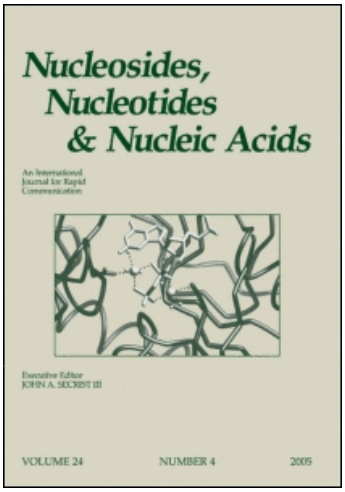

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An Efficient Method for the Isolation and Purification of Oligoribonucleotides

Brian Sproat[a]; Francesco Colonna[b]; Bashar Mullah[a]; Dean Tsou[a]; Alex Andrus[a]; Arnold Hampel[c]; Ravi Vinayak[a]
[a] EMBL, Meyerhofstrasse 1 Heidelberg 69117 Germany Applied Biosystems Division Perkin Elmer Corporation 850 Lincoln Centre Drive, Foster City, CA, USA [b] CNR, Via P. Gobetti, Bologna, Italy [c] Department of Biological Sciences, Northern Illinois University, DeKalb, IL, USA

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AN EFFICIENT METHOD FOR THE ISOLATION AND PURIFICATION OF OLIGORIBONUCLEOTIDES

Brian Sproat[1], Francesco Colonna[2], Bashar Mullah, Dean Tsou, Alex Andrus, Arnold Hampel[3] and Ravi Vinayak*. [1]EMBL, Meyerhofstrasse 1, Heidelberg, 69117 Germany; Applied Biosystems Division, Perkin Elmer Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404, USA; [2]CNR, Via P. Gobetti 101, 40129 Bologna, Italy; [3]Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA.

Abstract. Problems associated with the use of tetrabutylammonium fluoride like incomplete desilylation and removal of the tetrabutylammonium salts during large scale syntheses of oligoribonucleotides (RNA) have been eliminated by the use of triethylamine trihydrofluoride and precipitation of the RNA with 1-butanol. An efficient anion-exchange HPLC method has been developed for the purification of chemically synthesized RNA and the resulting product precipitated directly by the addition of 1-propanol. A new activator, 5-ethylthio-1*H*-tetrazole significantly enhances the synthesis quality and yield of oligoribonucleotides. RNA synthesized using these improvements has been shown to be biologically active by a comparative ribozyme-substrate assay.

Introduction

In this era of rational drug design much effort is being directed towards diagnostic and therapeutic agents based on oligonucleotides[1,2]. An increasing number of these investigations involve synthetic oligoribonucleotides (RNA). Interest in ribozymes and antisense RNA has propagated the demand for more efficacious methods for RNA synthesis[3,4]. The biological activity of chemically synthesized RNA, equivalent to that of RNA derived by transcription methods, is contingent upon efficient synthesis and purification systems[5-7]. Solid-phase chemistry currently

provides the most dynamic means for the scale-up of RNA synthesis. Progress in the methods for automated solid-support RNA synthesis has lagged behind DNA primarily due to problems of 2'-hydroxyl protection, slower internucleotide coupling kinetics, and the fragility of RNA to hydrolytic and enzymatic degradation[8]. Although oligoribonucleotides can be transcribed by using the phage T7 RNA polymerase method[9], only chemical synthesis provides the large quantities of RNA needed for physical studies (NMR and X-ray crystallography) and therapeutic ribozyme investigations. Also, the chemical synthesis of oligoribonucleotides is necessary to obtain RNA fragments that contain non-natural and modified nucleotides.

In this paper, we present improved post-synthesis protocols *viz.* a facile work-up procedure which simplifies the isolation of the crude product and an efficient anion-exchange purification and precipitation method for oligoribonucleotides. We also report new reagents for RNA synthesis that can be used on any automated DNA/RNA synthesizer to improve the yield and quality of synthetic oligoribonucleotides.

RESULTS AND DISCUSSION

The following RNA sequences were synthesized on automated synthesizers and subjected to post-synthesis protocols.

