

Labile exocyclic amine protection of nucleosides in DNA, RNA and oligonucleotide analog synthesis facilitating N-deacylation, minimizing depurination and chain degradation

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Summary — The use of *tert*-butylphenoxyacetyl for N-protection of nucleoside bases during oligonucleotide synthesis facilitates its removal under mild conditions. This protecting group reduces depurination of deoxyadenosine residues, and minimizes premature desilylation resulting in chain degradation during post-synthesis workup of synthetic RNA.

chemical synthesis of DNA, RNA / chimeric oligonucleotides / synthetic ribozyme / oligonucleotide methylphosphonate diesters

Introduction

Solid support assisted syntheses of DNA and to a certain extent RNA oligomers are routine processes, and with the help of an automated DNA synthesizer, DNA chains of up to 40–50 bases long can be achieved within a few hours. However, depending on the nature of the oligonucleotide chain and deprotection strategy (room temperature *vs* elevated temperature), post-synthesis steps can take much longer than chain assembly. Long exposures to concentrated ammonia does not affect the quality of normal synthetic deoxyoligonucleotides, but in the case of synthetic RNA, methylphosphonate oligonucleotide diesters, or oligonucleotides containing 4-thiothymidine or 6-thioguanosine, there is a need for milder deprotection conditions.

Since our introduction of β -cyanoethyl as a temporary phosphorous protecting group [1] for facilitating

post-synthesis workup and minimizing side reactions, recent efforts have been geared towards the use of more labile exocyclic amine protecting groups. Use of labile protecting groups not only facilitates DNA/RNA synthesis but also allows incorporation of modified and base sensitive nucleosides. Protecting groups such as phenoxyacetyl [2], and dimethylaminoformamidine [3,4] have been introduced or re-adapted for phosphoramidite and H-phosphonate chemistries. Use of the phenoxyacetyl group for exocyclic amine protection, especially for guanosine, remains problematic.

N-phenoxyacetyl 2'-deoxyguanosine phosphoramidites tend to form gelatinous precipitates in acetonitrile solution, thus requiring the use of DMF as a co-solvent. Dimethylformamide is frequently contaminated with free amines, which can interfere during amidite coupling. Similarly, the stability of dimethylamino formamidine derivatives of deoxyadenosine [4] towards acids or bases used during oligonucleotide synthesis is questionable and chain branching can result. Other protecting groups such as the tBDMSi-ether of 2-hydroxymethylbenzoyl [5] is not readily available for the synthesis of large amounts of nucleoside building blocks. The phthaloyl group for N-protection of deoxyadenosine has been successfully used [6], but it is difficult to introduce for C and G nucleosides.

Here, we describe the use of *t*-butylphenoxyacetyl [7] (tBPA) as a useful alternative for protection of

Abbreviations: tBDMSi, *t*-butyl dimethylsilyl; tBPA, *t*-butylphenoxy acetyl; THF, tetrahydrofuran; CPG, controlled pore glass; PAGE, polyacrylamide gel electrophoresis; DBU, diazabicyclo-undecene; DCA, dichloroacetic acid; TCA, trichloroacetic acid; DIPEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; tlc, thin layer chromatography; TEA, triethylamine; N-bz, *N*-benzoyl; N-dmf, *N*-dimethylformamidine; N-ibu, *N*-isobutyl; vac, vacuum; NaHCO₃ soln, sodium bicarbonate solution.

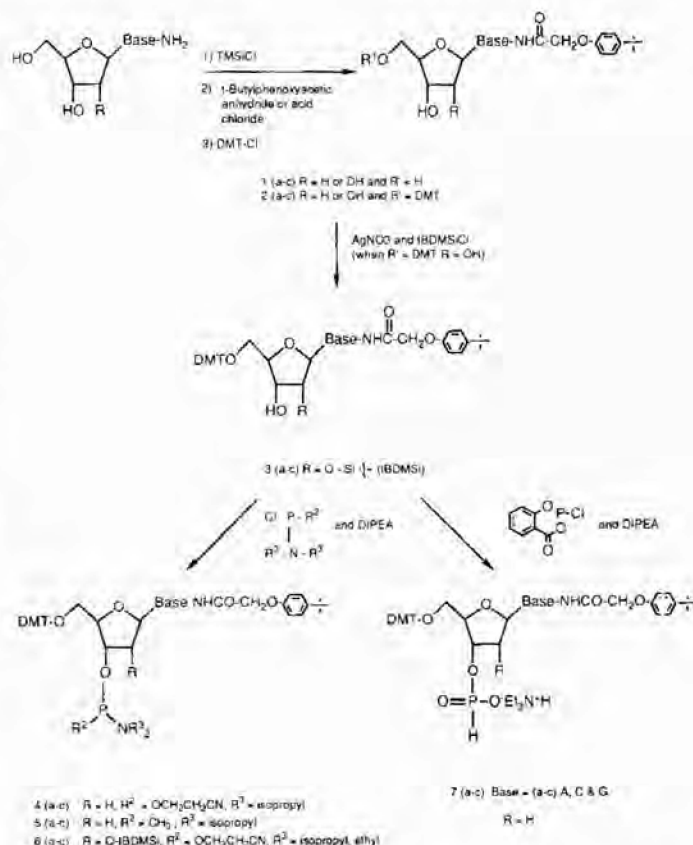
exocyclic amine groups of nucleosides including cytosine. This N-protecting group is stable towards typical acidic and basic reagents used in automated DNA/RNA synthesis following either standard phosphoramidite or H-phosphonate chemistries. Problems associated with solubility of deoxyguanosine-phosphoramidites using other labile N-protecting groups are eliminated with the use of the more lipophilic *t*-butylphenoxyacetyl groups. N-Deacylation is not only facilitated with the use of this protecting group but depurination of deoxyadenosine residues due to exposure to DCA or TCA solutions commonly used during detritylation is minimized. In addition, use of N-tBPA protection in RNA synthesis using *t*-BDMSi for 2'-hydroxyl protection minimizes chain cleavage due to premature desilylation [8] during prolonged incubation periods in concentrated ammonia solutions at 55°C, which is normally required when isobutryl- or benzoyl-protecting groups are used. N-tBPA protected nucleoside building blocks now allow synthesis of oligonucleotide chains containing base sensitive linkages and/or modified nucleosides.

