% average coupling yield) on the automated synthesizer, the synthesis column was removed and filled with thiophenoxide (thiophenol/triethylamine/dioxane, 1/2/2, 0.5 ml). After 45 min at ambient temperature, the column was extensively washed with ethanol (95%, 5x1ml). Ammonium hydroxide-ethanol solution (3:1, 1 ml) was introduced into the column via syringe and after 15 min transferred into a collection vial. This was repeated a total of four times. The ammonium hydroxide solutions were combined (4ml). Part of this solution (1 ml) was lyophilized and then desilylated and desalted to yield 6 A_{260} units of material. The remaining NH_4OH-ethanol solution was diluted with more 3:1 NH_4OH-ethanol (3 ml), sealed, and incubated (55°, 18 h). The solution was then lyophilized, desilylated and desalted to yield 8.5 A_{260} units of material. HPLC analysis was performed using 0.2 A_{260} units of material per analysis as described in Figure 1.

Preparation of N^6-phenoxyacetyladenosine (2a).

Trimethylsilyl chloride (375 mmol, 47.3 ml) was added to a suspension of adenosine (50 mmol, 13.35 g) in anhydrous pyridine (300 ml), followed after 2 h, by the addition of phenoxyacetic anhydride (150 mmol, 42.9 g). The reaction was stopped after another 2 h by addition of water (50 ml). 1M HF/pyridine solution (16) (200 ml) was added 5 min later and the solution was stirred for another 25 min. The mixture was poured into CH_2Cl_2 (800 ml), washed with saturated brine solution (3x500 ml), and dried over sodium sulfate. The solvents were evaporated to give a yellow gum. Crystallization of the residue from hot ethanol gave 11.0 g (55%) of **2a**. Some product remained in the mother liquor which was concentrated to a brown gum and purified by flash chromatography using a 5-7% MeOH/CH_2Cl_2 gradient to yield more **2a** (2.5 g, total yield = 65%). mp 132-134°; TLC (20% MeOH/CH_2Cl_2) Rf = 0.53; UV (H_2O) λmax 274, 210 nm; ^1H NMR (DMSO-d6, TMS as internal reference): 10.98 (s, 1,

NH); 8.75 (s, 1, H8); 8.70 (s, 1, H2); 6.03 (d, 1, J = 6 Hz, H1'); 4.63 (m, 1, H2'); 4.19 (m, 1, H3'); 4.00 (m, 1, H4'); 3.64 (m, 2, H5'& H5"); 5.04 (s, 2, $PhOCH_2CO$); 3.58 (s, 3, $-OCH_3$); 6.97 (m, 3, ArH); 7.32 (m, 2H, ArH). Anal. Calc. for $C_{18}H_{19}N_5O_6$: C, 53.86%; H, 4.74%; N, 17.46%. Found: C, 53.55%; H, 4.78%; N, 17.23%.

Preparation of 5'-Monomethoxytrityl-N^6-phenoxyacetyladenosine.

N^6-phenoxyacetyladenosine (11 g, 27.4 mmol) was dissolved in anhydrous pyridine (200 ml) and monomethoxytrityl chloride (10.12 g, 33 mmol) was added. After stirring at ambient temperature (4 h), TLC showed incomplete reaction and more monomethoxytrityl chloride (2 g) was added. After stirring (10 h), methanol (50 ml) was added and the mixture was poured into CH_2Cl_2 (600 ml). The solution was washed with aqueous $NaHCO_3$ (3x), water (1x) and dried with sodium sulfate. The solvent was removed and the residue was coevaporated with toluene (2x200 ml) to remove residual pyridine. The material was purified by flash chromatography using a 0-5% $MeOH/CH_2Cl_2$ gradient to yield 12 g of product (65%). mp 100-105°; TLC (10% $MeOH/CH_2Cl_2$) Rf = 0.36; UV (95% ethanol) λmax. 278, 234 nm; ^1H NMR ($CDCl_3$) 9.49 (s, 1H, NH), 8.75 (s, 1H, H8), 8.27 (s, 1H, H2), 6.74-7.35 (m, 19H, aryl), 6.23 (d, 1H, J = 0.6 Hz, H1'), 4.85 (m, 1H, H2'); 4.44 (m, 2H, H3', H4'); 3.30 (q, 1H, H5'); 3.45 (q, 1H, H5"); 4.84 (s, 2H, $PhOCH_2CO$), 3.77 (s, 3H, OCH_3). Anal. Calc. for $C_{38}H_{35}N_5O_7 \cdot 3/4\ H_2O$: C, 66.41%; H, 5.53%; N, 10.19%. Found: C, 66.66%; H, 5.81%; N, 9.57%.

Preparation of 2'-t-butyldimethylsilyl-5'-monomethoxytrityl-N^6-phenoxyacetyl-adenosine 3a.