Sequence 1: 5'> CUC AAU AAA GCU UGC CUU GAG <3' (21 mer)[10]
Sequence 2: 5'> GGA AUG GUU UUU UUA UCU UCG U <3' (22 mer)[11]
Sequence 3: 5'> GAG CCU GGG AGC UC <3' (14 mer)[12]
Sequence 4: 5'> ACA CAA CAA GA AGG CAA CCA GAG AAA CAC ACG UUG UGG UAU AUU ACC UGG UA <3' (52 mer)[13] and
Sequence 5: 5'> UGC CCG UCU GUU GUG U<3' (16 mer)[13]

Chemical synthesis of sufficiently pure RNA at scales appropriate for structural studies and therapeutic applications is still a major challenge. Although problems of efficient coupling and base-protecting group removal have been addressed by protecting group variation[14-20], an important problem remains: removal of the tetrabutylammonium salts after the 2'-silyl deprotection with tetrabutylammonium fluoride (TBAF)[21]. TBAF is used

most commonly for the removal of the 2'-silyl groups during oligoribonucleotide synthesis. The tetrabutylammonium salts are generally removed by size-exclusion chromatography on Sephadex media[22]. At larger scales (> 400 ODU, 12 mg of crude RNA) the use of TBAF becomes impractical as it demands several parallel chromatographies, or use of a large column for desalting purposes, and requires evaporation of large quantities of aqueous buffers. The TBAF procedure for large scale syntheses is cumbersome, requiring unusually large buffer volumes, excessive handling and extended concentration to remove water that degrades the quality of the final product. For oligoribonucleotides greater than 50 bases we seldom observed a complete desilylation. This could be due in part to poor solubility of RNA in a non-polar solvent like THF. It has also been proposed that the efficiency of desilylation by 1M TBAF/THF is adversely affected by the presence of excess water and that there are significant differences in desilylation rates between purine and pyrimidine nucleosides[21]. Our results concur with the previous findings that the silyl groups can be efficiently removed with neat triethylamine trihydrofluoride {Et3N(HF)3}, a mobile volatile liquid[23, 24] which generates a less hydrophobic and easily removable by-product. We have found that, the fully deprotected RNA can be precipitated directly from the Et3N(HF)3 solution by addition of excess 1-butanol. This procedure saves almost 9-10 hours of time after desilylation, compared to the isolation of RNA using Bu4NF. With Bu4NF, the time necessary for desalting and subsequent isolation of RNA increases with increasing amount of RNA, whereas with Et3N(HF)3 large quantities of RNA can be precipitated by a single addition of 1-butanol. An example of this is shown in FIG. 1 (a 25 μmole synthesis of sequence 1). This sequence represents the highly conserved region of the predicted structure of HIV-1 RNA immediately downstream of TAR and is HIV-1 genome's long terminal repeat as a molecular target for drug design[10]. We obtained approximately 2500 crude ODU of the crude RNA after cleavage from the support. It was deprotected with a 3 : 1 mixture of ammonium hydroxide : ethanol (4 hours at 55 °C), then desilylated with Et3N(HF)3 (24 hours at ambient temperature) and precipitated with 1-butanol. Isolation of the crude RNA after the desilylation reaction was complete in less than 1 hour. However, we required about 48 hours to completely isolate and desalt the RNA when using TBAF as a desilylating agent. Attempts to precipitate RNA with 1-butanol from a Bu4NF solution have not proven to be successful.