Materials and methods

Nucleosides, dimethoxytrityl chloride (DMT-Cl), trimethylchlorosilane, *t*-butyldimethylchlorosilane, and silver nitrate were obtained from Raylo Chemicals, Argyll Road, Edmonton, Canada and Aldrich Chemical Company, PO Box 355, Milwaukee, Wisconsin, USA. Solvents used were HPLC grade and were dried over a molecular sieve (4 Å). Tetrahydrofuran was dried and freshly distilled from sodium metal and benzophenone mixture. *t*-Butylphenoxyacetic anhydride, *t*-butylphenoxyacetyl chloride, and β -cyanoethyl-monochloro-N,N-diisopropylamino-phosphine were obtained from Biosyntech, Hamburg, Germany. DNA/RNA synthesis reagents were obtained from Millipore Corporation, MA USA. NMR spectra were taken on a Bruker Instrument. Automated DNA synthesizers used were Millipore's 8750 and 8400. Standard protocols were used for oligomer synthesis unless stated otherwise. Reaction steps involved in the preparation of suitably protected monomers have been outlined in scheme 1.

Synthesis of N-tBPA-deoxynucleoside/ribonucleoside (1 a-c)

Synthesis of N-protected nucleoside was carried out following transient protection strategy [9]. Typically nucleosides (10 mmol) were dried by coevaporating with pyridine (3 x 50 ml) under reduced pressure and suspended in dry pyridine (50 ml). To this suspension, trimethylsilyl chloride (30–40 mmol) was added through a syringe. After stirring at room temperature (RT) for 1 h, *t*-butylphenoxyacetic anhydride (15.0 mmol) for A and C or *t*-butylphenoxyacetyl chloride (12.5 mmol) for G was added dropwise at 0°C. The reaction mixture was stirred at RT for 3–4 h with monitoring by tlc (thin layer chromatography) (15% MeOH in CH₂Cl₂). On completion, the reaction mixture was treated with methanol (5 ml) followed by water (5 ml). The organic solvents and hydrolyzed silyl derivatives were removed by rotary evaporator under reduced pressure. The viscous mass was then treated with ice



Scheme 1. Synthesis of N-tBPA-protected nucleoside and reactive phosphorus derivatives.

cold water. Solid residue obtained was washed with hexane-ethyl acetate (1:1) mixture, and pale yellow solid was finally dried over phosphorus pentoxide under vacuum to yield about 7–8 mmol of N-protected nucleoside.

Synthesis of N-tBPA-5'-O-DMF deoxynucleoside/ribonucleoside (2 a-c)

Dimethoxytritylation reaction was performed following standard methods. The dried N-*t*-butylphenoxyacetyl nucleoside (10 mmol) was dissolved in dry pyridine (50 ml). Dimethoxytrityl chloride (1.1 eq) was added portionwise over 1 h at RT. After 3–4 h of stirring, when 90% of the material converted into desired product, reaction was quenched with ethanol. Pyridine was removed and viscous oil was taken up in ethyl acetate. Ethyl acetate solution was washed with cold saturated NaHCO₃ soln. Organic layer was washed once with saturated NaCl solution and dried over anhydrous Na₂SO₄. Concentration of ethyl acetate solution under reduced pressure gave a yellow foam. Purification by silica gel chromatography using ethyl acetate and increasing percentage of ethanol (0–10%) gave light yellow to colorless product in good yield (≥65%).

Synthesis of 2'-O-silyl-protected ribonucleoside(3 a-c):

Conversion of N-tBPA-5'-O-DMT-ribonucleosides into 2'-O and 3'-O-silyl derivatives were carried out according to our

previously published method [11], the nucleoside and solid silver nitrate (0.8–1.0 eq) were dried by co-evaporating in pyridine under reduced pressure. The mixture was taken up in dry THF (for A and C) or dry pyridine (for G) (solvent 5 ml/mmol), and *t*-butyldimethylsilyl chloride (1.0 eq) was added in two portions. The reaction mixture was then stirred overnight protected from light. To this mixture was added a moist paste of sodium bicarbonate and water and stirred for 30 min. This mixture was filtered through a celite packed sintered glass funnel and solid residue was washed thoroughly with ethyl acetate. The combined solution was concentrated and finally taken up in ethyl acetate, which was washed with cold sodium bicarbonate and saturated sodium chloride solutions. The dried organic solution was concentrated to a foam. This foam was dissolved in a small volume of methylene chloride and was pptd out as a solid from cold hexane. Finally the desired 2'-O-tBDMSi-protected nucleoside was obtained by chromatography using silica gel and hexane-ethyl acetate mixture. The pure 2'-O-silyl regio isomer for C and G were also obtained as a crystalline solid from ether-hexane-methylene chloride (1:4:1) (mps for C = 194–196°C, G = 161–163°C) in 45–55% isolated yield. Attempted crystallization of A-derivative was unsuccessful. The purity of the regio isomers was determined by proton NMR.

General method for the synthesis of reactive phosphorus-amidite derivative (4, 5, 6)

Synthesis of reactive phosphorus-amidite was carried out according to published methods [1, 11, 14]. Typically protected nucleoside was dried overnight under high vacuum and dissolved in freshly distilled THF (5 ml/mmol). To this solution, DIPEA (3.0 eq) and monochloro-*N,N*-diethylamino- β -cyanoethoxy/methyl phosphine (1.5 eq) were added. The reaction was monitored by tlc or by RP-HPLC for ribonucleoside derivatives. On completion of the reaction, solid hydrochloride was filtered under argon, the clear solution was concentrated and the residue taken up in ethyl acetate. Usual workup and purification by silica gel column chromatography gave the desired phosphoramidite in a 75–80% yield.

Synthesis of H-phosphonate (7 a–c)

These derivatives were also made according to a previously described method. Dried nucleoside (2 a–c) was treated with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (1.5 eq) and DIPEA (3.0 eq) in dry THF (5 ml/mmol). When all starting material was consumed, the reaction mixture was quenched with a cold pyridine-water mixture (1:1) at 0°C. After removal of solvent under reduced pressure, the reaction product was taken up in ethyl acetate and washed with cold 0.1 DBU-bicarbonate or 0.1 M TEA-bicarbonate solution. The product in organic solvent was dried and concentrated to a foam, which was purified by silica gel column using methylene chloride containing 0–10% methanol and 0.1% DBU or TEA. The product was characterized by phosphorus (chemical shift, A = 2.16, C = 1.73 and G = 1.17 ppm) and proton NMR.