To a solution of 5'-monomethoxytrityl-N^6-phenoxyacetyladenosine (11 mmol, 7.6 g) in anhydrous THF (30 ml) was added silver nitrate (15 mmol, 2.5 g) and anhydrous pyridine (45 mmol, 1.3 ml). Once the $AgNO_3$ dissolved, t-butyldimethylsilyl chloride (5.6 mmol, 3.4 g) was added. TLC (1:1 Et_2O/CH_2Cl_2) showed complete reaction after 4 h. The solution was filtered off into brine solution (400 ml) and extracted with CH_2Cl_2 (2x400 ml). The combined organic solutions were dried with sodium sulfate, and co-evaporated with toluene (2x200 ml) to remove pyridine. The 2'-silylated product was separated from the 3'-isomer by flash chromatography using 40% ethyl acetate/hexane to yield **3a** (5.5 g, 62%). TLC (1:1 ethyl acetate/hexane) Rf=0.35; UV (95% ethanol) λmax. 268, 222 nm; ^1H NMR ($CDCl_3$) 8.71 (s, 1H, H8), 8.23 (s, 1H, H2), 6.80-7.50 (m, 19H, ArH), 6.08 (d, 1H, J=5.4 Hz, H1'), 5.00 (t, 1H, H2'); 4.35 (q, 1H, H3'); 4.28 (m, 1H, H4'); 3.55 (q, 1H, H5'); 3.38 (q, 1H, H5"); 4.84 (s, 2H, $PhOCH_2CO$), 3.78 (s, 3H, OCH_3), 0.82 (s, 9H, $SiC(CH_3)_3$), -0.03 & -0.17

(s, 6H, $Si(CH_3)_2$). Anal. Calc. for $C_{44}H_{49}N_5O_7Si$: C, 67.07%; H, 6.27%; N, 8.89%. Found: C, 67.22%; H, 6.19%; N, 8.96%.

Preparation of 2'-t-butyldimethylsilyl-3'-(N,N-diisopropylmethoxy)-5'-monomethoxytrityl-N^6-phenoxyacetyladenosine phosphoramidite 4a.

A solution of **3a** (3.14 g, 4 mmol) in THF (6 ml) was slowly added to a stirred THF solution (9 ml) of N,N-diisopropylaminomethylphosphonamidic chloride (1 ml, 5.2 mmol), dimethylaminopyridine (0.1 g, 0.8 mmol) and diisopropylethylamine (2.8 ml, 16 mmol) in an Ar filled vial. A precipitate was formed after a few minutes and the mixture was stirred at room temperature (3 h) until TLC showed complete reaction. The mixture was added to ethyl acetate (150 ml, previously washed with aqueous $NaHCO_3$), extracted with brine (2 x 200 ml) and dried over sodium sulfate. The product was then purified by flash chromatography using Ch_2Cl_2/hexane/triethylamine, 50:47:3. mp 80-83°; TLC (20% ethyl acetate/CH_2Cl_2) Rf=0.48, 0.32 (two diastereomers); UV (95% ethanol) λmax. 274, 234nm; ^{31}P NMR ($CDCl_3$): 151.9, 150.1 ppm.

Preparation of N^2-phenoxyacetylguanosine 2a.

Guanosine (14.2 g, 50 mmol) was coevaporated with anhydrous pyridine (2x200 ml) and then suspended in pyridine (300 ml). Trimethylsilyl chloride (31.5 ml, 250 mmol) was transferred into the solution via syringe and the reaction was stirred for 2 h. Alternatively, guanosine (14.2 g, 50 mmol) was coevaporated to dryness with pyridine (2x200 ml) and anhydrous DMF (1x100 ml), suspended in DMF (80 ml) and hexamethyldisilazane (84 ml, 400 mmol) was added. Stirring at room temperature produced an almost clear solution within 15 min. Excess HMDSA and DMF were removed by evaporation and co-evaporation (1x) with pyridine and the oily residue was re-dissolved in pyridine.

The 2',3',5'-tri-trimethylsilylguanosine was acylated with either phenoxyacetic anhydride as previously described, or by adding phenoxyacetyl chloride (8.2 ml, 1.2 eq.) and stirring at room temperature (4 h). The reaction was cooled in ice and first water (50 ml) followed after 15 min by 30% NH_4OH (50 ml) was added. The slurry was vigorously stirred (10 min), and concentrated under vacuum to 200 ml and poured into water (1.7 l). The product crystallized out of the aqueous solution upon extraction with CH_2Cl_2 (300 ml) to yield 10.3 g of **2b** (49% yield). mp 168-170°; TLC (20% MeOH/CH_2Cl_2) Rf = 0.37; UV (water): λmax. 264 nm; ^1H NMR (DMSO-d6, TMS as reference): 8.29 (s, 1H, H8); 7.29-7.37 (t, 2H, Ar); 7.00-7.03 (m, 3H, Ar); 5.83 (d, 1H, H1', J = 6 Hz); 4.87 (s, 2H $PhOCH_2CO$); 4.45 (t, 1H, H2'), 4.14 (t, 1H, H3'); 3.93 (m, 1H, H4'); 3.58 (m, 2H, H5',H5"). Anal. Calc. for $C_{18}H_{19}N_5O_7 \cdot 3/4\ H_2O$: C, 50.17%; H, 4.64%; N, 16.26%. Found: C, 50.09%; H, 4.76%; N, 15.81%.

Preparation of 5'-monomethoxytrityl-N^2-phenoxyacetylguanosine.

N^2-phenoxyacetylguanosine (16 g, 38 mmol) was coevaporated with anhydrous pyridine (300 ml) and then dissolved in pyridine (200 ml). Monomethoxytrityl chloride (14.2 g, 1.2 eq.) was added and after 6 h the reaction was complete. The reaction was quenched with methanol (50 ml), concentrated to 100 ml and added to CH_2Cl_2 (500 ml). The solution was washed with brine (2x300 ml) and dried over sodium sulfate. The product was purified by flash chromatography using a 0-5% MeOH/CH_2Cl_2 gradient to yield pure product (19 g, 72% yield). mp 144-145°; TLC (10% MeOH/CH_2Cl_2) Rf = 0.23; UV (95% ethanol) λmax. 278, 256, 236 nm; ^{1}H NMR (DMSO-d6, TMS as reference): 8.09 (s, 1H, H8); 7.19-7.38 (m, 14H, ArH); 6.82-7.00 (m, 5H, ArH); 5.85(d, 1H, H1', J=4.5Hz); 4.81 (s, 2H, PhOCH_2CO); 4.51(t, 1H, H2'); 4.19 (m, 1H, H3'); 4.05 (m, 1H, H4'); 3.72 (s, 3H, OCH_3); 3.21 (m, 2H, H5', H5"). Anal. Calc. for $C_{38}H_{34}O_8N_5 \cdot 1/2\ H_2O$: C, 65.42; H, 5.02; N, 10.16. Found: C, 65.52%; H, 5.14%; N, 9.99%.

