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Synthesis, deprotection, analysis and purification of RNA and ribozymes

Francine Wincott, Anthony DiRenzo, Chris Shaffer, Susan Grimm, Danuta Tracz, Christopher Workman, David Sweedler, Carolyn Gonzalez, Stephen Scaringe[1] and Nassim Usman*

Department of Chemistry and Biochemistry, Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, CO 80301, USA and [1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

Improvements in the synthesis, deprotection and purification of oligoribonucleotides are described. These advances allow for reduced synthesis and deprotection times, while improving product yield. Coupling times are reduced by half using 5-ethylthio-1*H*-tetrazole (*S*-ethyltetrazole) as the activator. Base and 2′-*O*-t-butyldimethylsilyl deprotection with methylamine (MA) and anhydrous triethylamine/hydrogen fluoride in *N*-methylpyrrolidinone (TEA·HF/NMP), respectively, requires a fraction of the time necessitated by current standard methods. In addition, the ease of oligoribonucleotide purification and analysis have been significantly enhanced using anion exchange chromatography. These new methods improve the yield and quality of the oligoribonucleotides synthesized. Hammerhead ribozymes synthesized utilizing the described methods exhibited no diminution in catalytic activity.

INTRODUCTION

The discovery of ribozymes (1) and the general interest in the structural elements of RNA (2) have engendered a need for larger amounts and numbers of oligoribonucleotides. This increased demand has illustrated that recent advances in the biological applications of nucleic acids has not been accompanied by equal improvements in the chemical arena. Progress has been made in improving methods for DNA synthesis that have enabled the production of large amounts of antisense oligonucleotides for structural and therapeutic applications. On the other hand, large quantities of RNA have been more difficult to obtain using present methods.

Generally, RNA is synthesized and purified by the same methodologies used to produce DNA: tetrazole to activate the amidite, ammonium hydroxide in ethanol (NH_4OH/EtOH) (3) to remove the exocyclic amino protecting groups and gel or HPLC (4–8) purification and analysis of the deprotected oligonucleotide. For the synthesis of RNA an additional step of tetra-*n*-butylammonium fluoride (TBAF) (3,9) to remove the 2′-*O*-t-butyldimethylsilyl (TBDMS) protecting groups is required. Although RNA can be produced using this process, these steps have not been completely optimized. As a rule, additional time is required for all phases of RNA synthesis. Activation of the RNA amidites is less efficient, thus requiring additional coupling time that can increase the formation of side products. Base deprotection with NH_4OH is quite often destructive. Desilylation with TBAF is very sensitive to water (10) and produces salts that must be removed prior to analysis. Although triethylamine trihydrogen fluoride (TEA·3HF) has been used as an alternative to TBAF (11,12), in our hands use of this reagent results in degradation of some oligoribonucleotides and a reduction in yield (D.Sweedler and S.Scaringe, unpublished results).

Given the overall problems associated with the synthesis of RNA, purification techniques for larger (>0.5 mg) amounts have not been developed. Gel electrophoresis has been widely used, however, it is a very low capacity procedure for purification and is not reproducible as an analytical tool. HPLC has not been used as a single purification step for RNA, except for short oligomers (4–8).

We have determined that the synthesis of long [>30 nucleotides (nt)] RNA in high yield and quantity is dependent upon certain critical steps used during its preparation. Specifically, we show that RNA phosphoramidite coupling efficiency is dependant on activator, activator concentration and time. The use of improved deprotection reagents for both the exocyclic amine and 2′-*O*-TBDMS hydroxyl protecting groups increase both the yield and recovery of the synthetic RNA.

MATERIALS AND METHODS

General

Automated small scale (2.5 μmol) RNA syntheses were carried out on an ABI 394 DNA/RNA synthesizer. Columns (2.5 μmol scale) and empty OPC columns were obtained from ABI. Automated large scale ($\geq$25 μmol) RNA syntheses were carried out on an ABI 390Z DNA/RNA synthesizer. Derivatized aminomethyl polystyrene (ABI) was used as the solid support. RNA phosphoramidites, 5′-*O*-DMT-N^6-(phenoxyacetyl)-2′-*O*-TBDMS-adenosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite, 5′-*O*-DMT-