Results and discussion

Introduction of the tBPA protecting group onto exocyclic amines of nucleosides was carried out following the transient hydroxyl protection strategy developed

by Jones *et al* [9]. Yields of *N*-protected nucleosides were in the range of 65–85%. Dimethoxytrityl- and *t*-butyldimethylsilyl in the case of ribonucleosides, were introduced according to standard methods [10, 11]. *N*-tBPA-5'-O-DMT-ribonucleosides resulted in a mixture of 2'-O- and 3'-O-*t*-butyldimethylsilyl ethers, using *tert*-butyldimethylsilyl chloride and silver nitrate. However, some selectivity towards the formation of the desired 2'-O-silyl ether (60%) was observed in the reaction mixture. The crude reaction products, after precipitation from hexane or low boiling petroleum ether in order to remove unreacted species of *t*-butyldimethylsilyl derivatives, were purified on silica gel columns using ethyl acetate-hexane mixtures to yield the pure 2'-O-silyl derivative (45–50% yield). Regio-isomerically pure 2'-O-silyl cytosine and guanosine nucleosides were crystallized from hexane-methylene chloride-ether mixture (4:1:1, v/v/v). The purity of these 2'-O-silyl isomers was determined by melting points and spectroscopic properties.

Deoxynucleoside- β -cyanoethyl-*N,N*-diisopropyl-amino-phosphoramidites were obtained using our previously described method [1] and successfully purified on large scales in high purity ($\geq 98.5\%$) using silica gel column chromatography or a Waters Prep 500 HPLC. Methylphosphoramidites of *N*-tBPA dA, *N*-tBPA dC and *N*-tBPA dG derivatives were also similarly prepared using monochloro-*N,N*-diisopropyl-amino-methyl phosphine [14] (1.5 eq) and *N,N*-diisopropylethylamine (3.0 eq) in dry THF. These derivatives were purified on silica column using ethyl acetate-hexane or ethylacetate. Products were characterized for purity and regiospecificity by proton and phosphorus NMR.

In order to prepare various alkyl-amino phosphoramidites, the desired monochlorocycanoethoxy-*N,N*-dialkylamino (*N,N*-dimethylamino/*N,N*-diethylamino/*N,N*-diisopropylamino-/and *N*-morpholino)-phosphines were prepared according to published methods [1, 11]. Then *N*-tBPA-ribonucleosides were converted into these various phosphoramidites in dry THF using diisopropylethylamine (3.0 eq) and the monochlorophosphine (1.5 eq). The reaction of the free 3'-hydroxyl group was complete within 4–8 h of stirring at RT. Isolation of *N,N*-dimethylamino phosphoramidite by aqueous bicarbonate extraction followed by purification on a silica gel column was achieved in good yield (50%). Interestingly, the stability of this species in acetonitrile solution was short (approximately 3 h). All other dialkylaminophosphoramidites were isolated in pure form ($\geq 98\%$ according to ^{31}P -NMR and $\geq 99.5\%$ 3'-isomer). Of these, the morpholino phosphoramidites were the most stable in solution, yet least reactive in couplings to support bound nucleoside residues.

N-tBPA deoxynucleoside-3'-O-H phosphonate salts of triethylamine or diazobicyclo-undecene (DBU)

were prepared in dry THF using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one [12, 13] and the products were subsequently converted into the more stable ammonium salt with either triethylammonium bicarbonate or DBU-bicarbonate solution (0.1 M). The crude H-phosphonate salts were purified by silica gel column chromatography using methylene chloride-methanol mixtures (5–15%) containing DBU or triethylamine (0.1%). High purity and good yield (60–65%) of dA and dC H-phosphonate salt were obtained after purification. Although reasonably good yield of dG-H-phosphonate DBU salt (50%) was obtained, purity, according to phosphorus NMR, was only 96–97%.

CPG derivatives functionalized with N-tBPA protected nucleosides were synthesized *via* their 3'-O-succinate nitrophenyl esters. Almost identical loadings of up to 40 μ mol nucleoside per gram of support were obtained.

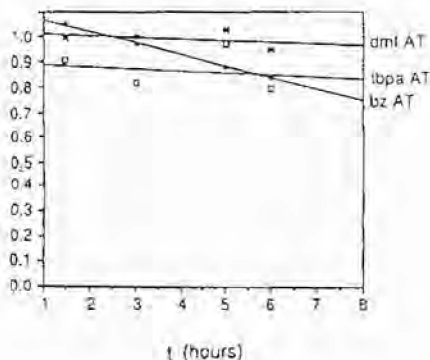
Stabilities of N-t-butylphenoxyacetyl protected nucleosides towards chemical reagents used during oligonucleotide synthesis were determined. A small amount of the N-tBPA protected nucleosides were treated with various reagents used in DNA synthesis.

N-tBPA deoxycytidine was stable towards all these reagents. In a mixture of acetic anhydride N-methylimidazole pyridine it gave the desired 3',5'-O-diacylated product as expected. N-t-BPA-Deoxyadenosine and N-t-BPA-deoxyguanosine were stable in iodine, acetic anhydride and N-methylimidazole solutions. However, during acetic anhydride / N-methylimidazole / pyridine treatment, some conversion of t-BPA deoxyguanosine into 3'-5'-O-, N-triacetylated-deoxyguanosine was observed. This was confirmed by similar treatment of 3'-5'-di-O-tetraisopropylsiloxy N-t-BPA deoxyguanosine. This transamidation of N-tBPA deoxyguanosine was prevented by the use of t-butyl phenoxyacetic anhydride (t-BPA anhydride (10% in THF)), which was as effective as acetic anhydride in capping non-coupled 5'-hydroxyl groups.

A small amount of depurination of N-tBPA deoxyadenosine in DCA solution was observed, this effect was investigated in detail by synthesizing the dimer d(TA) and trimers d(TTA) and d(CAT) on CPG supports *via* phosphoramidites and compared with syntheses utilizing N-bz or N,N-dimethylformamido deoxyadenosine phosphoramidites. The extent of glycoside bond cleavage was determined by measuring