Preparation of 5'-monomethoxytrityl-N^2-phenoxyacetyl-2'-triisopropylsilylguanosine 3b.

5'-Monomethoxytrityl-N^2-phenoxyacetylguanosine (11 g, 16 mmol), and imidazole (4.4 g, 4 eq.) were dissolved in anhydrous DMF (50 ml). Triisopropylsilyl chloride (6.85 ml, 2eq.) was added and the solution was stirred at room temperature (12 h). The solution was poured into CH_2Cl_2 (300 ml), washed with brine (2x200 ml), and dried with sodium sulfate. The 2'-silylated product was separated from the 3'-isomer by flash chromatography using 20% ethyl acetate/CH_2Cl_2 to yield 6 g of **3b** (44.5% yield). mp 103-105°; TLC (40% ethyl acetate/CH_2Cl_2) Rf = 0.6; UV (95% ethanol): λmax. 276, 254, 230, 214 nm; ^{1}H NMR ($CDCl_3$): 7.93 (s, 1H, H8); 5.94 (d, 1H, H1', J=5.8); 4.89(q, 1H, H2'); 4.29 (m, 1H, H3'); 4.24 (m, 1H, H4'); 3.48 (q, 1H, H5'); 3.33 (q, 1H, H5"); 4.57 (s, 2H, PhOCH_2CO); 3.76 (s, 3H, OCH_3); 0.99,0.92,0.90 (s, 18H, iPr_3). Anal. Calc. for $C_{47}H_{54}O_8N_5Si$: C, 66.83%; H, 6.40%; N, 8.29%. Found: C, 66.56%; H, 6.58%; N, 8.22%.

Preparation of 3'-(N,N-diisopropylaminomethoxy)-5'-monomethoxytrityl-N^2-phenoxyacetyl-2'-triisopropylsilylguanosine phosphoramidite 4b.

A solution of **3b** (0.84 g, 1.0 mmol) in THF (1.5 ml) was added to a stirred solution of dimethylaminopyridine (0.04 g, 0.2 eq.), triethylamine (1.6 ml, 9 eq.) and N,N-diisopropylaminomethylphosphonamidic chloride (0.61ml, 3 eq.) in THF (2 ml). The reaction was stirred at room temperature overnight. The solution was added to ethyl acetate (100 ml, prewashed with aq. $NaHCO_3$), washed with brine (3x100 ml) and dried with anhydrous sodium sulfate. The

solution was concentrated and purified by flash chromatography using 20:75:5 ethyl acetate/CH_2Cl_2/triethylamine to yield pure **4b** (0.9g, 90%). TLC (50% ethyl acetate/CH_2Cl_2) Rf=0.62; UV (95% ethanol) λmax. 278, 256, 208 nm; ^{31}P NMR ($CDCl_3$): 152.3, 150.4 ppm.

Preparation of N^4-Phenoxacetylcytidine 2c.

Cytidine (50 mmol, 12.2 g) was co-evaporated with anhydrous pyridine (3x) and suspended in pyridine (300 ml). Chlorotrimethylsilane (400 mmol, 51 ml) was added and the mixture stirred (1 h). Phenoxyacetyl chloride (62.5 mmol, 8.6 ml) was added via syringe and an orange colour appeared. The reaction was stirred at room temperature (2.5 h) before the addition of first ice and H_2O (150 ml) followed after 20 min by 30% NH_4OH (2 ml). The solution was concentrated to remove pyridine, redissolved in H_2O (700 ml) and extracted with $CHCl_3$. The product precipitated from the aqueous layer and after further concentration of the aqueous solution a total of 12.1 g (32 mmol, 64% yield) of **2c** was obtained. This was used without further purification. TLC (10% MeOH/$CHCl_3$) Rf = 0.28; UV (MeOH) λmax. 303, 277, 248 nm. Anal. Calc. for $C_{17}H_{19}N_3O_7$: C, 54.11%; H, 5.07%; N, 11.14%. Found: C, 54.43%; H, 5.21%; N, 10.63%.

Preparation of 5'-monomethoxytrityl-N^4-phenoxyacetylcytidine.

