Enzymatic and NMR analysis of oligoribonucleotides synthesized with 2'-tert-butyldimethylsilyl protected cyanoethylphosphoramidite monomers

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ABSTRACT

The regioisomeric integrity of the internucleotide phosphate linkage in synthetic RNA using 2'-tert-butyldimethylsilyl protection was examined using enzymatic and NMR techniques. Two sets of DNA-RNA hybrid nonamers, T_3XT_5 and T_5XT_3 (where X = rA, rC, rG and U) and the tetramer AGCU were analyzed. Enzyme catalyzed hydrolysis of the nonamers with ribonuclease T2 showed that the linkage at the ribonucleotide was the desired 3'-5'. A control nonamer with a 2'-5' linkage was subjected to the enzyme, and showed no cleavage. High-resolution proton NMR of the tetramer also gave a favorable comparison with the same molecule obtained by non-chemical means.

INTRODUCTION

Physical studies of RNA molecules require efficient methods for synthesis. Polymerization catalyzed by polynucleotide phosphorylase allowed production of a limited variety of oligomers for thermodynamic[1,2] and NMR analysis[3,4]. The development of techniques involving RNA ligase[5] and T7 RNA polymerase[6] greatly extend the variety of sequences available, but are most efficient for making oligomers longer than 10–15 nucleotides.

Solid phase chemical synthesis is the most promising approach for making milligram quantities of short oligoribonucleotides. Chemical synthesis of RNA has been slower to evolve than DNA because of the complications introduced by the necessity to reversibly block the 2'-OH group and the ease with which the deblocked RNA can be hydrolyzed by base or nucleases that commonly contaminate the preparations. Early work used solution phase or solid-phase supported synthesis with 2'-O-tetrahydropyranyl blocking groups[7,8], 2'-O-silyl groups[9,10], as well as a variety of others[11–15].

We wanted to prepare milligram quantities of RNA for NMR structural studies, using methods for RNA synthesis developed by Olgilvie[10]. First the disposition of the internucleotide phosphate linkage had to be better established, and then physical methods which would purify small RNA molecules adequately for high resolution NMR studies had to be developed.

The nonamer studies described in this work are specifically designed to analyze the presence of 2'-5' internucleotide linkages. The isocratic HPLC methods used by others to establish the correct internucleotide linkages[16] might not necessarily reveal unhydrolyzed oligoribonucleotides, which may contain the undesired linkages.

A detailed analytical study of synthetic RNA has been published, but the RNA was synthesized with 2'-tetrahydropyranyl protecting groups[8]. We wanted to establish the purity of products made with 2'-trialkylsilyl protection. This is the first study which uses a control of known 2'-5' internucleotide phosphate configuration using these protecting groups.

In addition, we wanted to establish physical methods for isolating short RNA molecules of 98% or greater purity. The present work indicates that these goals can be achieved.

MATERIALS AND METHODS

Reagents for Synthesis:

N^6benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tertbutyldimethylsilyladenosine-3'-O-N,N-diisopropylbetacyanoethylphosphoramidite (A RNA amidite), N^4benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tertbutyldimethyl-silylcytidine-3'-O-N,N-diisopropylbetacyanoethylphosphoramidite (C RNA Amidite), N^2benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tertbutyldimethylsilylguanosine-3'-O-N,N-diisopropylbetacyanoethylphosphoramidite (G RNA Amidite) and 5'-(4,4'-dimethoxytrityl)-2'-O-tertbutyl-dimethylsilyl uridine-3'-O-N, N-diisopropylbetacyanoethylphosphoramidite (U RNA Amidite) were made by first synthesizing the nucleoside monomers according to the literature[17] and converting these, after they were first checked for purity by proton NMR, to the betacyanoethylphosphoramidites[18] using published procedures for DNA. A small amount of N^6benzoyl-5'-O-(4,4'dimethoxy-trityl)-3'-O-tertbutyldimethylsilyl-adenosine-2'-O-N,N-diisopropylbetacyan oethyl-phosphoramidite (A RNA 2'amidite) was prepared using N^6 benzoyl-5'-O-(4,4'dimethoxytri-tyl)-3'-O-tertbutyldimethylsilyl adenosine formed in the synthetic reactions leading to A RNA amidite. The chemical shifts ($CDCl_3$, trimethylphosphate ref.) of the diastereomeric ^{31}P NMR signals for the RNA amidites