* To whom correspondence should be addressed

N^2-(isopropylphenoxyacetyl)-2′-*O*-TBDMS-guanosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite, 5′-*O*-DMT-N^4-(acetyl)-2′-*O*-TBDMS-cytidine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite and 5′-*O*-DMT- 2′-*O*-TBDMS-uridine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite were purchased from Pharmacia. 2′-*O*-Methylphosphoramidites, 5′-*O*-DMT-N^6-(t-butylphenoxyacetyl)-2′-*O*-methyl-adenosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite, 5′-*O*-DMT-N^2-(t-butylphenoxyacetyl)-2′-*O*-methyl-guanosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite, 5′-*O*-DMT-N^4-(t-butylphenoxyacetyl)-2′-*O*-methyl-cytidine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phos phoramidite and 5′-*O*-DMT-2′-*O*-methyl-uridine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropylamino) phosphoramidite were purchased from PerSeptive Biosearch. The 1 M TBAF, TEA·3HF, *N*-methylpyrrolidinone and 40% aqueous MA were purchased from Aldrich, NH_4OH was purchased from Baker and $NaClO_4$ was purchased from Fluka. All analytical HPLC analyses were performed on a Hewlett Packard 1090 HPLC with a Dionex NucleoPac® PA-100 column, 4 × 250 mm, at 50°C. Flow rate was 1.5 ml/min (buffer, A 20 mM $NaClO_4$; buffer B, 300 mM $NaClO_4$) and the gradient was as follows: 0.00 min, 0.0% buffer B; 5.00 min, 38.0% buffer B; 20.00 min, 73.0% buffer B; 21.00 min, 100.0% buffer B; 23.00 min, 100.0% buffer B; 24.00 min, 0.0% buffer B.

Synthesis of RNA and ribozymes using 5-ethylthio-1*H*-tetrazole as activating agent

The general procedures for RNA synthesis have been described previously (3,9). Small scale syntheses were conducted on a 394 (ABI) synthesizer using a modified 2.5 µmol scale protocol with a 5 min coupling step for 2′-*O*-TBDMS-protected nucleotides and 2.5 min coupling step for 2′-*O*-methylated nucleotides. Table 1 outlines the amounts and the contact times of the reagents used in the synthesis cycle. A 6.5-fold excess (163 µl, 0.1 M = 16.3 µmol) of phosphoramidite and a 24-fold excess of *S*-ethyltetrazole (238 µl, 0.25 M = 59.5 µmol) relative to polymer-bound 5′-hydroxyl was used in each coupling cycle. Average coupling yields on the 394, determined by colorimetric quantitation of the trityl fractions, were 97.5–99%. Other oligonucleotide synthesis reagents for the 394: detritylation solution, 2% TCA in methylene chloride (ABI); capping reagent, 16% *N*-methylimidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution, 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Biosearch). B and J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyltetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical Inc.

Large scale syntheses were conducted on a modified (eight amidite port capacity) 390Z (ABI) synthesizer using a modified 25 µmol scale protocol with a 10–15 min coupling step for alkylsilyl-protected RNA and a 5–15 min coupling step for 2′-*O*-methylated RNA (Table 3). A 6-fold excess (1.5 ml, 0.1 M = 150 µmol) of phosphoramidite and a 45-fold excess of *S*-ethyltetrazole (2.25 ml, 0.5 M = 1125 µmol) relative to polymer-bound 5′-hydroxyl was used in each coupling cycle. Average coupling yields on the 390Z, determined by colorimetric quantitation of the trityl fractions, was 97.5–99%. Oligonucleotide synthesis reagents for the 390Z: detritylation solution, 2% DCA in methylene chloride (ABI); capping reagent, 16% *N*-methylimidazole in THF and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution, 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Biosearch). B and J Synthesis Grade acetonitrile was used from a 200 l tank. *S*-Ethyltetrazole solution (0.5 M in acetonitrile) was made up from the solid obtained from American International Chemical Inc.

Table 1. 2.5 µmol synthesis cycle

Reagent	Equivalents	Amount (µl)	Wait time[a] (s)
Phosphoramidites	6.5	163	300/150
S-Ethyltetrazole	23.8	238	300/150
Acetic anhydride	100	233	5
N-Methylimidazole	186	233	5
TCA	83.2	1.73×10^3	21
Iodine	8.0	1.18×10^3	45
Acetonitrile	NA	6.67×10^3	NA

[a]Wait time does not include contact time during delivery. Where two coupling times are indicated the first refers to RNA coupling and the second to 2′-*O*-Me coupling.

RNA and ribozyme deprotection of exocyclic amino protecting groups using methylamine (MA) or NH_4OH/methylamine (AMA)

The polymer-bound oligoribonucleotide was transferred from the synthesis column to a 4 ml screw top glass vial and was suspended in either a solution of methylamine (MA) or NH_4OH/MA 1:1 (AMA) at 65°C for 10 min to remove the exocyclic amino protecting groups. After cooling to –20°C the supernatant was removed from the polymer support. The support was washed three times with 1.0 ml EtOH/MeCN/H_2O 3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

Trityl-off RNA and ribozyme deprotection of 2′-*O*-silyl groups using anhydrous TEA·3HF

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA·HF/NMP solution (250 µl of a solution of 1.5 ml *N*-methylpyrrolidinone, 750 µl TEA and 1.0 ml TEA·3HF to provide a 1.4 M HF concentration) and heated to 65°C for 1.5 h. The resulting fully deprotected oligomer was either precipitated directly from the desilylation reaction or quenched with 50 mM TEAB (9 ml) prior to anion exchange desalting.

The precipitation protocol required the addition of 3 M NaOAc (25 µl) followed by addition of *n*-BuOH (1 ml). The mixture was cooled to –70°C for 1 h and then centrifuged at 4°C, 10 000 *g* for 30 min. The solution was decanted, the pellet washed with 70% EtOH and then dried.