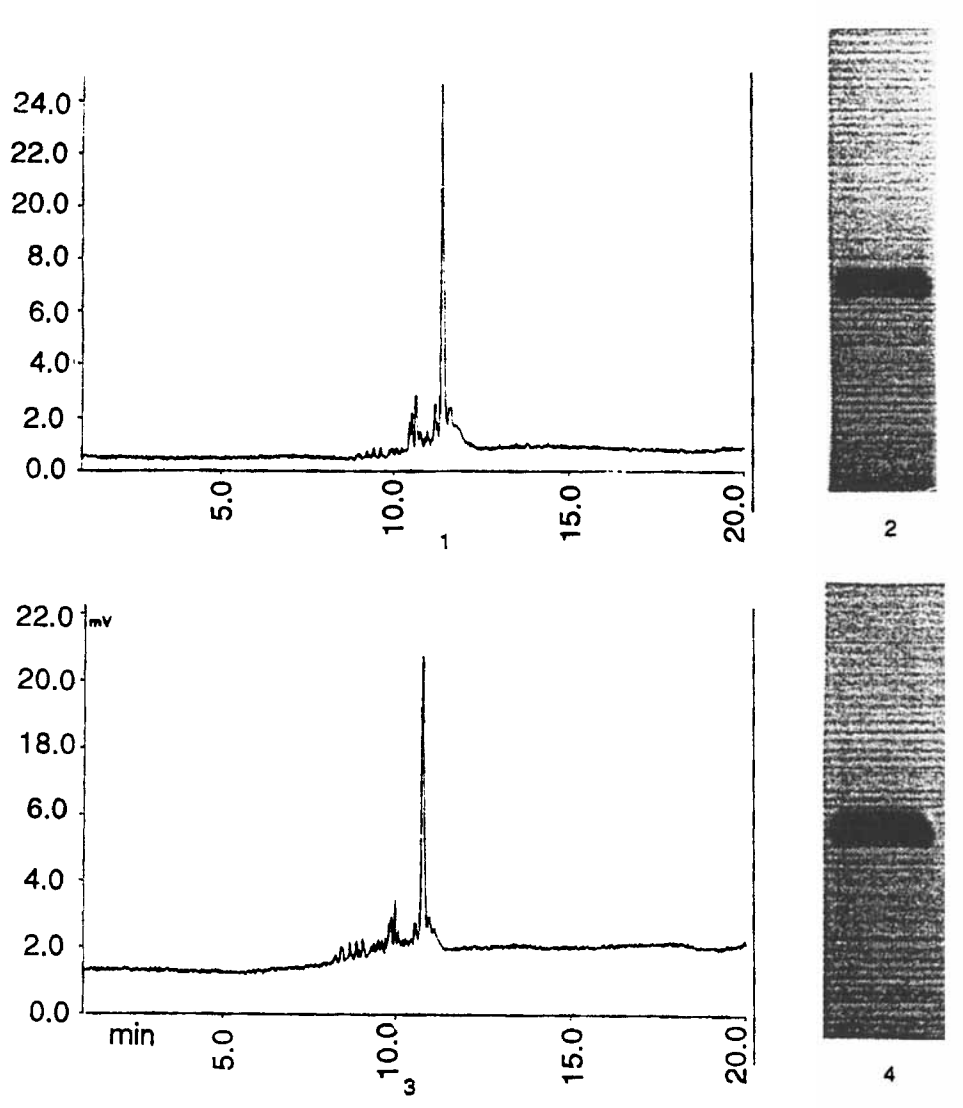


FIG.1. Micro Gel CE (1) and PAGE (2) analysis of the crude 21 mer RNA (sequence 1) obtained after desilylating with TBAF, followed by G-25 Sephadex desalting. MicroGel CE (3) and PAGE (4) analysis of the same sequence obtained after desilylation with TEA.3HF, followed by precipitation with butanol.

Figure 2 shows that the quality of the product obtained after using different desilylation and desalting methods are essentially comparable as analyzed by anion exchange HPLC. This 22 mer RNA (sequence 2, FIG. 2) represents the 3' terminus of the genomic RNAs of vesicular stomatitis virus (VSV) of either the Indiana or New Jersey serotypes. The 22 mer oligoribonucleotide was purified and encapsidated with the appropriate VSV nucleocapsid protein which could be transcribed by VSV RNA dependent RNA polymerase [11].