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were similar to those reported in the literature for the 5'(4-methoxy-trityl) methyl RNA phosphoramidites[16]. They were; A RNA amidite, 148.622, 146.699; A RNA 2'amidite, 147.960, 147.829; C RNA amidite, 147.746, 146.817; G RNA amidite, 149.934, 145.857; U RNA amidite, 147.438, 147.146. The amidites used for the tests described below were all over 98% pure by ^{31}P NMR. The support for solid phase supported synthesis was the same as that described in the literature[16], except that the unattached ribose hydroxyl functionality was capped with acetyl, rather than trialkylsilyl. Tetrabutyl ammonium fluoride (TBAF) was purchased from Aldrich, RNase T2 from Sigma and acetonitrile from J.T. Baker.

Oligomer synthesis:

All of the syntheses were done with one micromole of immobilized nucleoside on the support. The syntheses of mixed DNA/RNA oligomers and AGCU were carried out on MilliGen/Biosearch model 8700 Series DNA synthesizers. The concentration of amidites was 50 mg/ml in acetonitrile and the amidite consumption was 33 mg per coupling. The following generalized cycle for RNA synthesis was used:

Name of Step	*Duration*	*Reagents Used*
Wash	10 sec.	Acetonitrile
Deblock	60 sec.	3% Dichloroacetic acid in Methylene Chloride
Wash	35 sec.	Acetonitrile
Couple	14 min.	0.025 M RNA amidite, 0.33 M Tetrazole in Acetonitrile
Wash	10 sec.	Acetonitrile
Oxidize	10 sec.	0.015 M Iodine, 5.5 M water, 0.13 M pyridine in Tetrahydrofuran
Wash	25 sec.	Acetonitrile
Cap	10 sec.	0.3 M Pyridine, 0.3 M N-methylimidazole, 0.5 M Acetic Anhydride in Tetrahydrofuran
Wash	25 sec.	Acetonitrile

Purification

Gloves, sterile tubes and glassware were used during purification and handling. Aqueous solutions were prepared with HPLC water which was heated at 115°C for 2 hrs in an autoclave.

For the 9-mers, the last 5'-0-(4,4'-dimethoxytrityl) (DMT) was removed and the oligomer cleaved from the support by treatment with 1 ml of (3:1 NH_3/EtOH). After 1 hr, the ammonia solution was decanted from the support and heated at 55°C in a sealed tube for 16 hrs. The sample was dried *in vacuo* and treated with 0.5 ml TBAF (1M solution in THF) at room temperature for 12 hrs. The sample was then treated with 0.5 ml 1 M triethylammonium acetate buffer pH 7.5 (TEAA), dried to approximately 0.5 ml, and passed through a 2 by 23 cm Sephadex G-10 column, (40−120 micron particles (Sigma) eluted with 0.1 M ammonium acetate. Fractions were collected and assayed by U.V. absorbance at 260 nm, and those containing the RNA were dried, dissolved in approximately 0.2 ml of water and injected onto the prep HPLC system described below, collecting the 9-mer peak. The product bearing fractions were reduced to 0.5 mL *in vacuo* and desalted by passing through the G-10 column (as above). 1 mL fractions were collected, and those containing oligonucleotide were pooled and dried.

For the tetramer AGCU, the 5'-DMT group was left on, and the procedure was the same as above up to HPLC injection. The crude tetramer obtained from a one micromole synthesis was dissolved in 1 mL of 0.1 M TEAA and injected onto an 'OPC reverse phase cartridge' (Applied Biosystems) which had been prepared by rinsing with 10 mL of acetonitrile and 5 mL of 2 M TEAA. The sample was loaded on slowly, repeating the loading 2 additional times. Directionality of flow was maintained. The cartridge was rinsed with 5 ml of 0.1 M TEAA slowly (the flow rate was 1−2 drops/sec), followed by 15 ml of water. 5 mL of 2% TFA in water was pushed through, and the column was left for 3 min to detritylate the sample. The cartridge was rinsed with 15 mL of water, and the RNA was eluted with 5 mL of 20% Acetonitrile in water. 1 ml fractions were collected and assayed by U.V., and those containing the RNA were pooled and dried. The material obtained was then further purified by the prep. HPLC method described below.