For anion exchange desalting of the deprotected oligomer the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) pre-washed with 50 mM TEAB (10 ml). After washing the loaded cartridge with 50 mM TEAB (10 ml) the RNA was eluted with 2 M TEAB (10 ml) and dried to a white powder (9).

Trityl-on RNA and ribozyme deprotection of 2′-*O*-silyl protecting groups using anhydrous TEA·3HF

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA·HF/NMP solution (250 µl of a solution of 1.5 ml *N*-methylpyrrolidine, 750 µl TEA and 1.0 ml TEA·3HF to provide a 1.4 M HF concentration) and heated to 65°C for 1.5 h. The reaction was quenched with 1.5 M ammonium bicarbonate (NH_4HCO_3) (9 ml). The resulting solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) and pre-washed with 16 mM NH_4HCO_3 (10 ml). After washing the cartridge with 16 mM NH_4HCO_3 (10 ml) the RNA was eluted with 1.5 M NH_4HCO_3 (10 ml) and dried to a white powder.

RNA and ribozyme purification

For a small scale (2.5 µmol) synthesis, the crude material was diluted to 5 ml with RNase-free water. The sample was injected onto either a Pharmacia Mono Q® 16/10 mm or Dionex NucleoPac® PA-100, 22 × 250 mm, column with 100% buffer A (10 mM $NaClO_4$). A gradient of 180–210 mM $NaClO_4$ at a rate of 8 ml/min for a Pharmacia Mono Q® anion exchange column or 100–150 mM $NaClO_4$ at a rate of 15 ml/min for a Dionex NucleoPac® anion exchange column was used to elute the RNA. Fractions were analyzed by HPLC and those containing full-length product ≥ 80% by peak area were pooled for desalting. The pooled fractions were applied to a SepPak cartridge (C_{18}) pre-washed successively with CH_3CN (10 ml), CH_3CN/MeOH/H_2O 1:1:1 (10 ml) and RNase-free H_2O (20 ml). Following sample application, the cartridge was washed with RNase-free H_2O (10 ml) to remove the salt. Product was then eluted from the column with CH_3CN/MeOH/H_2O 1:1:1 (10 ml) and dried.

For trityl-off large scale (≥25 µmol) synthesis the crude material was desalted by applying the solution that resulted from quenching of the desilylation reaction to a Pharmacia HiLoad 26/10 mm Q-Sepharose® Fast Flow column. The column was thoroughly washed with 10 mM $NaClO_4$ buffer. The oligoribonucleotide was step eluted from the column with 300 mM $NaClO_4$. This step replaces the anion exchange desalting in the small scale synthesis. The eluent was quantitated and an analytical HPLC was run to determine the percent full-length material in the synthesis. The eluent was diluted 4-fold in RNase-free H_2O to lower the salt concentration and applied to a Pharmacia Mono Q® 16/10 mm column. A gradient of 10–185 mM $NaClO_4$ was run over 4 column volumes (CVs) to elute shorter sequences, the full-length product was then eluted in a gradient of 185–214 mM $NaClO_4$ in 30 CVs. The fractions of interest were analyzed by HPLC and fractions containing >85% full-length material were pooled. The pool was applied to a Pharmacia Source RPC® 16/10 mm column for desalting. The column was washed with RNase-free H_2O to remove the salt, monitored by conductivity, at which time product began to elute. A gradient of 0–50% MeOH in 2 CVs completed the desalting process. The solution was then dried.

For trityl-on large scale (≥25 µmol) synthesis the crude material was desalted by applying the solution that resulted from quenching of the desilylation reaction to a Pharmacia HiLoad 26/10 Q-Sepharose® Fast Flow column. The column was thoroughly washed with 20 mM NH_4CO_3H/10% CH_3CN buffer. The oligoribonucleotide was step eluted from the column with 1.5 M NH_4CO_3H/10% acetonitrile. The eluent was quantitated and an analytical HPLC was run to determine the percent full-length material present in the synthesis. The oligonucleotide was then applied to a Pharmacia Source RPC 16/10 column. A gradient of 20–55% buffer B (buffer A, 20 mM NH_4CO_3H/10% CH_3CN; buffer B, 20 mM NH_4CO_3H/25% CH_3CN) was run over 35 CVs. The fractions of interest were analyzed by HPLC and those fractions containing >60% full-length material were pooled. The pooled fractions were then submitted to manual detritylation with 80% acetic acid, dried down immediately, resuspended in RNase-free H_2O, dried down and resuspended in H_2O again. This material was analyzed by HPLC. The material was then purified by anion exchange chromatography as in the trityl-off scheme (*vide supra*).

Ribozyme activity assay

Ribozymes and 5′-^{32}P-labeled substrate were heated separately in reaction buffer (50 mM Tris–HCl, pH 7.5/10 mM $MgCl_2$) to 95°C for 2 min, quenched on ice and equilibrated to 37°C prior to starting the reactions. Reactions were carried out in enzyme excess and were started by mixing ~1 nM substrate and 40 nM ribozyme to a final volume of 50 µl. Aliquots of 5 µl were removed at 1, 5, 15, 30, 60 and 120 min, quenched in formamide loading buffer and loaded onto 15% polyacrylamide–8 M urea gels. The fraction of substrate and product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics PhosphorImager®. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining versus time using a double exponential curve fit (Kaleidagraph, Synergy Software).