A) TA - DEPURINATION

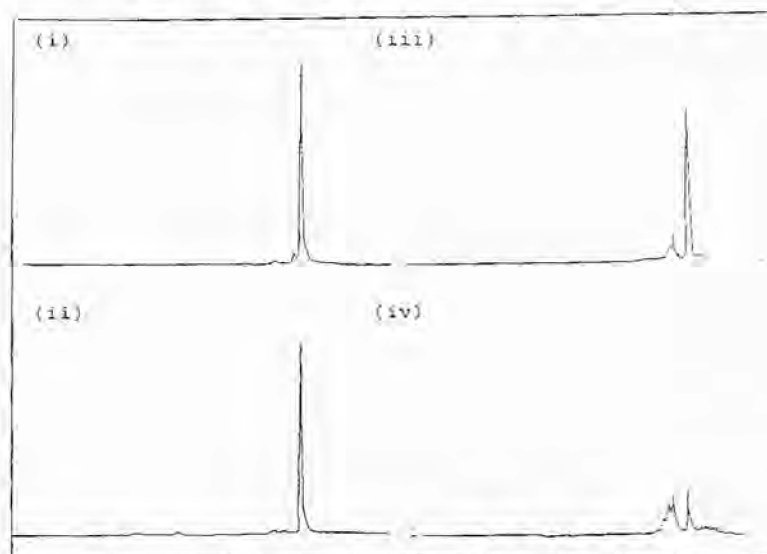
Depurination of 3'-terminal dA



t 1/2 OF DEPURINATION

N-bz dA = 2.5 days
N-tBPAdA = 8.0 days
N-dmfdA = 9.0 days

B) TTA SUBJECTED TO DCA SOLUTION, DEPROTECTED AND ANALYZED BY RP-HPLC



- (i) MADE FROM tBPAdA AND DMT-GR. REMOVED ON THE INSTRUMENT
- (ii) MADE FROM bz dA AND DMT-G REMOVED ON THE INSTRUMENT
- (iii) MADE FROM tBPAdA, INCUBATED IN DCA FOR 64 HR.
- (iv) MADE FROM bz dA, INCUBATED IN DCA FOR 64 HR.

Fig 1. Effect of 3% DCA solution on deoxyadenosine bound to solid supports.

the amount of full length dimer and trimer present after incubating the support in DCA solution for various lengths of time followed by cleavage from the support and deprotection with concentrated ammonium hydroxide prior to HPLC analysis. Similar experiments were carried out using N-bz and N-dmf deoxyadenosine derivatives. The comparative rates of depurination and quality of product are shown in figure 1. No significant depurination during a normal 1.0 μ mol scale synthesis cycle is observed with any of the above deoxyadenosine derivatives. Nevertheless these derivatives did show increasing amounts of depurination when the dimers and trimers were incubated for longer periods with acid. The rate of depurination was more pronounced for N-bz dA as compared to N-tBPA or N-dmf dA derivatives.

Removal of the N-protecting group from the synthetic oligonucleotide

It is well known that removal of the N-protecting group from deoxyguanosine is the limiting step in deprotection; therefore, its removal with NH_4OH solution at RT using various N-protected deoxyguanosines anchored to CPG beads was studied. Complete removal of tBPA from deoxyguanosine was achieved in 1 h at RT as shown in figure 2. This removal was also complete within 15 min at 55°C. In addition to aqueous concentrated ammonium hydroxide solution, N-deacylation was also carried out with: i) methanol-ammonium hydroxide (1:3, v/v) solution; ii) ethanol/methanol saturated with ammonia gas; and iii) an anhydrous methanolic solution of potassium carbonate (50 mM); obtained by warming up the suspension. These milder deprotection conditions were also able to completely remove the t-butylphenoxyacetyl group.

Concentrated Ammonia,
Room Temperature

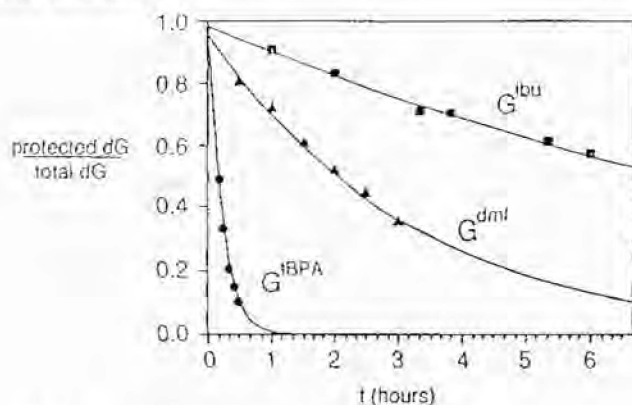


Fig 2. Deprotection of G protected nucleoside.

Oligonucleotide syntheses

Syntheses of DNA and RNA oligomers were carried out on Millipore automated Cyclone or Model 8750 DNA synthesizers using phosphoramidite or H-phosphonate chemistries and standard protocols. In syntheses using tBPA nucleoside phosphoramidites, tBPA anhydride was used instead of acetic anhydride in the capping reactions. Concentrations of amidite used in the syntheses were the same as those for standard N-bz and N-ibu protected monomers (50 mg/ml).

DNA synthesis

Another major concern in DNA synthesis is 0-6 modification of deoxyguanosine residues during synthesis and ammonia deprotection. We synthesized $\text{d}(\text{GT})_{10}$ oligomer using different orders for the oxidation (ox) and capping (cap) steps (eg ox-cap, cap-ox, cap-ox-cap). Following these syntheses using tBPA phosphoramidites and deprotection with conc NH_4OH at 55°C for 15 min, products were analyzed by UV, HPLC and enzymatic degradations. In each case, we found that product quality, quantity, UV spectra, HPLC and enzymatic degradations gave results which were almost identical (table I).

Although we did not see any significant difference in the quality of the product, slightly better yields of purified material were obtained when an extra capping step was introduced in the synthesis cycle. Several oligonucleotides ranging from 16–100 bases long were synthesized in good quality using tBPA phosphoramidites. We therefore, prefer the cap-ox-cap protocol in our syntheses. Oligodeoxynucleotides were also synthesized in good yield using H-phosphonate salts of appropriately protected N-tBPA deoxynucleosides.

Synthetic oligonucleotides obtained from N-tBPA-protected nucleosides following either phosphoramidite or H-phosphonate chemistry were analyzed for base modifications by complete degradation into deoxynucleosides by snake venom phosphodiesterase (SVPD) and alkaline phosphatase (BAP) as shown in figure 3. These analyses all gave the four expected deoxynucleosides in the appropriate ratios for the sequences synthesized. DNA synthesized from N-tBPA protected nucleoside monomers were also effective in DNA sequencing and polymerase chain reaction (PCR) studies (data not shown).

RNA synthesis

In RNA synthesis using tBDMSi for 2'-O-hydroxyl protection, it is considered that long exposures to concentrated ammonia at elevated temperatures results in some desilylation which promotes chain cleavage and ultimately gives poor yields of the desired RNA product. To investigate the effect of

Table I. The results of d(GT)10 synthesis on 0.2 mmol scale with different orders of capping and oxidation after each coupling step: 1) oxidation-capping; 2) capping-oxidation; 3) capping-oxidation-capping.