RNase T2 Hydrolysis

0.25 A_{260} units of oligomer which had been purified by the method described above were hydrolyzed in 0.25 mL 0.05 M NaOAc, pH 4.5, 0.002 M EDTA, 100 u/mL RNase T2 by heating at 37°C for 15 min. The RNase T2 was removed by Sep-

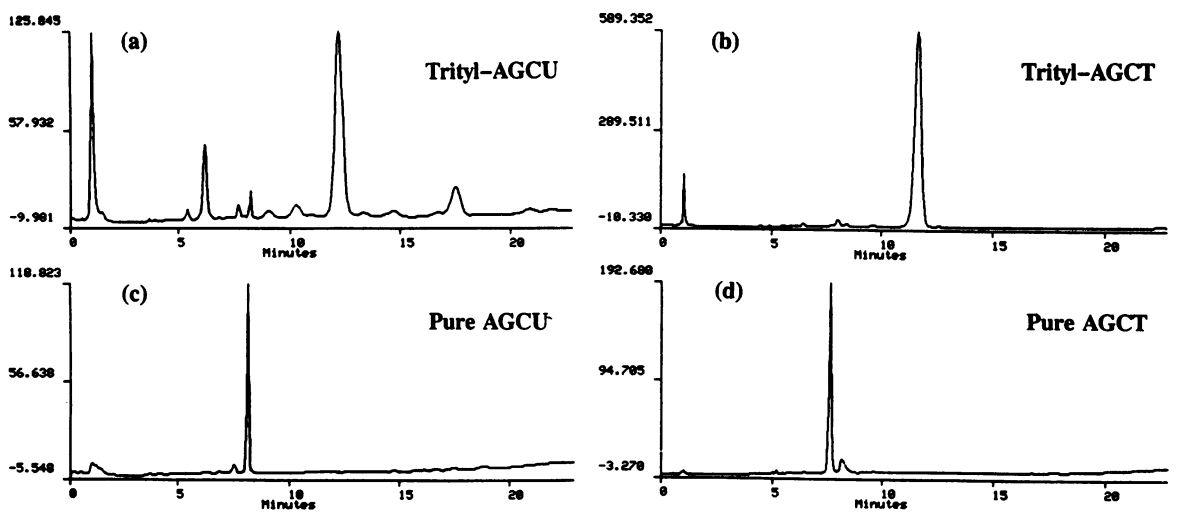

Figure 1. Analytical-scale HPLC of crude reaction mixtures for tritylated AGCU (a), and d(AGCT) (b); also (c,d) show traces for the oligomers after purification on an 'OPC' cartridge followed by preparative DEAE HPLC.

Table 1. Integration of the hydrolysis products after AX100 HPLC Analysis.

Sample	T_5AT_3	T_5CT_3	T_5GT_3	T_5UT_3
Peak	$T_5Ap\ T_3$	$T_5Cp\ T_3$	$T_5Gp\ T_3$	$T_5Up\ T_3$
*Ratio	1.08:1	0.97:1	1.04:1	1.20:1**
Factor	2.24	1.95	2.07	2.02
Sample	T_3AT_5	T_3CT_5	T_3GT_5	T_3UT_5
Peak	$T_3Ap\ T_5$	T_3Cp T5	$T_3Gp\ T_5$	$T_3Up\ T_5$
*Ratio	1.18:1	1.01:1	0.94:1	0.96:1
Factor	0.96	0.79	0.86	0.80

*Ratio = (Area 2 / Area 1)x(Factor) in which Area(1) is for the first eluting peak, Area(2) is for the second peak (see Figures 2 and 3). The factor is based on estimated molar absorbancies of the products based on the absorbancies of the dimer and mononucleotide units[19]. ** In this synthesis there was a substantial side-product peak eluting near T_5Up.