RESULTS AND DISCUSSION

Analytical HPLC

To accurately assess the success or failure of a new synthetic or deprotection protocol, a standard assay system must be developed. We have found that analysis of the crude synthesis by anion exchange HPLC and spectrophotometric quantitation provide the most accurate measurement of the amount of full-length product generated. A Dionex NucleoPac® PA-100 column, 4 × 250 mm, at 50°C, used with $NaClO_4$ buffers, gave optimal resolution. Figure 1 shows a typical chromatogram of a 2.5 µmol scale 37mer, ribozyme **1**, synthesized and deprotected according to the methods described herein. Spectrophotometric analysis indicated a crude yield of 422 AU. Integration by area provides a value of 42% (177 AU) for the full-length product. The synthetic improvements did not have an appreciable effect on the overall crude yield of the syntheses; they did however, result in greater amounts of full-length product. This increase in full-length product yield also improved the efficiency of purification, resulting in a higher recovery of purified product (*vide infra*).

Synthesis

In the case of chemical RNA synthesis the use of tetrazole as an activator of RNA phosphoramidites is known (3,9). The tetrazole reacts with incoming amidite to produce a reactive intermediate that couples to the 5′-hydroxyl of the growing polymer chain. In previous reports a 0.5 M solution of tetrazole was allowed to react with the RNA phosphoramidite and couple with the polymer-bound 5′-hydroxyl group for 10 min (9). Unfortunately, this reaction is not ideal for stepwise synthesis. Changes in concentration and or coupling time only result in additional side products, not

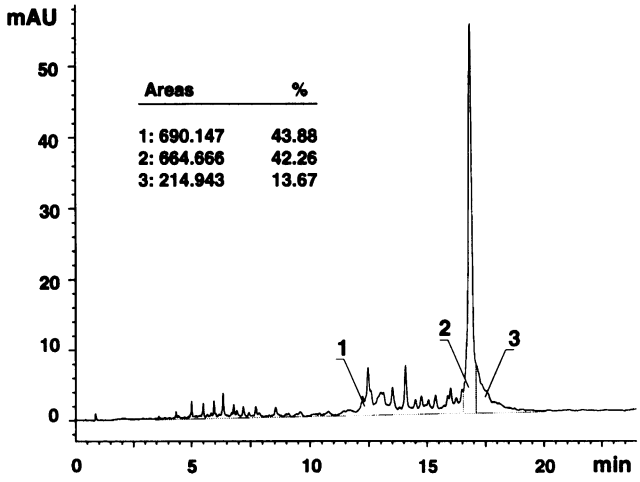

Figure 1. Chromatogram of crude ribozyme **1**, analyzed on a Hewlett Packard 1090 HPLC with a Dionex NucleoPac® PA-100 column at 50°C using $NaClO_4$ buffers. Area 2 represents the full-length 37mer. Rz **1**, ucu cca ucu GAu Gag gcc gaa agg ccG aaA auc ccu T. Lower case, 2′-*O*-Me; T, 3′-3′ thymidine.

increased coupling yields. The use of lower alkyl-substituted phosphoramidites, such as diethylamino instead of diisopropylamino, has been reported to improve coupling yields in RNA synthesis when measured by dimethoxytrityl cation quantitation (13). However, these compounds have not been used extensively due to their instability.

Recently the use of substituted tetrazoles (14) as activators for DNA and RNA synthesis has been reported (15–17). *S*-Ethyltetrazole has been found to be a more effective activator, due to its higher solubility properties and greater acidity (17). We tested this reagent by synthesizing a variety of ribozymes and homopolymers on both small (2.5 μmol) and large (25 μmol) scales. The oligomers were synthesized with both tetrazole and *S*-ethyltetrazole. All syntheses referred to in this manuscript were carried out on a highly cross-linked polystyrene support (18), since we found this to be the optimal matrix for RNA synthesis. The results of these experiments are shown in Table 2. Ribozyme **2** is a 36mer ribozyme consisting of all RNA nucleotides. Ribozyme **3** has the same sequence as ribozyme **2**, but 10 of the ribose residues have been replaced with 2′-*O*-Me residues. Oligomers were synthesized using standard conditions (see Materials and Methods), varying only the activator and the coupling times. In each case, oligonucleotides synthesized with *S*-ethyltetrazole gave greater full-length product than the polymer synthesized with tetrazole. This trend was corroborated regardless of the scale of synthesis. In addition, *S*-ethyltetrazole was used at a lower concentration, 0.25 M, and for shorter coupling times, 5 min, than recently reported (17).