Monomethoxytrityl chloride (13.25 g, 1.2 eq.) was added to a solution of **2c** in pyridine (13.5 g, 36 mmol). After stirring 4 h, methanol (50 ml) was added and the solution was concentrated to a brown gum. This was redissolved in CH_2Cl_2 (600 ml), washed with brine, dried over sodium sulfate and coevaporated with toluene (2x) to remove pyridine. The mixture was then purified by flash chromatography using a 0-4% MeOH/CH_2Cl_2 gradient to 14 g of pure product (60% yield). mp 115-117°; TLC (5% MeOH/CH_2Cl_2) Rf = 0.2; UV (95% ethanol) λmax. 304, 238, 208 nm; ^1H NMR ($CDCl_3$) 8.13 (d, 1, H6, J=7.3 Hz), 6.75-7.33 (m, 19, ArH), 6.91 (d, 1, H1', J=1.1 Hz), 4.56 (s, 2, CH_2O), 4.37 (m, 3, H2', H3', H4'), 3.73 (s, 3, $-OCH_3$), 3.28-3.36 (m, 2, H5', H5"). Anal. Calc. for $C_{37}H_{35}O_8N_3$: C, 68.40%; H, 5.43%; N, 6.47%. Found: C, 68.09%; H, 5.42%; N, 6.51%

Preparation of 2'-t-butyldimethylsilyl-5'-monomethoxytrityl-N^4-phenoxyacetylcytidine 3c.

Silver nitrate (4.4 g, 1.2 eq.), pyridine (7 ml, 4 eq.) and 5'-monomethoxytrityl-N^4-phenoxyacetylcytidine (14 g, 21.6 mmol) were dissolved in anhydrous THF (250 ml) and then t-butyldimethylsilyl chloride (4.1 g, 1.2 eq.) was added. After 4 h, TLC indicated incomplete reaction and more silver nitrate (0.37 g, 0.1 eq.) and t-butyldimethylsilyl chloride (0.34 g, 0.1 eq.)

were added. After stirring 2h, the mixture was filtered into 5% aqueous $NaHCO_3$ (400 ml). This was extracted with CH_2Cl_2 (2x300 ml) and the combined organic extracts were dried with sodium sulfate. The solution was coevaporated with toluene (2x200 ml) to remove pyridine and then purified by flash chromatography using a 5-10% Et_2O/CH_2Cl_2 gradient to yield **3c** (8 g, 49%). mp 107-109°; TLC (20% Et_2O/CH_2Cl_2) Rf = 0.69; UV (95% ethanol) λmax. 306, 238, 206 nm; ^1H NMR ($CDCl_3$): 8.43 (d, 1, H6, J=7.4 Hz), 6.76-7.35 (m, 19, ArH), 5.82 (s, 1, H1'), 4.50 (s, 2, $-CH_2O$), 4.20 (m, 2, H2', H3'), 4.02 (m, 1, H4'), 3.48 (m, 2, H5', H5"), 3.73 (s, 3, $-OCH_3$), 0.84 (s, 9, $SiC(CH_3)_3$), 0.23 (s, 3, $-SiCH_3$), 0.10 (s, 3, CH_3Si-). Anal. Calc. for $C_{43}H_{49}O_8N_3Si$: C, 67.60%; H, 6.47%;, N, 5.50%. Found: C, 67.43%; H, 6.42%; N, 5.54%.

Preparation of 2'-t-butyldimethylsilyl-3'-(N,N-diisopropylaminomethoxy)-5'-monomethoxytrityl-N^4-phenoxyacetylcytidine phosphoramidite 4c.

A solution of **3c** (3.82 g, 1 eq.) in THF (9 ml) was added dropwise via syringe to a solution of dimethylaminopyridine (0.12 g, 0.2 eq.), diisopropylethylamine (3.5 ml, 4 eq.) and N,N-diisopropylmethylphosphonamidic chloride (1.26 ml, 1.3 eq.) in THF (12 ml). After stirring 6 h at room temperature, the reaction was added to ethyl acetate (200 ml), washed with 5% aqueous $NaHCO_3$ and dried over sodium sulfate. The material was purified by flash chromatography using 50:48:2 hexane/CH_2Cl_2/triethylamine. The fractions containing **4c** were combined, concentrated under vacuum and coevaporate with absolute ethanol to yield **4c** (4.0 g, 86.5%). mp 100-102°; TLC (50:48:2 hexane/CH_2Cl_2/triethylamine) Rf = 0.2; UV (95% ethanol) λmax. 306, 238, 214 nm; ^{31}P NMR: 150.1, 148.7 ppm.

RESULTS AND DISCUSSION

The synthesis of oligoribonucleotides has always been more difficult than the synthesis of oligodeoxyribonucleotides because of the need for 2'-hydroxyl group protection. In DNA synthesis, relatively severe conditions (30% NH_4OH, 55°, 16-24 h) can be used to cleave the N-acyl protecting groups from the final sequence since this is the last step in the synthesis (12). However, in oligoribonucleotide synthesis more moderate conditions are required because the 2'-hydroxyl positions are still protected. In our previous solid-phase syntheses (1-2, 7) the oligoribonucleotides were de-acylated using the less severe conditions of 3:1 ammonium hydroxide-ethanol (55°, 16-18 h). These conditions have recently been confirmed to be superior to the standard ammonium hydroxide conditions used in oligodeoxyribonucleotide synthesis by an