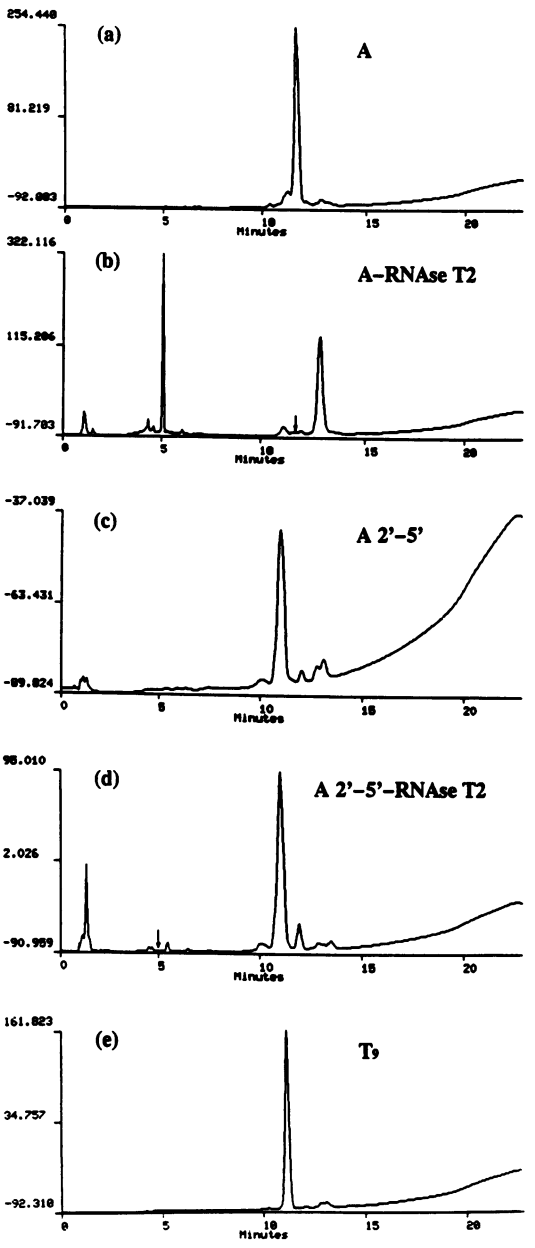

Figure 2. Analytical-scale HPLC of purified T_5XT_3 oligomers along with corresponding RNase T2 hydrolysates. X=A(a,b), 2'-5'A(c,d), and T(e). The small arrow in the hydrolysate traces is drawn at the retention time of the parent 9-mer, (except for (d) where the arrow indicates the position for T_3).

pak C18 (Waters Corp.) cartridge purification following this procedure: Four solutions were prepared: A = CH_3CN; B = 2% CH_3CN, 0.02M NH_4OAc; C = 1% CH_3CN, 0.01M NH_4HCO_3; D = 20% CH_3CN. The cartridge was attached to a 10 ml glass syringe and flushed with 5 ml A. It was then flushed with 5 ml B. The sample was diluted 1:1 with B and loaded on the cartridge. Next, 10 ml of C was washed through. Using a syringe uncontaminated by RNase, the sample was eluted from the other end of the cartridge with D, collecting 1 to 2 ml fractions. Fractions were combined according to A_{260} and reduced under vacuum for analytical HPLC injection.

HPLC system

The purification and analysis were carried out on a Waters HPLC instrument. The preparative-scale purifications were done on a Machery-Nagel Nucleogen-DEAE (diethlyaminoethyl) 60−7 10×125mm column with a 30 min linear gradient from 10 to 70% B (buffer A = 20% CH_3CN, 0.02M NaOAc/HOAc pH = 5.5, buffer B = A, but 1M LiCl), pumped at 4ml/min, approximately 750 psi. The analytical HPLC of oligomers was done on a Synchropak AX100 4.6×100mm column. The AX100 analysis was carried out with a 20 min linear gradient from 0 to 50% B at 1.5 ml/min (buffer A = 20% CH_3CN, 0.0032M phosphate, pH = 6.8; buffer B is the same as A except for 0.64M phosphate).