In order to establish the unambiguity of the two desilylation and desalting methods, the oligoribonucleotides were analyzed by anion-exchange HPLC, PAGE and by MicroGel capillary electrophoresis[25-28]. Analyses were performed on oligoribonucleotides synthesized trityl-Off, since the acidic nature of reagents that remove the final 5'-O-DMT group may cause phosphate migration. We have routinely performed anion-exchange HPLC analysis of synthetic oligoribonucleotides on a NucleoPac PA-100 anion exchange column[29, 30]. We have developed a gradient system that employs $LiClO_4$ in the mobile phases with the RNA obtained in the lithium salt form. Figure 3 shows the anion exchange HPLC traces of the 21 mer RNA (sequence 1). This sequence was analyzed by heating the analytical column to 50 °C to minimize the secondary structure effect (compare traces 1 and 3 in FIG. 3). It was purified by anion-exchange HPLC (trace 5, FIG.3) on a semi-preparative anion-exchange column using the same gradient as the analytical method and then desalted and precipitated by the addition of 1-propanol. This method of desalting and isolation of purified RNA avoids the necessity of evaporating large amounts of aqueous solutions and subsequent time-consuming size exclusion chromatography. We have routinely precipitated 3000-4000 ODs of the oligoribonucleotides in this fashion. The use of lithium perchlorate for anion-exchange based HPLC purifications allows the RNA to be easily isolated salt free by precipitation directly from the product peak fraction by adding 4 volumes of 1-propanol. Lithium perchlorate is also much more soluble in organic solvents than other perchlorates we have examined and this obviates a final desalting column. The purified 21 mer RNA was analyzed with negative ion mode electrospray mass spectroscopy (FIG. 4). The measured molecular mass of 6657.0 by mass spectrometry was in total agreement with the molecular

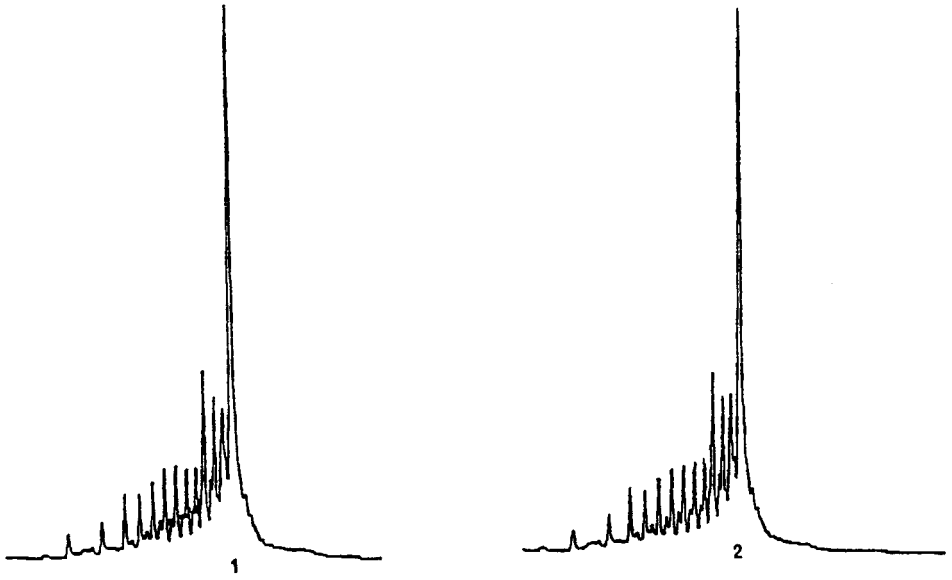


FIG.2. Anion-exchange HPLC of the crude 22 mer RNA (sequence 2) obtained after TBAF desilylation and G-25 Sephadex desalting (1) and TEA.3HF desilylation and butanol precipitation (2).

weight of the purified 21 mer ($C_{199}H_{247}N_{77}O_{145}P_{20}$, calculated molecular mass 6657.04).

We have performed the oligoribonucleotide syntheses on an optimized type of polystyrene support. These (FIG. 5) were prepared by coupling the 2'-O-acetyl-3'-O-succinate nucleoside esters with non-swelling aminomethyl polystyrene beads using published procedures[31-33]. The rigid non-swelling polystyrene beads of 50-70 micron particle size retain less water and wet more thoroughly with organic solvents than CPG. The drier environment provides for a more efficient synthesis. Acetyl was found to be the preferred 2'-OH protecting group for RNA supports, instead of the sterically bulky 2'-O-*t*-butyldimethyl silyl group[14] since it enables a more complete cleavage of the RNA from the support within 2 hours on the instrument with a mixture of ammonium hydroxide : ethanol (3:1)[33]. In general, we obtained 90-95% of the oligoribonucleotide after 2 hours of cleavage on the instrument when we used the 2'-O-acetyl protected RNA support as compared with 60-65% when using the 2'-O-*t*-butyldimethyl silyl support.