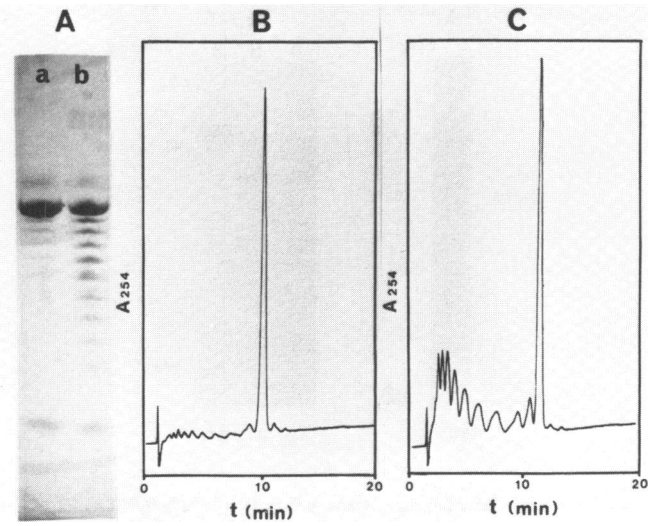


Figure 1. A, 20% Polyacrylamide/8M urea gel electrophoresis of unpurified U_{15}: **Lane a,** sample without 3:1 ethanol-NH_4OH (18 h, 55°) treatment; **Lane b,** sample after treatment with 3:1 ethanol-NH_4OH (18 h, 55°). **B,** HPLC analysis of the U_{15} sample from lane a. **C,** HPLC analysis of the U_{15} sample from lane b. HPLC conditions: column, RP300 (4.6x100mm); solvent, linear gradient (0-30 min) of 7-20% acetonitrile in 0.1M triethylammonium acetate (pH 7); flow, 1 ml/min.

independent investigation (9). However, we have found that a small amount of silyl hydrolysis and subsequent chain cleavage still occurs under these conditions.

The problem of silyl group cleavage under severe ammonium hydroxide conditions can best be investigated using oligouridylic acid sequences since no exocyclic amino protecting groups are present. Thus it is possible to directly compare preparations which differ only in their exposure to ammonia hydrolysis. A pentadecameric homopolymer of uridine, U_{15}, was synthesized using methylphosphoramidite derivatives according to our previously described method (1). The average coupling yield obtained was 97%, as determined by trityl colour analysis. After synthesis, the product was treated with thiophenoxide (thiophenol/dioxane/triethylamine, 1/2/2) to remove the methyl groups from the phosphate linkages and was then cleaved from the solid support by treatment with 3:1 ammonium hydroxide-ethanol (1 h) at room temperature. One half of this product was desilylated without any further treatment. The other half was incubated in 3:1 ammonium hydroxide-ethanol for 18h at 55° and then desilylated. The crude products from these two trials were then analyzed

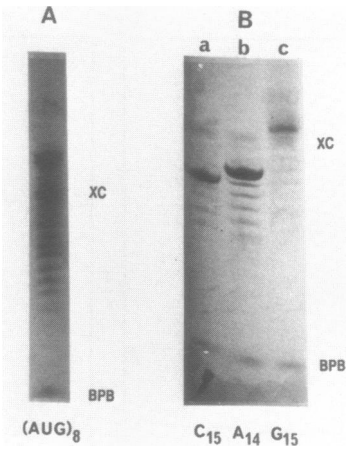


Figure 2. 20% Polyacrylamide/8M urea gel electrophoresis of oligoribonucleotides prepared using the 3:1 ethanol-NH_4OH (18 h, 55°) hydrolysis conditions. **A**, $(AUG)_8$, **B: lane a** C_{15}; **lane b** A_{14}; **lane c** G_{15}.

by both polyacrylamide gel electrophoresis (Figure 1a) and reversed-phase HPLC (Figures 1b-c).

The sample which did not receive strong ammonium hydroxide-ethanol treatment contained the full-length product predominantly, as expected from the trityl coupling yields. The sample which received the 18 h, 55° ammonium hydroxide-ethanol treatment contained noticeably higher amounts of shorter chain fragments. These can be seen as a ladder of shorter bands in the gel (Figure 1a, lane b) or as multiple early eluting peaks in the chromatogram (Figure 1c). The increased chain cleavage in the ammonium hydroxide treated sample is the immediate result of silyl group hydrolysis, since RNA linkages with exposed 2'-hydroxyl groups are quite susceptible to cleavage under alkaline conditions. Clearly, however, the desired product was by far the major component even with the severe ammonium hydroxide treatment. Given the fourteen phosphate linkages in this chain, the amount of cleavage which occurred at any single internucleotide bond was quite small. This was in agreement with the study by Stawinski et. al. (9) on a uridine dinucleotide, which found that the amount of chain cleavage occurring under these conditions was barely detectable.

Additional oligoribonucleotides, C_{15}, A_{14}, G_{15}, and $(AUG)_8$, which contained N-benzoylated bases were also prepared. Average coupling yields of 96-97% were obtained for all sequences except A_{14} which had average coupling

Table 1. Hydrolysis of t-butyldimethylsilyl groups from 2',3'-di-t-butyldimethylsilyluridine in 3:1 ammonium hydroxide-ethanol, as determined by HPLC analysis on a 4.6x250 mm Whatman C8 column (60:40 MeCN:H_2O, 1 ml/min).

Hydrolysis Products	Hydrolysis Conditions 55°, 18 h	R. T., 12 h
2'-t-butyldimethylsilyluridine	0.7%	0.3%
3'-t-butyldimethylsilyluridine	0.7%	0.3%
uridine	7.6%	0.7%
2',3'-di-t-butyldimethylsilyl-uridine (unreacted)	91.0%	98.7%

yields of 94%. The presence of the benzoyl protecting groups in these sequences made hydrolysis in 3:1 ammonium hydroxide-ethanol (18 h, 55°) a mandatory step of the deprotection sequence. Thus the crude deprotected products from these syntheses, when examined by gel electrophoresis (Figure 2a-b), all showed a pattern of default sequences similar to the above results. The least amount of cleavage was observed with the G_{15} sequence since the triisopropylsilyl protecting group used on guanosine was less susceptible to hydrolysis than the t-butyldimethylsilyl group used on the other nucleosides.