<i>Order of capping and oxidation</i>	<i>Crude material OD at 260 nm</i>	<i>Purified material OD at 260 nm</i>	<i>Coupling efficiency based on DMT-color</i>	<i>Base integrity after enzyme digest</i>
Ox-cap	30	13.5	98.6	>99%
Cap-ox	26	14.6	96.6	>99%
Cap-ox-cap	31	16.1	97.5	>99%

longer ammonia or ethanol-ammonia treatments for removing N-bz and N-ibu, we studied the 2'-O-silyl protected dimers rAT, rCT and rGT. These dimers were synthesized using N-tBPA, 2'-O-TMBSi ribonucleoside phosphoramidites. After synthesis, the 5'-DMT-group was removed and the dimer products were isolated in pure form by HPLC. The products

were then treated with ethanolic ammonium hydroxide overnight at 55°C. We observed that prolonged treatment of silyl derivatives with this solution results in significant desilylation and/or chain cleavage (fig 4). Although it is not clear, we observed that this desilylation is nucleoside dependent. Therefore, we believe that milder N-deacylation methods are

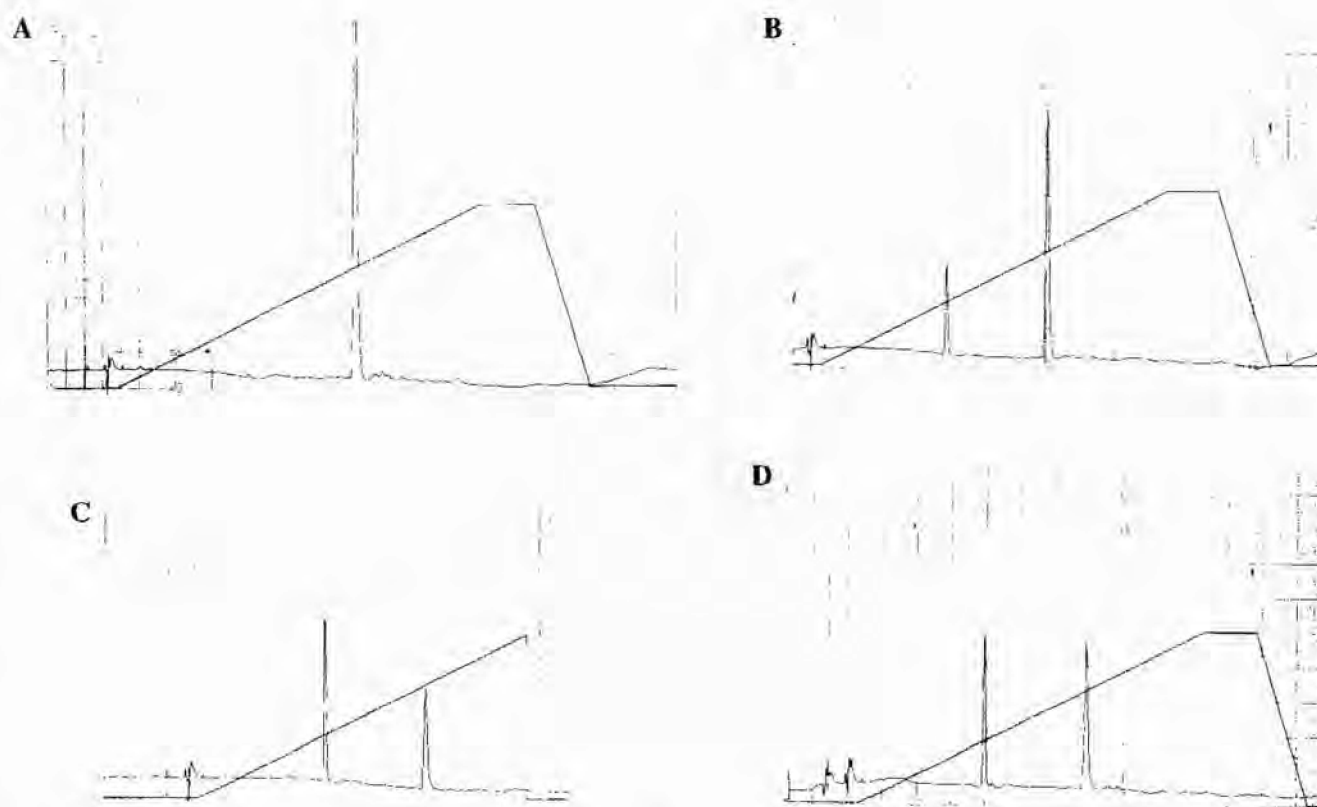


Fig 4. Effect of ammonia solution on 2'-O-TBDMSi of rAT dimer. **A.** Purified 2'-O-TBDMSi-rAT. **B.** Incubated with ethanolic ammonia for 1 h at 55°C. **C.** Incubated for 3 h at 55°C. **D.** Incubated for 20 h at 55°C.

24-MER CONTAINED 6A, 4C, 10G AND 4T.
HPLC DATA SHOW 99.5% AUTHENTICITY.

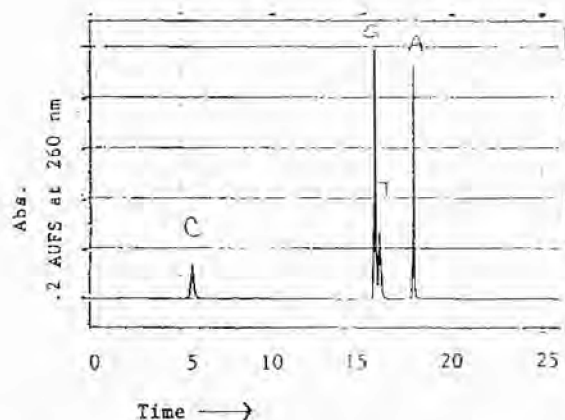


Fig 3. Enzymatic degradation of HPLC-purified 24-Mer.

desirable for RNA syntheses using tBDMSi as the 2'-hydroxyl protecting group and this labile group should be highly beneficial for RNA synthesis.