On a small scale, 1.0–2.5 μmol, this activator can be used at 0.25 M concentration. Within the framework of our cycle (Table 1) 24 equivalents (eq.) of *S*-ethyltetrazole were used in each coupling step. The activator was mixed with 6.5 eq. amidite to provide a 3.65:1 ratio of activator to amidite. These changes did not result in a diminution of full-length product, rather, the same or a slightly better result was achieved. For a large scale synthesis, 25–100 μmol, the effective or final concentration of the activator was more significant than the initial concentration of the solution. An effective concentration of 0.35 M for activation of the RNA amidite during the coupling step was optimal. As in the small scale syntheses, coupling times could be reduced, in this case to 10 min, for RNA amidites without a loss in full-length product.

Table 2. Coupling optimization

Sequence	Scale (μmol)	Activator [added/final] (M)	Amidite [added/final] (M)	Time[a] (min)	Percent full-length product
Rz **2** (36mer)	2.5	T [0.5/0.28]	[0.1/0.04]	5	21
Rz **2** (36mer)	2.5	S [0.25/0.14]	[0.1/0.04]	5	31
Rz **3** (36mer)	2.5	T [0.5/0.28]	[0.1/0.04]	10/5	29
Rz **3** (36mer)	2.5	S [0.25/0.14]	[0.1/0.04]	5/2.5	30
Rz **3** (36mer)	2.5	S [0.25/0.14]	[0.1/0.04]	5/2.5	30
A_9T	25	T [0.50/0.33]	[0.1/0.02]	15	85
A_9T	25	S [0.25/0.17]	[0.1/0.02]	15	89
$(GGU)_3GGT$	25	T [0.50/0.33]	[0.1/0.02]	15	78
$(GGU)_3GGT$	25	S [0.25/0.17]	[0.1/0.02]	15	81
C_9T	25	T [0.50/0.33]	[0.1/0.02]	15	90
C_9T	25	S [0.25/0.17]	[0.1/0.02]	15	97
U_9T	25	T [0.50/0.33]	[0.1/0.02]	15	80
U_9T	25	S [0.25/0.17]	[0.1/0.02]	15	85
Rz **3** (36mer)	25	T [0.50/0.33]	[0.1/0.02]	15/15	21
Rz **3** (36mer)	25	S [0.25/0.17]	[0.1/0.02]	15/15	25
Rz **3** (36mer)	25	S [0.50/0.24]	[0.1/0.03]	15/15	25
Rz **3** (36mer)	25	S [0.50/0.18]	[0.1/0.05]	15/15	38
Rz **3** (36mer)	25	S [0.50/0.18]	[0.1/0.05]	10/5	42

[a]Where two coupling times are indicated the first refers to RNA coupling and the second to 2′-*O*-Me coupling. S, *S*-ethyltetrazole, T, tetrazole activator. Rz **2**, UCU CCA UCU GAU GAG GCC GAA AGG CCG AAA AUC CCU. Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Lower case, 2′-*O*-Me.

Base deprotection

To obtain a correct synthesis in terms of yield and biochemical activity of a large RNA molecule (*i.e.* 30–40 nt), the protection of the amino functions of the bases requires an acyl protecting group. These groups must be, on the one hand, stable enough to survive the conditions of synthesis and, on the other hand, easily cleaved at the end of the synthesis. These requirements are traditionally met by the acyl protecting groups; benzoyl for adenosine, isobutyryl or benzoyl for cytidine and isobutyryl for guanosine, which may be removed at the end of the synthesis by incubating the ribozyme with either NH_4OH/EtOH 3:1 (3) or NH_3/EtOH (9) for ~20 h at 55–65°C.

We, and others (11,19–21), have found that the more labile phenoxyacetyl-type protecting groups on guanosine and adenosine and acetyl protecting groups on cytidine are superior to the standard acyl protecting groups. Phenoxyacetyl and acetyl protecting groups require an incubation in NH_4OH/EtOH 3:1 for 4 h at 65°C to acheive complete removal. These groups are completely stable under the conditions of oligonucleotide synthesis (21).

It has been reported (22) that AMA at 65°C fully deprotects oligodeoxyribonucleotides in 10 min if acetyl is used as the protecting group for cytidine. This mixture, and MA alone, were examined for their efficacy as base deprotection reagents for RNA polymers. Table 3 summarizes the results. A homopolymer of C, a GGU heteropolymer and two ribozymes were synthesized on a 25 μmol scale. Following synthesis, multiple aliquots of 100 mg of polymer-bound oligomer were subjected to treatment with one of three reagents; the standard deprotecting agent NH_4OH/EtOH 3:1 at 65°C for 4 h, MA at 65°C for 10 min or AMA at 65°C for 10 min. Base deprotection was followed by desilylation using TBAF at room temperature for 24 h. In all cases treatment with MA alone gave greater full-length product than all other methods tested.

The oligonucleotides were subjected to base compositional analysis to determine if treatment with MA caused modification of the bases. Upon digestion and subsequent HPLC analysis no mutations were detected (data not shown). Thus these results indicate that MA provides a highly efficient method for deprotecting RNA. The reagent requires minimal incubation time, is fully compatible with oligoribonucleotides, causes no premature removal of the silyl protecting groups and does not result in base modification (22).