We investigated the stability of the t-butyldimethylsilyl group on the secondary hydroxyl positions by using 2',3'-di-t-butyldimethylsilyluridine. This nucleoside was dissolved in 3:1 ammonium hydroxide-ethanol and incubated at either room temperature or 55°. The solutions were then analyzed by reversed-phase HPLC for the desilylation products, 2'-t-butyldimethylsilyluridine, 3'-t-butyldimethylsilyluridine and uridine (Table 1). These results

Table 2. Half-time for N-Deacylation of Various Nucleosides in Aqueous or Methanolic Ammonia at Room Temperature.

Nucleoside	28-30% NH_4OH[a]	Saturated NH_3/MeOH[b]
N^6-Benzoyladenosine	11 h	1 h
N^6-Phenoxyacetyladenosine	7 min	<1 min
N^4-Benzoylcytidine	3 h	30 min
N^4-Phenoxyacetylcytidine	2 min	<1 min
N^2-Benzoylguanosine	10 h	8-10 h
N^2-Phenoxyacetylguanosine	8 min	<1 min

a - Determined by UV monitoring
b - Determined by HPLC analysis on an Aquapore RP300 (4.6x100mm) column using 15-25% MeOH in H_2O gradient over 30 min, 1 ml/min.

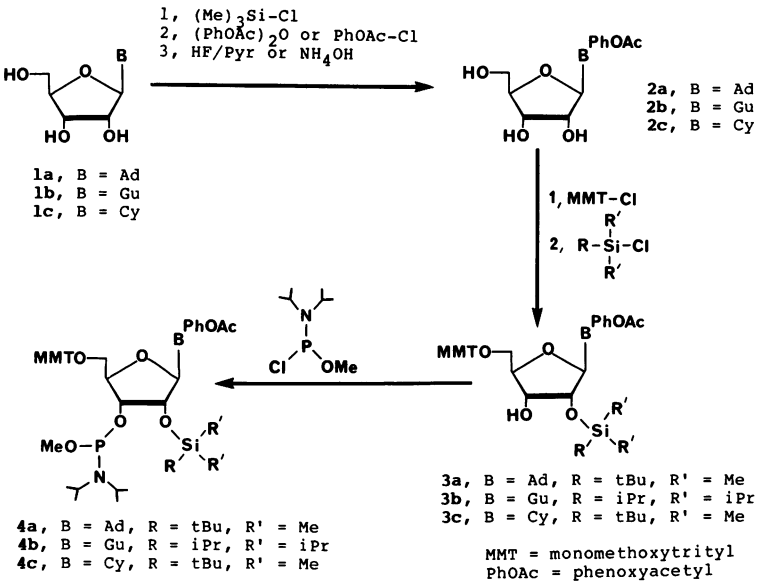
showed that the 18 h, 55° hydrolysis caused loss of one or two silyl groups from 9% of the material. This cleavage was reduced to only 1.3% when a milder 12 h room temperature hydrolysis was performed. These results clearly demonstrated that the the degree of desilylation was determined by the vigor of the conditions used.

The N-benzoyl protecting groups can also be removed by anhydrous methanolic ammonia (saturated), a procedure introduced by Lohrmann and Khorana (13) and previously used in our solution-phase oligoribonucleotide syntheses (5, 14). We therefore subjected 2',3'-di-t-butyldimethylsilyluridine to saturated ammonia in methanol (prepared at 0°) for 12 h at room temperature. HPLC analysis, in contrast to the previous two experiments, showed no detectable peaks corresponding to uridine or monosilylated uridine. These results established that the t-butyldimethylsilyl group was stable under the methanolic ammonia conditions. Studies on the rate of deprotection (Table 2) also showed a significantly increased rate of hydrolysis in the methanolic ammonia solution compared to the aqueous ammonium hydroxide.

The above studies suggested that the use of N-protecting groups that can be removed under mild conditions could completely eliminate the loss of alkylsilyl protecting groups and the resulting chain cleavage. However, it must be recognized that there is a significant difference among the rates of removal of the N-benzoyl group from cytidine compared to that for adenosine and guanosine (15), as can be seen from the data in Table 2. With anhydrous methanolic ammonia at room temperature, the half-lives of N-benzoylated cytidine, adenosine, and guanosine are 0.5 h, 1 h, and 8-10 h, respectively. Thus the limiting steps are firstly, the deacylation of N^2-benzoylguanosine and secondly, the deacylation of N^6-benzoyladenosine.

The phenoxyacetyl group has been recently introduced into oligodeoxyribonucleotide synthesis for protection of the exocyclic amino groups of deoxyadenosine and deoxyguanosine (16). This protecting group has been shown to be much more labile to base than the benzoyl group, being generally removed within 4h in ammonium hydroxide at room temperature. We therefore decided to adapt the phenoxyacetyl group to oligoribonucleotide synthesis by preparing the N-phenoxyacetyl- adenosine, guanosine and cytidine nucleosides, **2a-c** (Scheme 1).

The transient protection method of Jones et. al. (17) using trimethylsilyl chloride was used to block the hydroxyl positions of adenosine. Acylation was then performed using phenoxyacetic anhydride, prepared as previously described (11, 18). This route was preferred over the full acylation method



Scheme I

(16) previously used to make N^6-phenoxyacetyldeoxyadenosine **2a** because it required less of the expensive acylating reagent. After the completion of acylation, the trimethylsilyl groups were removed with 1M hydrogen fluoride-pyridine complex (19). The final acylated product was purified by crystallization from absolute ethanol (65% yield).