NMR spectrum of AGCU

The NMR sample was 0.1mM in AGCU, 0.1M NaCl, 0.01M phosphate buffer (pH 7), 0.1mMEDTA, and 0.01% NaN_3. The exchangeable protons were replaced by drying the sample 3x with glass-distilled 99.8% D_2O. The sample was then dissolved in 0.4ml 99.96% D_2O (Aldrich). The NMR spectrum was taken on a GE GN-500 at 41°C.

RESULTS AND DISCUSSION

Two sets of RNA-DNA hybrids were synthesized, T_5XT_3 and T_3XT_5, where X is rA, rC, rG and U. For the first set the DNA

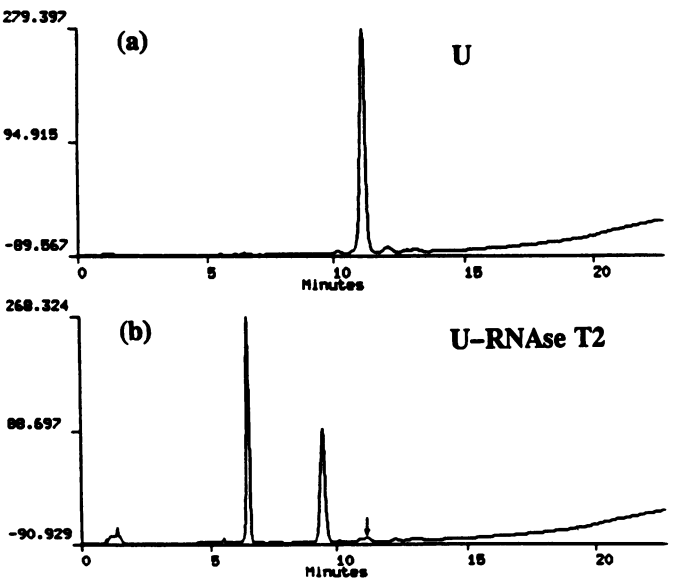

Figure 3. Analytical-scale HPLC of purified T_3UT_5 oligomer along with corresponding RNase T2 hydrolysate. The small arrow in (b) denote the retention time for the parent nonamer.

coupling protocol was used for the T parts of the sequences and the RNA coupling protocol for the incorporation of the RNA base, and for the second set the RNA synthesis program was used throughout. Finally, AGCU and d(AGCT) were prepared using the RNA coupling protocol.

When the 14 min coupling time was used for the incorporation of Thymidine instead of the 2 min coupling time, inferior results are obtained compared to when the short coupling time is used to incorporate T. The reason for this is unknown but similar observations have been noted by us for other mixed DNA/RNA sequences.

The recovery of the purified 9 mers was an average of 40% of the crude A_{260} units obtained. The average crude yield was 28 A_{260} units, and the average purified yield was 11 A_{260} units. The yield of the tetramer AGCU purified by 'OPC' and DEAE HPLC was only about 13%. We also purified d(AGCT) with the cartridge and HPLC, and found that the yield was only about 20%. Analytical-scale HPLC of the purified products indicates over 90% purity, in all cases. Four separate 1 micromole scale syntheses of the tetramer were done, with an average crude yield of 27.5 A_{260} units. The material from all four 'OPC' treatments was pooled and purified by prep HPLC. The final amount obtained was 14 A_{260} units.

Because the DNA tetramer also gives a low yield, the yields are probably due in part to the 'OPC' method and not the fact that we are purifying RNA. A significant amount of U.V. absorbing material, both aromatic compounds from the exocyclic amine deprotection and also failure strands is also lost during the Sephadex size exclusion chromatography. Because of the need to remove the TBAF, it is difficult to analyze the exact composition of the crude material produced, in contrast to DNA synthesis.

Figure 1 compares analytical-scale HPLC (AX100) plots of crude AGCU (a), and d(AGCT) (b), as well as the 'OPC' and DEAE-purified tetramers in panels (c,d). The HPLC plots (Figs. 2 and 3) show that pure products of the type T_5XT_3 and T_3XT_5 can be prepared.