In comparison to DNA synthesis, RNA synthesis coupling times are much longer for N,N-diisopropylamino phosphoramidites. We investigated the use of other dialkylamino and N-morpholino phosphoramidites for faster coupling times. N,N-Dimethylaminophosphoramidites were the most reactive but at the same time were very unstable in solution. N,N-diethylaminophosphoramidites had slightly better coupling kinetics compared to N,N-diisopropylamino phosphoramidites. We synthesized several RNA oligomers using either N,N-diethylamino or N,N-diisopropylamino phosphoramidites which were then used in studies of ribozyme activity of synthetic RNA. Three chimeric (DNA-RNA-DNA) oligomers were also synthesized using N-tBPA-2'-O-tBDMSi-5'-O-DMT-ribonucleoside-3'-O-(N,N-diethylamino)-phosphoramidite or N-tBPA-5'-O-DMT-deoxynucleoside-3'-O-(N,N-diisopropylamino)-phosphoramidite. The coupling time for ribonucleoside monomer phosphoramidite was 12 min with intermittent pulsing of fresh

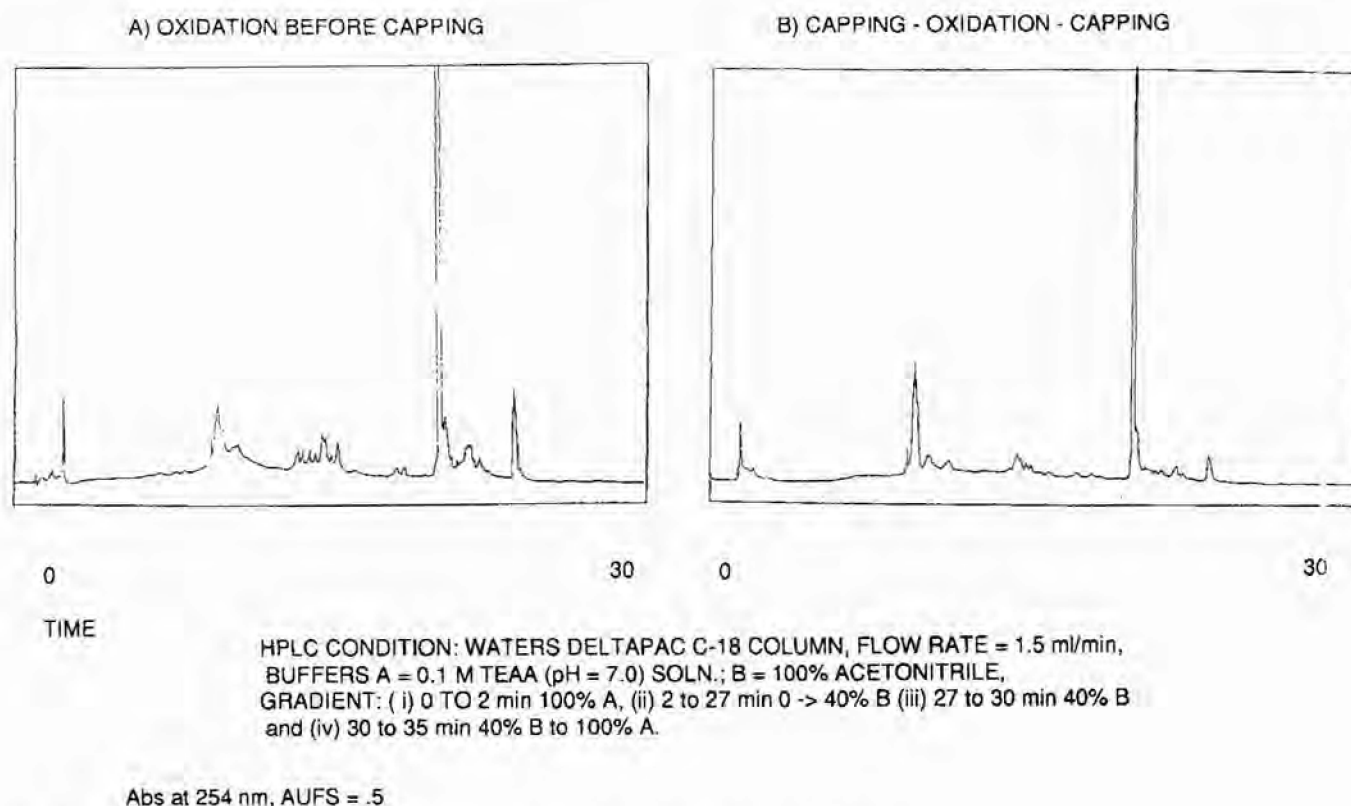


Fig 5. RP-HPLC profiles of crude synthetic DMT-rAUC-UGC-ACU-CGC-GAU.

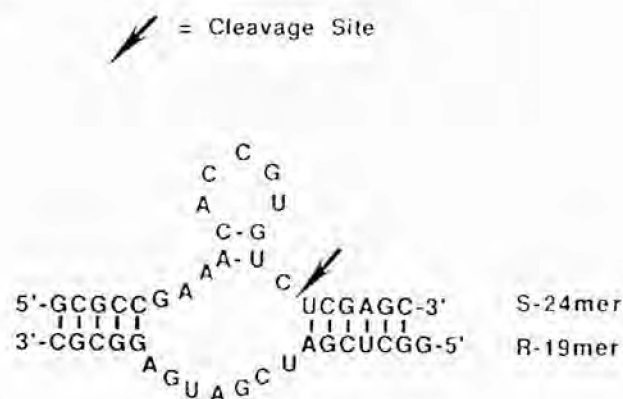
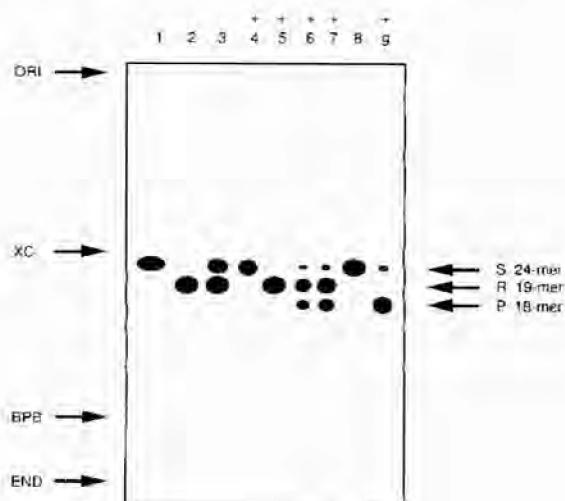


Fig 6. S24-R19 hammer head ribozyme.

tetrazole and amidite solutions. Deoxynucleoside phosphoramidite coupling times were much shorter. In this case coupling was followed by the capping, oxidation and capping protocol. The final 5'-O-DMT was left on the chain for reverse phase HPLC purification. The removal of N-tBPA protecting groups was achieved either with a mixture of concentrated ammonium hydroxide and ethanol (3:1, v/v) or with a saturated ethanolic ammonia at RT within 2 h.

Prior to removal of silyl protecting groups, the ammonia solution was completely evaporated. The



Lanes (L → R) 1 = 24 mer, 2 = 19 mer, 3 = 24 & 19 mers mixture, 4 = 24 mer and 5 = 19 mer with Mg^{2+} ion, 6 & 7 = 24 & 19 mers mixture with Mg^{2+} ion at 35 and 50 °C, 8 & 9 = 24 & 19 mers mixture with Mg^{2+} , 19 mer unlabelled.