The transient protection method was also effective in preparing N^2-phenoxyacetylguanosine **2b**, and N^4-phenoxyacetylcytidine **2c**. For guanosine, it was found that either phenoxyacetic anhydride (yield 56%) or phenoxyacetyl chloride (yield 49%) could be used with comparable efficiency as the acylating reagent. However, more coloured impurities were formed when the latter reagent was used. With cytidine, phenoxyacetyl chloride was a satisfactory acylating reagent and a yield of 60-64% was obtained. The trimethylsilyl groups in both the guanosine and cytidine reactions were removed by brief treatment with ammonium hydroxide.

The N-phenoxyacetylated compounds **2a-c** were tritylated with monomethoxytrityl chloride and silylated with t-butyldimethylsilyl chloride (triisopropylsilyl chloride for guanosine) using the standard procedures (20) to give the 2'-t-butyldimethylsilyl (triisopropylsilyl for guanosine)-5'-monomethoxytrityl-N-phenoxyacetylnucleosides **3a-c**. Phosphorylation of **3a-c** was performed using N,N-diisopropylmethylphosphonamidic chloride, dimethylamino-

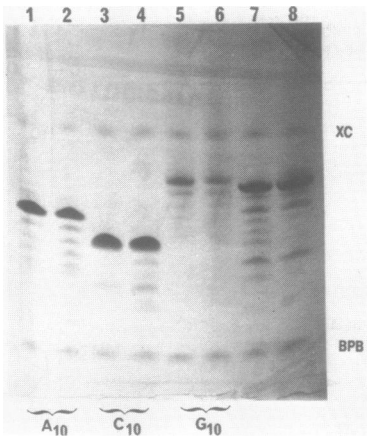


Figure 3. 24% Polyacrylamide/8M urea gel electrophoresis of unpurified oligoribonucleotides prepared using either anhydrous methanolic ammonia (8 h, room temperature, lanes 1, 3, 5, and 8) or 3:1 ethanol-NH_4OH (18 h, 55°, lanes 2, 4, 6, and 7). **Lanes 1 and 2:** A_{10}. **Lanes 3 and 4:** C_{10}. **Lanes 5 and 6:** G_{10}. **Lanes 7 and 8:** CACUUGACUAGCC.

pyridine (0.2 eq), and diisopropylethylamine in THF to produce the phosphoramidite derivatives **4a-c** in 83-90% yield. The ^{31}P NMR of each phosphoramidite with the new amino protecting group showed two signals characteristic of a pair of diastereomers (see experimental).

The lability of the N-phenoxyacetylated compounds **2a-c** relative to the N-benzoylated derivatives was then verified by either HPLC or UV analysis. The approximate half-lives of the N-protected nucleosides in aqueous or methanolic ammonia at room temperature are shown in Table 2. These results show that the half-lives of phenoxyacetylated nucleosides were very much less than the half-lives of the benzoylated nucleosides. The half-lives of **2a** and **2b** in aqueous ammonia (4-8 min) were similar to the half-lives (7-15 min) reported for the N-phenoxyacetylated deoxyadenosine and deoxyguanosine compounds (16). The half-life for the removal of the benzoyl group on cytidine using anhydrous methanolic ammonia, at 30 min, was longer than for either N-phenoxyacetylated adenosine or guanosine but was still very reasonable. Clearly, treatment with anhydrous methanolic ammonia at room temperature (8-12 h) should be sufficient for the deacylation of oligoribonucleotides using this combination of protecting groups. Under such condition the silyl group used to protect the 2'-hydroxyl will not be affected and chain cleavage will be eliminated.

The N-phenoxyacetylated phosphoramidite compounds **4a-c**, were evaluated in the solid-phase synthesis of oligoribonucleotides. However, difficulty was

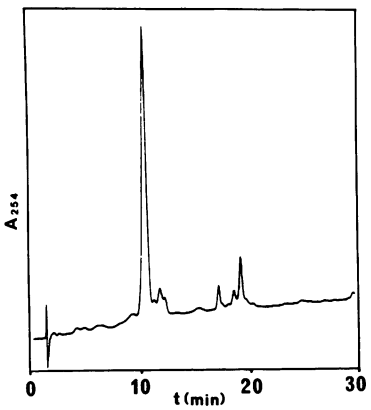


Figure 4. HPLC profile of crude oligoribonucleotide CACUUGACUAGCC prepared using the phenoxyacetyl amino protecting group on adenosine and guanosine and the benzoyl amino protecting group on cytidine. HPLC conditions: column, Aquapore RP-300 (4.6x100mm); solvent, linear gradient (0-30 min) of 7-20% acetonitrile in 0.1M triethylammonium acetate (pH 7); flow, 1 ml/min.

observed with the cytidine derivative **4c** since this compound was only poorly soluble in acetonitrile. Compound **4c** tended to crystallize out of acetonitrile solutions after standing for a few minutes. This problem precluded further use of **4c** and N-benzoylcytidine was used in its place.

The effectiveness of this combination of protecting groups was demonstrated through the synthesis of decanucleotide homopolymers containing N^6-phenoxyacetyladenosine, N^4-benzoylcytidine, and N^2-phenoxyacetylguanosine, as well as the synthesis of the tridecanucleotide, CACUUGACUAGCC. The syntheses were performed using our standard automated coupling cycles (1-2) and average coupling yields of 95-99% were obtained. After synthesis, the supports were treated with thiophenoxide to remove the methyl protecting groups. Then the supports were removed from the synthesizer and treated with methanolic ammonia at room temperature (1 h) to cleave the sequences from the support.