RNase T2 specifically hydrolyzes 3'-5' linked oligomers where the 2'-OH is not blocked and does not cleave 2'-5' phosphodiesters. Plots of the RNase T2 hydrolysates are shown in Figs. 2 and 3. The peaks corresponding to the two hydrolysis products should occur in a ratio near 1:1 after correction for

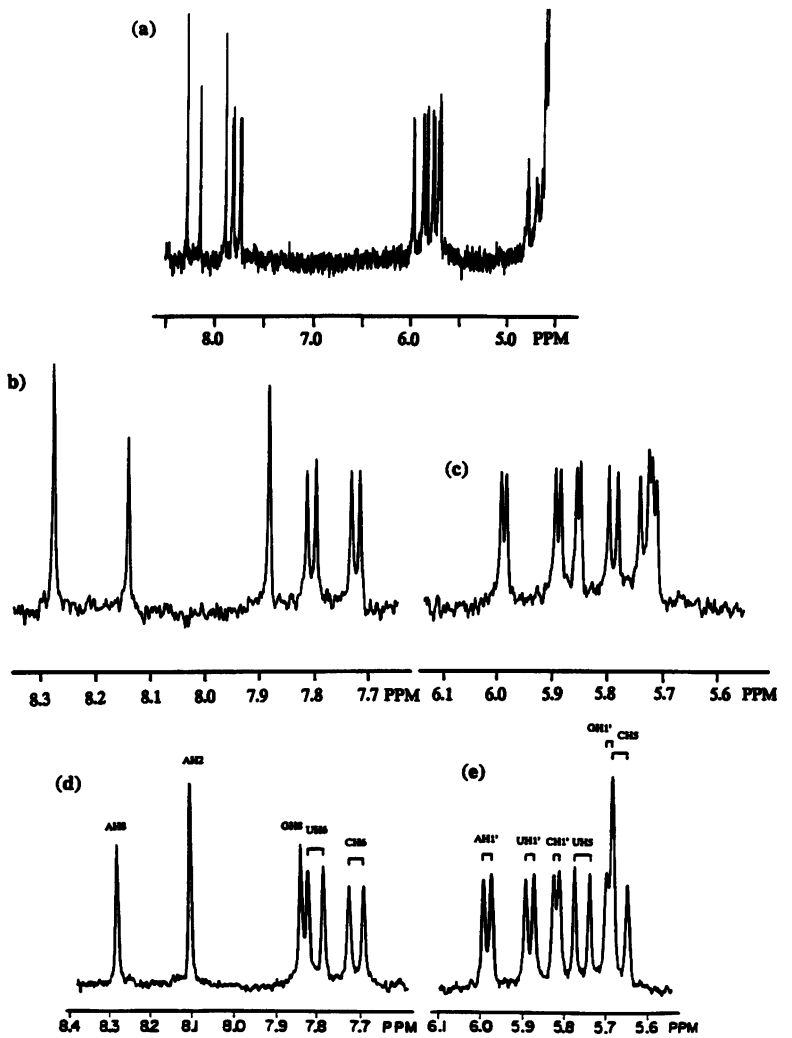

Figure 4. ^{1}H-NMR Spectrum of AGCU at 41°C; (a) the region downfield from HDO, in the 500 MHz spectrum, (b) expansion of the base H8, H6, H2 region, (c) expansion of the H1' and H5 region, (d,e) corresponding expansions from the 220 MHz spectrum[21] at 41°C.

extinction coefficients. The correlation is excellent in most cases(see table 1). The deviations that are seen probably arise from the method for estimating the extinction coefficients (subject to +/− 10% error)[19] and the presence of an impurity peak in the T_5UT_3 chromatogram (not shown). TTTTTA-(2′-5′)-TTT was also treated with RNase T2. Figure 2 (a,b) compares the RNase T2 susceptibility of $T_5A(3'-5')T_3$ with the 2′-5′ linked isomer (Figures 2 c,d). It appears that 100% of 2′-5′ linked monomer is RNase T2 resistant, as expected. The HPLC analysis with the high resolution AX100 column shows that T_5XT_3 and T_3XT_5 are >99% hydrolyzed at the XpT linkage, whereas T_3GT_5 and T_3UT_5 are >98% hydrolyzed. Figs 2 and 3 show representative chromatograms, with a T_9 control in fig. 2 e. These results demonstrate both the absence of 2′-5′ linkages and of 2′-O-silyl protecting groups.