2′-*O*-Silyl deprotection

The second step of the deprotection of RNA molecules, removal of the 2′-*O*-alkylsilyl protecting group, is usually accomplished using TBAF for 8–24 h (3,9). Unfortunately, the use of this deprotecting agent produces salts which must be removed prior to analysis and purification. In addition, the long exposure time required for complete removal of the protecting group, coupled with the reagent's sensitivity to adventitious water (10), makes it a less than ideal reagent. Neat TEA·3HF has been used to desilylate oligoribonucleotides at room temperature in 16 h (11). A more recent paper (12) reported that neat TEA·3HF fully deprotects a $(Up)_{20}U$ in 1 h, even with the addition of ~5.8% water (w/w).

In our hands, neat TEA·3HF was not a viable deprotection reagent, resulting in degradation of full-length product. We investigated several mixtures of aqueous HF in the presence of TEA and NMP at elevated temperatures and found that complete deprotection of the oligomer was effected only after 24 h exposure. We therefore prepared an anhydrous reagent of TEA·3HF in NMP with TEA. This reagent gave better results in terms of yield, full-length product and reaction time. The data for all subsequent experiments is shown in Table 4. Homopolymers of A, C and U, a GGU heteropolymer and two ribozymes were synthesized on a 25 μmol scale. Aliquots of 100 mg of polymer-bound oligomer were base deprotected with NH_4OH/EtOH 3:1 at 65°C for 4 h. After drying the samples were subjected to desilylation with either 1 M TBAF at room temperature or a mixture of TEA·3HF in NMP with TEA at 65°C. Aliquots were removed from the TEA·HF/NMP samples at 0.5, 1.5, 2, 8 and 24 h and analyzed by HPLC. All the samples were fully deprotected after 0.5 h. No degradation was apparent at 8 h, however, a small amount of breakdown could be observed at the 24 h time point. For longer oligomers 1.5 h was found to be the optimal reaction time.

The use of anhydrous TEA·HF/NMP for 0.5–1.5 h at 65°C gives equivalent or better results than TBAF. In all cases the TEA·HF/NMP solution gave the same or a greater amount of full-length product in 1/16th the time. In addition, the resulting oligomer can be isolated by anion exchange desalting or by direct precipitation from the deprotection mixture using 3 M sodium acetate and butanol (see Materials and Methods).

Table 3. Base deprotection

Sequence	Deprotection reagent	Time	*T* (°C)	Percent full-length product
$(GGU)_4$	NH_4OH/EtOH	4 h	65	50
	MA	10 min	65	70
	AMA	10 min	65	65
C_9U	NH_4OH/EtOH	4 h	65	75
	MA	10 min	65	80
	AMA	10 min	65	77
Rz **3** (36mer)	NH_4OH/EtOH	4 h	65	30
	MA	10 min	65	35
Rz **1** (37mer)	NH_4OH/EtOH	4 h	65	36
	MA	10 min	65	41

Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Rz **1**, ucu cca ucu GAu Gag gcc gaa agg ccG aaA auc ccu T. Lower case, 2′-*O*-Me; T, 3′-3′ thymidine.

Table 4. 2′-*O*-alkylsilyl deprotection

Sequence	Deprotection reagent	Time	*T* (°C)	Percent full-length product
A_9T	TBAF	24 h	20	85
	TEA·HF/NMP[a]	30 min	65	81
$(GGU)_4$	TBAF	24 h	20	65
	TEA·HF/NMP	30 min	65	70
C_{10}	TBAF	24 h	20	86
	TEA·HF/NMP	30 min	65	89
U_{10}	TBAF	24 h	20	82
	TEA·HF/NMP	30 min	65	85
Rz **2** (36mer)	TBAF	24 h	20	31
	TEA·HF/NMP	1.5 h	65	34
Rz **3** (36mer)	TBAF	24 h	20	33
	TEA·HF/NMP	1.5 h	65	36

[a]HF concentration 1.4 M.

Rz **2**, UCU CCA UCU GAU GAG GCC GAA AGG CCG AAA AUC CCU. Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Lower case, 2′-*O*-Me.

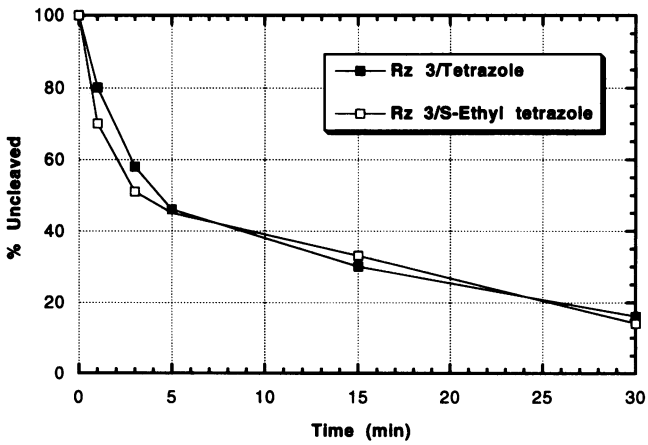

Figure 2. Comparative cleavage activity of ribozyme **3** synthesized using tetrazole (■) or *S*-ethyltetrazole (□). Both oligomers were base deprotected with NH_4OH/EtOH 3:1, desilylated with TBAF and gel purified. Assays were carried out in ribozyme excess. Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Lower case, 2′-*O*-Me.