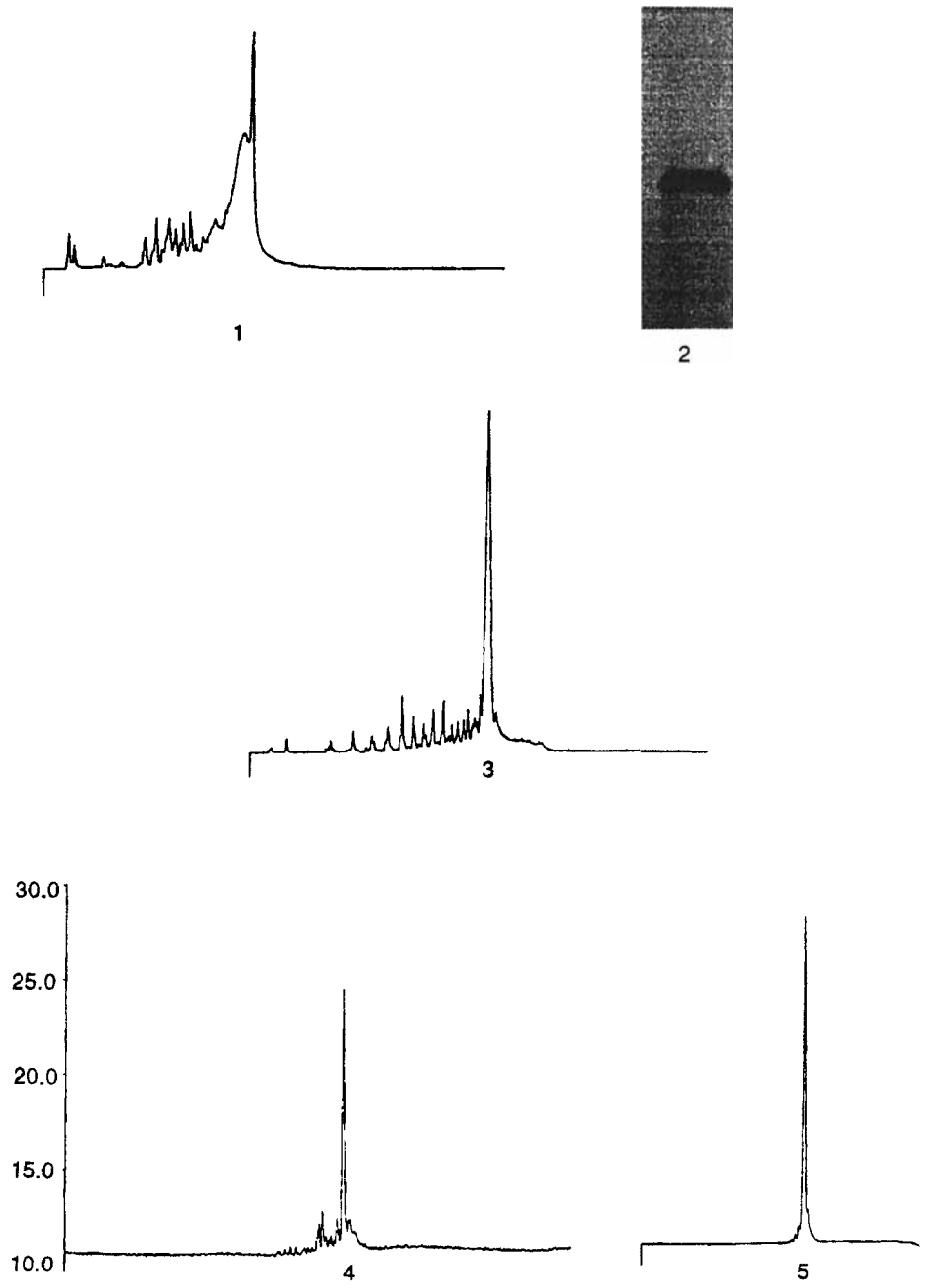


FIG.3. Anion-exchange HPLC (at ambient temp.) (1), PAGE analysis (2), Anion-exchange HPLC (at 50 °C) (3), Micro Gel CE (4) of the crude 21 mer RNA (sequence 1) synthesized at 25 μmole scale. The anion-exchange HPLC trace of the combined purified fraction (at 50 °C) is shown in trace (5).