Fig 7. S24-R19 hammer head ribozyme cleavage reaction.

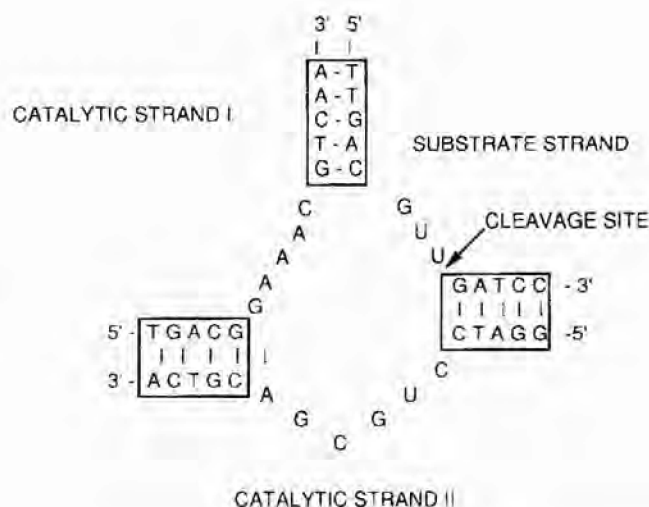


Fig 8. Three-piece chimeric ribozyme.

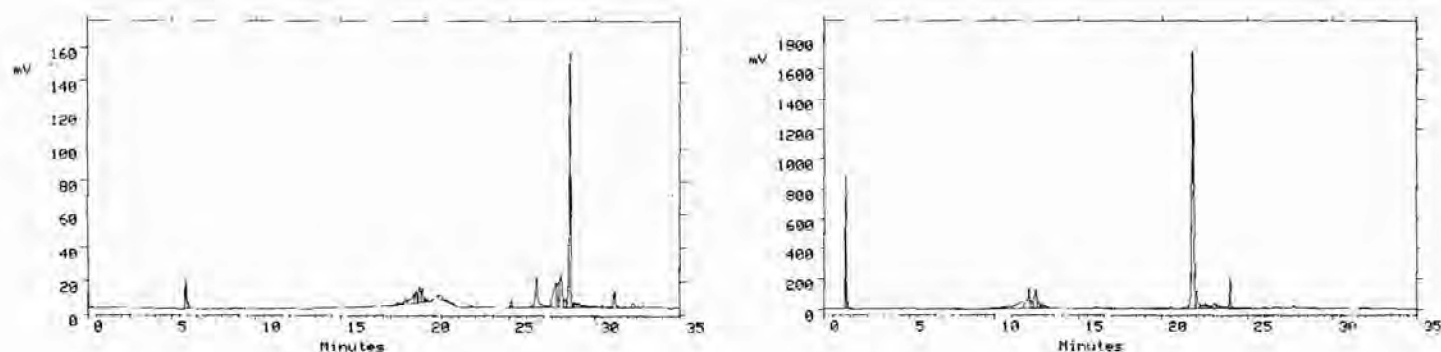
dried residue was then treated with tetrabutylammonium fluoride solution (TBAF in THF; 1.0 M, 600 μ l) for 20 h. Excess TBAF reagent was quenched with ammonium acetate (0.1 M, pH 7.0; 600 μ l) and products were analyzed by RP-HPLC for complete deprotection as indicated in figure 5. The desired material (45–55 O.D.) for 1 mmol scale run was isolated by Oligo-Pak column purification, and further purified by preparative PAGE (20%). In our hands, we did not notice a significant difference in either yield or quality of the product using anhydrous ethanolic ammonia or mixtures of ethanol and concentrated ammonium hydroxide. Thus synthesized hammerhead ribozyme sequences (19, 24 mers), as shown in figure 6 were found to be effective in cleaving substrate sequences in figure 7. The three piece chimeric oligonucleotide sequences (13, 15 and 17 mers) were able to form ribozyme structures as shown in figure 8.

Modified oligonucleotides

In order to exploit the benefit of milder deprotection, we utilized N-tBPA building blocks for the synthesis of base labile oligonucleotide chains (oligodeoxynucleosidemethylphosphonate diesters [14, 15]) and RNA chains containing 5-bromouridine and other labile modified nucleoside such as 4-thiouridine.

A) METHYLPHOSPHONATE DIESTER OLIGOMER

B) REGULAR DIESTER OLIGOMER



HPLC METHOD: STATIONARY PHASE = WATERS DELTA PAK (C-18 (300A), MOBILE PHASE = (A) TEAA SOLN (0.1 M, pH = 7.0), (B) = ACETONITRILE, FLOW RATE = 1.5 ML/MIN, GRADIENT: (i) 100% A FOR 2 MIN, (ii) 30% B IN 15 MIN, (iii) 50% B IN 3 MIN, (iv) 50% B FOR 10 MIN, (v) 100% A IN 3 MIN AND (vi) EQUILIBRATE FOR 5 MIN.

Fig 9. RP-HPLC chromatogram of TTTCCGTCCGGAAT oligomer with methyl phosphonate diester and regular phosphate diester linkages.