To further verify our procedures, the A_{10}, C_{10}, G_{10}, and CACUUGACUAGCC sequences were divided into two portions and each portion was treated with either 3:1 ammonium hydroxide-ethanol (55°, 18 h) or anhydrous saturated methanolic ammonia (room temperature, 8 h). After evaporation of the ammonia, the sequences were desilylated in 1M tetrabutylammonium fluoride/THF (12 h) and desalted on Sephadex G-25. The crude completely deprotected sequences from both the aqueous and methanolic ammonia treatments were run side by side on an

electrophoresis gel (Figure 3). This experiment showed that the anhydrous methanolic ammonia treatment resulted in substantially cleaner products for all four sequences than did the ammonium hydroxide-ethanol treatment.

The crude CACUUGACUAGCC product was further characterized by HPLC analysis (Figure 4), which showed that the full-length sequence was the predominant product. The purified tridecanucleotide was also enzymatically degraded into mononucleosides and subjected to HPLC analysis to confirm the correct nucleoside ratio and the absence of unprotected nucleosides.

The combination of adenosine and guanosine nucleosides protected with the labile phenoxyacetyl group and use of more effective anhydrous methanolic ammonia solutions for deacylation has thus been proven to further enhance the suitability of 2'-silylated nucleosides in oligoribonucleotide synthesis. This combination not only eliminates the small amount of unwanted chain cleavage which previously occurred but also continues the trend towards the use of milder and more selective conditions in organic synthesis.

CONCLUSION

It is clear that ammonium hydroxide treatment of protected synthetic oligoribonucleotide chains leads to some cleavage of the product resulting in the formation of shorter, default sequences. The cleavage is brought about by the removal of a small percentage of the t-butyldimethylsilyl protecting groups at 2'-hydroxyl positions. This cleavage is most severe in the aqueous ammonium hydroxide conditions routinely employed for oligodeoxyribonucleotide synthesis and greatly reduced but still detectable in the 3:1 ammonium hydroxide-ethanol conditions used in our previous oligoribonucleotide syntheses.

The problem can be virtually eliminated by using the milder deacylation conditions made possible by the easily removable phenoxyacetyl protecting group. Using N^2-phenoxyacetylguanosine, N^6-phenoxyacetyladenosine and N^4-benzoylcytidine (which remains adequate), deacylation can be satisfactorily performed using anhydrous methanolic ammonia at room temperature without causing any detectable chain cleavage. The use of these improved conditions should therefore increase the ease and yield in which long synthetic oligoribonucleotide molecules can be purified, since fewer default sequences will be present.

ACKNOWLEDGMENTS

We are indebted to the National Sciences and Engineering Council of Canada and the Medical Research Council of Canada for financial support of this research.

*To whom correspondence should be addressed

REFERENCES

1, N. Usman, K.K. Ogilvie, M.-Y. Jiang and R. J. Cedergren, J. Am. Chem. Soc. **109**, 7845-7854 (1987).
2, K. K. Ogilvie, N. Usman, K. Nicoghosian and R. J. Cedergren, Proc. Natl. Acad. Sci. USA. **85**, 5764-5768 (1988).
3, K.K. Ogilvie, N. Theriault and K.L. Sadana, J. Am. Chem. Soc. **99**, 7741-7743 (1977).
4, K. K. Ogilvie, S. L. Beaucage, A. L. Schifman, N. Y. Theriault, and K. L. Sadana, Can. J. Chem. **56**, 2768-2780 (1978).
5, K. K. Ogilvie, A. L. Schifman, and C. L. Penney, Can. J. Chem. **57**, 2230-2238 (1979).
6, K. K. Ogilvie and R. T. Pon, Nucl. Acids Res. **8**, 2105-2115 (1980).
7, K. K. Ogilvie, M. J. Damha, N. Usman and R. T. Pon, Pure & Appl. Chem. **59**, 325-330 (1987).
8, P. J. Garegg, I. Lindh, T. Regberg, J. Stawinski and R. Stromberg, Tetrahedron Lett. **27**, 4055-4058 (1986).
9, J. Stawinski, R. Stromberg, M. Thelin and E. Westman, Nucl. Acids Res. **16**, 9285-9298 (1988).
10, Pon, R. T., Tetrahedron Lett. **28**, 3643-3646 (1987).
11, T. Wu, K.K. Ogilvie and R. T. Pon, Tetrahedron Lett. **29**, 4249-4252 (1988).
12, K. Itakura, J. J. Rossi, and R. B. Wallace, Ann. Rev. Biochem. **53**, 323-356 (1984).
13, R. Lohrmann and H.G. Khorana, J. Am. Chem. Soc. **86**, 4188-4194 (1964).
14, K. K. Ogilvie and N. Y. Theriault, Can. J. Chem. **57**, 3140-3144 (1979).
15, H. Koster, K. Kulikowski, T. Liese, W. Heikens and V. Kohli, Tetrahedron **37**, 363-369 (1981).
16, J. C. Schulhof, D. Molko and R. Teoule, Nucl. Acid Res. **15**, 397-416 (1987).
17, X. Gao, B. L. Gaffney, S. Hadden and R. A. Jones, J. Org. Chem. **51**, 755-758 (1986).
18, R.K. Smalley and H. Suschitzky, J. Chem. Soc., 755-757 (1964).
19, L. W. McLaughlin, N. Piel and T. Hellmann, Synthesis, 322-323 (1985).
20, G. H. Hakimelahi, Z. A. Proba and K. K. Ogilvie, Can. J. Chem. **60**, 1106-1113 (1982).