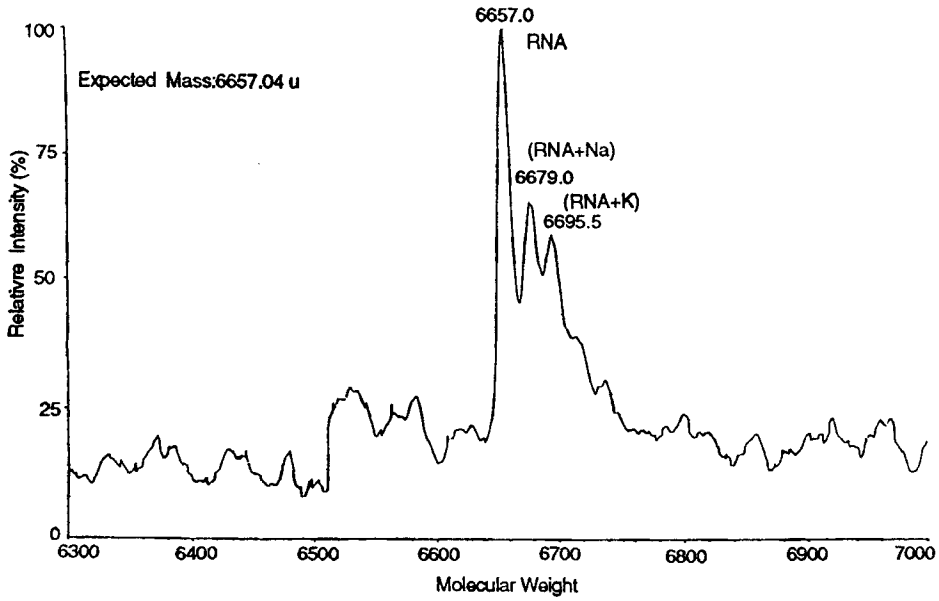
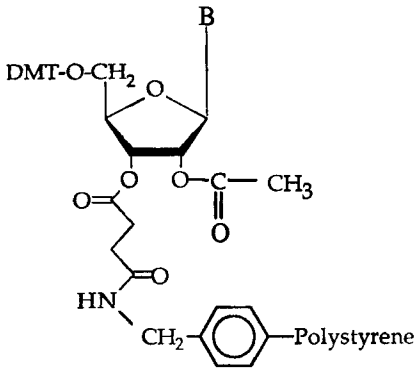


FIG.4. Negative ion mode electrospray mass spectrum of the purified 21 mer (sequence 1).



B= A ^{Pac}, G ^{dmf}, C ^{iBu} and U

FIG.5. Polystyrene support for RNA (1000 Å pore size, 50-70 μm particle size);
Pac : Phenoxyacetyl,
dmf : N-(dimethylamino)methylene
and iBu : isobutyryl.