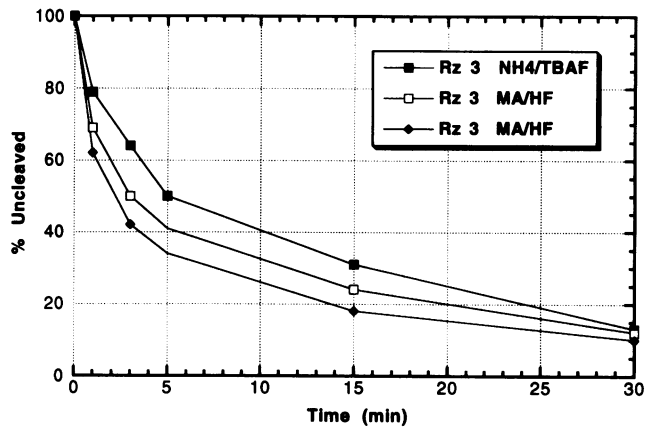

Figure 3. Comparative cleavage activity of ribozyme **3** deprotected using the standard and new methods. One ribozyme was base deprotected with NH_4OH/EtOH 3:1 followed by desilylation with TBAF (■). The two remaining ribozymes were base deprotected with MA followed by desilylation with anhydrous TEA·HF/NMP (□, ◆). All samples were HPLC purified. Assays were carried out in ribozyme excess. Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Lower case, 2′-*O*-Me.

Catalytic activity

Following development of these new protocols for the synthesis and deprotection of oligoribonucleotides it was necessary to ascertain the effect these changes might have on the catalytic activity of our ribozymes. The first experiment assayed the effect of coupling with *S*-ethyltetrazole as the activator. Ribozyme **3** was synthesized using the old (tetrazole) coupling strategy and the new (*S*-ethyltetrazole) protocol. Both ribozymes were deprotected under the standard conditions using NH_4OH/EtOH 3:1 to remove the base protecting groups, followed by desilylation with TBAF. The samples were gel purified and assayed for their cleavage rate. The results are illustrated in Figure 2. The graph depicts cleavage of the substrate over time by the ribozymes. Both ribozymes, regardless of the method of synthesis, exhibited the same catalytic activity.

It was also necessary to determine the impact of our new deprotection protocols on catalytic activity. Ribozyme **3** was synthesized three times using *S*-ethyltetrazole as the activator, which had already been shown to have no adverse effect on catalytic activity. One sample was treated with the standard deprotection protocol, base deprotection with NH_4OH/EtOH 3:1 followed by desilylation with TBAF. The remaining ribozymes were exposed to MA followed by the anhydrous TEA·HF/NMP reagent. The samples were HPLC purified and assayed for their cleavage rate. As shown in Figure 3, the method of deprotection had no effect on the catalytic activity of the ribozyme.

These results demonstrate that our new protocols for synthesis and deprotection of oligoribonucleotides provide ribozymes that are chemically and biochemically identical to the oligomers produced under standard conditions.