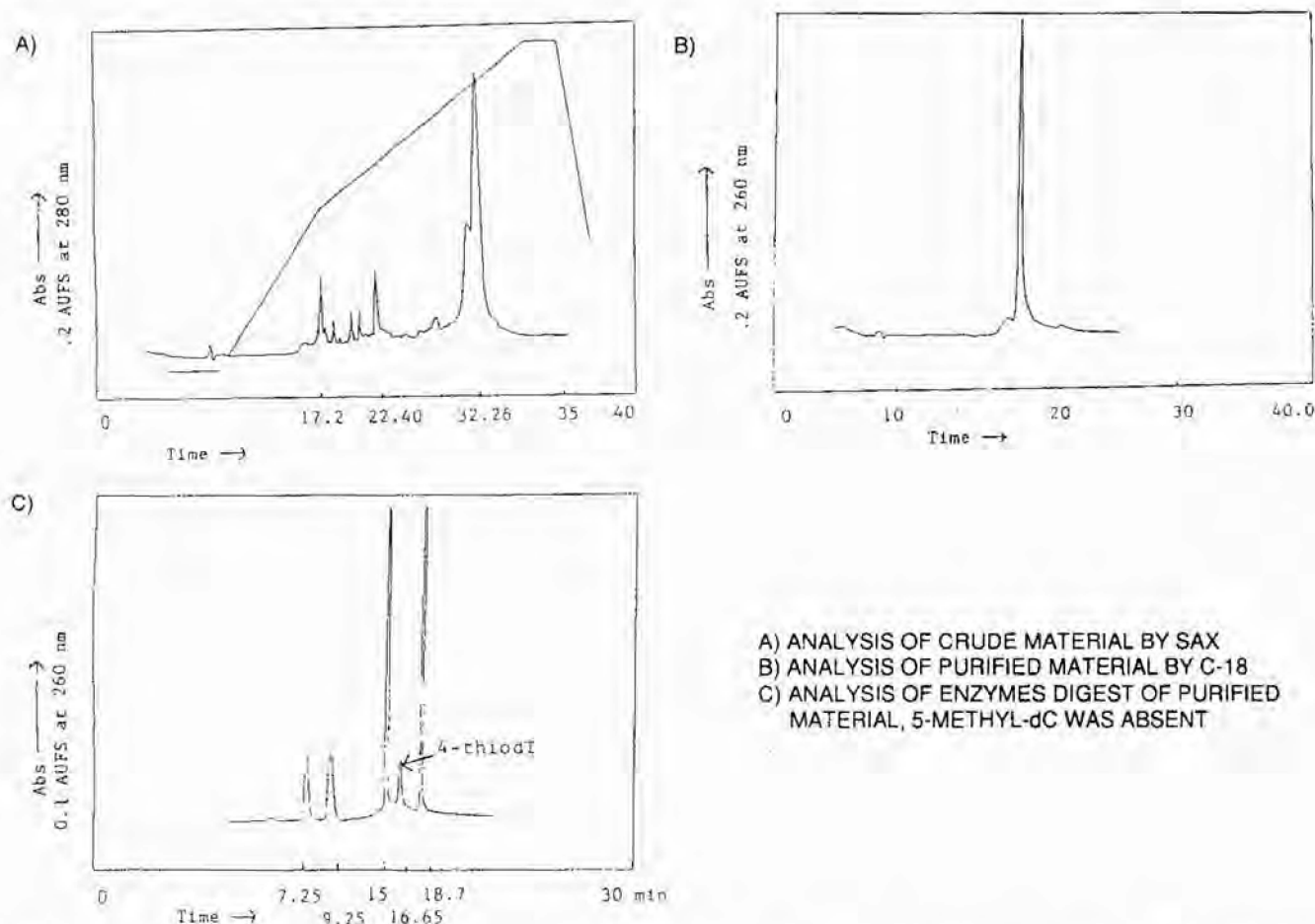
Base labile oligomers

One of the major demerits of existing methods for post-synthesis work-up of methylphosphonate oligomers is the use of hydrazine followed by ethylenediamine or ethylenediamine treatment alone [15]. Hydrazine is very toxic and somewhat tends to degrade pyrimidine bases as used in Maxam-Gilbert sequencing, and ethylenediamine tends to convert cytosine into aminoethylcytosine. Furthermore, standard N-deacylation with concentrated ammonium hydroxide solution for N-benzoyl and N-isobutyryl removal in oligonucleoside methyl phosphonates leads to severe chain degradation [15]. In order to utilize milder deprotection condition, several different strands of methyl phosphonate oligomers were synthesized using N-tBPA protected nucleoside methylphosphoramidites in acetonitrile. These were deprotected with concentrated ammonium-hydroxide ethanol mixture (3:1) or methanol saturated with ammonia gas for 15 min at 55°C or 2 h at RT. The oligomers were separated from the CPG support and lyophilized in a speed vac. Products were analyzed by reverse phase HPLC (fig 9) and in every synthesis, the full length product was the major peak. Similarly three different ribonucleotide chains containing 5-bromouridine and 4-thiothymidine were synthesized

using N-tBPA protected phosphoramidites. These were incubated in ethanol-ammonium hydroxide mixture (1:3) for 2 h at RT. After removal of ammonium hydroxide, the materials were subjected to TBAF solution in THF (1.0 M, 20 h) for the removal of the 2'-TBDMSi group. The crude material was purified by strong anion exchange column chromatography in good yield (M Gait, C Pritt, personal communication). Enzymatic digest of the purified material resulted in the five expected nucleosides in the appropriate ratio (fig 10).

Backbone modified oligonucleotide

In order to assess stability of N-tBPA protection towards treatment with Beaucage's [16], Reese's [17] reagents or with sulphur in carbon/disulphide-pyridine mixture, we also synthesized several polyphosphorothioated oligonucleotides using phosphoramidites and H-phosphonate monomers. After synthesis, the chain was cleaved from the support and N-deacylated using concentrated ammonium hydroxide at RT for 2 h. The products were analyzed by HPLC and ^{31}P -NMR. In each case these products were identical to those made from standard N-benzoyl or N-isobutyryl protected nucleoside monomers.



A) ANALYSIS OF CRUDE MATERIAL BY SAX
 B) ANALYSIS OF PURIFIED MATERIAL BY C-18
 C) ANALYSIS OF ENZYMES DIGEST OF PURIFIED MATERIAL, 5-METHYL-dC WAS ABSENT

Fig 10. HPLC profiles of a synthetic oligoribonucleotide containing 4-thiothymidine. **A.** Before purification. **B.** After purification. **C.** Enzymes digest (SVPD and ALK phosphatase).

Conclusion

The following conclusions were drawn: i) *t*-butylphenoxyacetyl (tBPA), is useful for exocyclic amine protection for A, G and even C nucleosides; ii) N-tBPA protection is stable towards most DNA/RNA synthesis reagents; iii) N-tBPA protected deoxyguanosine phosphoramidite is highly soluble in acetonitrile; iv) although acetic anhydride- and N-methylimidazole solutions cause displacement of tBPA to a certain extent by the acetyl group, use of tBPA anhydride in place of acetic anhydride prevents this problem; v) H-phosphonate DBU/TEA salts containing the t-BPA protecting group can be synthesized in high purity and good yield; vi) complete removal of the t-BPA protecting group with ammonia solution is achieved within 2 h at RT or 15 min at 55°C; vi) oligonucleo-

tides obtained using this labile protecting group possess 99% base integrity (by HPLC) and are also effective in biological studies; viii) rapid deprotection of the t-BPA group allows synthesis of oligoribonucleotides in good yield with minimal chain degradation; ix) t-BPA protected nucleosides facilitate syntheses of more labile oligonucleoside methylphosphonate diesters, other base sensitive oligonucleotides containing modified nucleosides, and polyphosphorothioate oligonucleotides.

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