Some other conclusions which can be drawn from this data are that the enzyme is performing properly and that there is no isomerization occurring during workup. Most important is the confirmation that regioisomerically pure amidites give the proper internucleotide linkage in the products.

The reason that the hydrolysate in some of the traces elutes later than the parent 9-mers is that the 3′ terminal phosphates on some of the fragments[20] have a stronger influence in the mobility of the fragment in the strong anion exchange (SAX) environment than internal phosphates would.

NMR Spectrum of AGCU

Figure 4 is the region downfield of HDO in the 500 mHz ^{1}H-NMR spectrum of AGCU. Figures 4b and c show expansions that can be compared with similar regions (Figures 4d,e) in the 220 MHz spectrum of AGCU prepared using polynucleotide phosphorylase[21]. The spectra are identical except for differences attributable to increased dispersion in the 500 MHz spectrum, and small changes in chemical shift that may be due to slightly different temperature, ionic conditions and strand concentration. Comparison of Figure 4a with the ^{1}H-NMR spectrum of A2′p5′G[22] shows that any A2′p5′G linkage is absent in r(AGCU) since there is no AH2′ quintet around 5.06 ppm. The NMR spectra present strong evidence that the chemical method can produce oligomers with the correct sequence and phosphodiester linkage.

Preparative Purification of RNA oligomers

In this work, we were primarily interested in demonstrating the fidelity of RNA synthesis using a 2′-silyl blocking group and standard solid-phase supported chemistry. However, efficient purification is an issue still to be resolved. Chou, et. al. (1989) report difficulties finding suitable ion exchange or reverse phase HPLC conditions for purification of RNA dodecamers prepared by a similar solid phase supported chemistry. They attributed the difficulties to aggregation of the RNA strands, and report efficient purification using a TLC (thin layer chromatography) technique. However, close examination of their published NMR data (e.g., see Figure 9, Chou et. al [23]) reveals substantial amounts of some impurity resonances visible as extra peaks at about the 5−10% level and broad, unresolved disturbances near the baseline in what ought to be blank regions of the spectrum.

Our studies show that the T_5XT_3 and T_3XT_5 oligomers could be purified with a single chromatographic step (final trityl removed on the synthesizer, followed by deblocking and DEAE 60−7 HPLC). This preparative procedure fails for AGCU. Extra peaks are seen in the 500 MHz NMR spectrum and upon rechromatography on an analytical-scale AX100 column (data not shown). However, use of the 'OPC' cartridge method for removal of untritylated failure chains, combined with a DEAE 60−7 step and G-10 desalting provides a method for isolating pure AGCU. However, recovery is only about 13% for tritylated AGCU and 20% for d(AGCT) from the OPC cartridge followed by HPLC. Thus, the 'OPC' / ion exchange method is adequate for preparing small quantities of pure RNA, but not sufficient as a general procedure for preparing multi-milligram amounts for NMR structure analysis.

Furthermore, the need for removal of failure sequences by HPLC after the reverse phase 'OPC' purification suggests that some cleavage of the tetramer has occurred during workup. DMT positive failure chains could also be caused by either inefficient capping during the synthesis cycle, or partial hydrolysis by unwanted RNases.

It has been established that ammonia treatment of 2′-O-silyl protected ribonucleotides results in a small amount of de-silylation followed by strand cleavage[24]. This is an important limitation of this chemistry which may restrict its ability to make long sequences.

SUMMARY AND FUTURE PROSPECTS

We have demonstrated that RNA with the correct 3′-5′ internucleotide phosphate linkages can be prepared on solid supports using commercially available betacyanoethyl phosphoramidites having a 2′-tertbutyl silyl blocking group. The 500 mHz ^{1}H spectrum of a synthetic tetramer shows its high purity and identity with enzymatically produced material.

We have established an analytical technique, using enzymatic hydrolysis studies, which confirms the integrity of the RNA synthesized with 2′-O-tertbutyldimethylsilyl protection. We are looking at improving the physical methods for purification of short RNA strands, as well as establishing reliable methods for the synthesis of longer (20 + base) oligoribonucleotides.

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