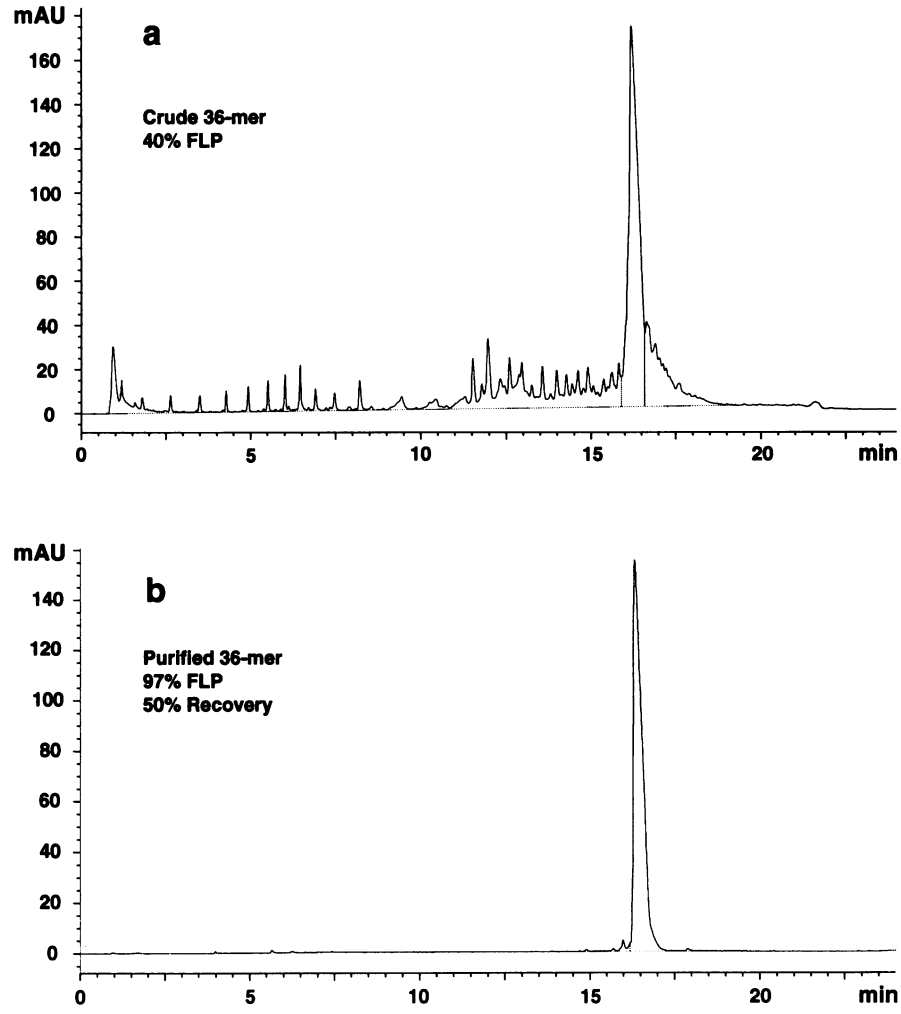

Figure 4. (**a**) Chromatogram of crude 100 μmol synthesis of ribozyme **3** analyzed on a Hewlett Packard 1090 HPLC with a Dionex NucleoPac® PA-100 column at 50°C using $NaClO_4$ buffers. (**b**) Chromatogram of ribozyme **3** purified by the two-step HPLC process, reversed phase, trityl-on, followed by anion exchange, trityl-off. Rz **3**, ucu ccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccu. Lower case, 2′-*O*-Me.

Purification

The most quantitative procedure for recovering fully deprotected RNA molecules is by ethanol precipitation or anion exchange cartridge desalting. Once an oligomer has been desalted, the sample is typically analyzed by HPLC to determine the extent of full-length material produced. Although many methods exist for reversed phase HPLC (4,5,7) purification of oligonucleotides, these protocols are generally suited to shorter (<10 bases) oligonucleotides. Purification by anion exchange chromatography has been reported (6,8), however, it has also been limited to shorter sequences or it has been used as a preliminary purification step (23). We have found that anion exchange chromatography is particularly well suited to the analysis and purification of long RNA sequences on both small and large scales.

The purification of long RNA sequences may be accomplished by either a two-step or a single-step chromatographic procedure (24). The two-step procedure requires an initial purification of the molecule on a reversed phase column with the trityl group at the 5′-position conserved. The trityl group is removed by the addition of an acid followed by neutralization. The final purification is carried out on an anion exchange column. For the single-step purification, the molecule is purified on an anion exchange column, trityl-off. A final desalting step on a small reversed phase cartridge completes both purification procedures.

In the case of the two-step purification procedure, in which the first step is a reversed phase purification followed by an anion exchange step, the reversed phase purification is best accomplished using polymeric, *e.g.* polystyrene-based, reversed phase media, using a 5′-trityl-on method. This purification is achieved using standard methods, consisting of an acetonitrile gradient with aqueous ammonium bicarbonate as the mobile phase. The molecule is recovered using this reversed phase method and then, once detritylated, subjected to an anion exchange purification step.

The utility of this two-step purification procedure for large amounts ($\geq$ 25 μmol) of RNA is illustrated in Figure 4. Ribozyme **3**, a 36mer with five 2′-*O*-methyl groups at both the 3′- and 5′-ends, was the test sequence. Synthesis on a 100 μmol scale yielded 17 000 AU of crude total RNA containing 40% (6800 AU) full-length product. The oligonucleotide was subjected to reversed phase trityl-on purification. Detritylation of the isolated product was followed by anion exchange chromatography. The pooled fractions containing the full-length ribozyme were desalted and

concentrated. Analysis of the product indicated a purity of 97%, with an overall recovery of 50% of the full-length product (3400 AU) for the two-step purification procedure.

Anion exchange chromatography is crucial for the purification of long RNA molecules, particularly in conjunction with alkali perchlorate salts (17). Anion exchange purification may be used in conjunction with an initial reversed phase purification, as described above, or alone. This method results in the formation of the sodium salt of the ribozyme during the chromatography. This salt is preferred for biological applications, since it is not toxic.

CONCLUSIONS

The methods presented in this paper provide an efficient protocol for the synthesis, deprotection and purification of oligoribonucleotides. The use of *S*-ethyltetrazole as the coupling activator reduces the reaction time by one-half. Base deprotection with MA requires 4% of the time required by standard methods. Similarly, the desilylation process has been optimized by reducing both the time and salts produced during deprotection with the anhydrous TEA·HF/NMP solution. The combination of the improved coupling and deprotection methods described reproducibly shortens the time of synthesis and deprotection of a 36mer oligoribonucleotide from >36 to ~6 h. Finally, the ease of analysis and purification has been greatly improved through the use of anion exchange chromatography. These improved protocols enhance both the yield and quality of the final product.

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