The key step in the automated synthesis of an oligonucleotide by the phosphoramidite approach is the 1*H*-tetrazole mediated coupling reaction of a 5'-O-DMT-nucleoside-3'-O-phosphoramidite with the 5'-hydroxyl group of the growing chain anchored to the polymeric support[34]. Activation of phosphoramidites by tetrazole creates a highly electrophilic intermediate that reacts with the nucleophilic 5'-hydroxyl group of the oligonucleotide. It became quite obvious during large scale synthesis on the model 390Z DNA/RNA synthesizer that tetrazole was not the activator of choice, as we routinely obtained average-stepwise-trityl yields in the 94-95% range. Modifications in the synthesis cycles and/or greater excess of nucleoside phosphoramidites and tetrazole did not have any significant beneficial effect. Other activators such as 5-(p-nitrophenyl)-1*H*-tetrazole[35] and 1-hydroxybenzotriazole[36] *etc.* were not useful. During our systematic search for an ideal activator, it was found that a previously reported activator 5-ethylthio-1*H*-tetrazole (FIG. 6)[37, 38] was a better choice. Several comparison syntheses with both 5-ethylthio-1*H*-tetrazole and 1*H*-tetrazole gave 2-3 % higher average yield per cycle with the former, resulting in higher yields and purity of the oligonucleotide product[39,40]. The effect was especially pronounced at scales ≥ 25 μmole, and at lower phosphoramidite excess. The greater acidity and solubility of 5-ethylthio-1*H*-tetrazole in acetonitrile perhaps accounts for the superior performance. Sequence 3, synthesized at 25 μmole scale on a 390Z DNA/RNA synthesizer, was analyzed by anion-exchange HPLC (FIG. 7). This 14 mer RNA sequence represents the hairpin loop of the TAR sequence of the HIV-1 genome[12]. With 1*H*-tetrazole as activator we required a minimum 10 fold excess of phosphoramidite per coupling and a 20 minute coupling wait time per cycle (trace 2, FIG. 7). 5-Ethylthio-1*H*-tetrazole as activator gave a superior product with only a 6 fold excess of phosphoramidite and a 15 minute coupling wait time (trace 1, FIG. 7). A 0.75 M solution of 5-ethylthio-1*H*-tetrazole in acetonitrile and a 7 fold excess of phosphoramidites (at 25 μmole scale) over the 3'-nucleoside support consistently gave good quality syntheses with average-stepwise-trityl yield being >98%.

During this work on large scale RNA synthesis, we found that *t*-butyl hydroperoxide (TBHP) can be used as an alternative to iodine for oxidation of the P(III) species to P(V) species during oligonucleotide chain propagation[41-46]. The reagent was prepared by combining 37 mL of the TBHP solution in

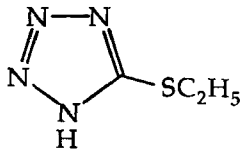


FIG.6. 5-Ethylthio-1*H*-tetrazole, activator used in large scale RNA synthesis

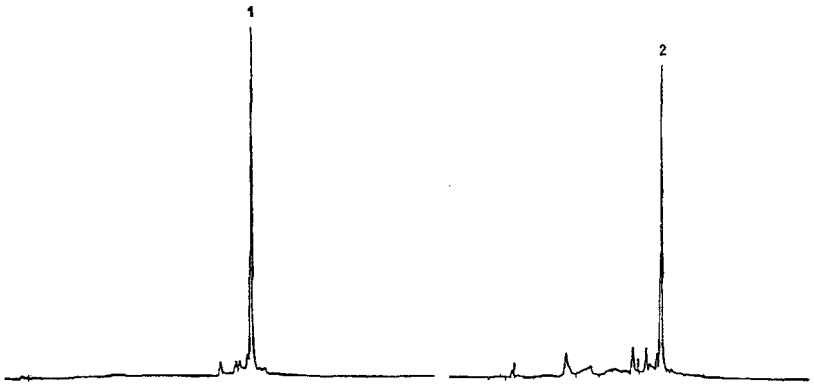


FIG.7. Anion-exchange HPLCs of the crude 14 mer RNA (sequence 3) synthesized at 25 μmole scale using (1) 5-ethylthio-1*H*-tetrazole and (2) tetrazole.

isooctane with 63 mL of dichloromethane. The time of oxidation per cycle was extended to 6 minutes compared with 2 minutes for iodine at the 25 μmole scale. The oxidation time was previously determined by ^{31}P NMR spectroscopy. TBHP gave slightly better yields and a slightly higher overall yield than iodine. This is perhaps due to the fact that the iodine reagent is in a mixture of lutidine/THF/water, and polystyrene, by nature being hydrophobic, wets comparatively less with iodine solution than with TBHP/dichloromethane. TBHP could be used as an oxidizing agent for oligonucleotides with functionalities susceptible to aqueous conditions.

In an effort to demonstrate that the RNA synthesized using these improvements is biologically active we synthesized a HIV-1 directed hairpin

ribozyme (sequence 4) and the corresponding substrate (sequence 5)[13]. This ribozyme, designed to cleave the 5' leader region of HIV-1, has been shown to reduce levels of HIV-1 infection and expression by a factor of from 10^3-10^4. It currently is the most effective *in vivo* engineered ribozyme known[47]. The 52 mer ribozyme was synthesized at 1 μmole scale on a polystyrene support using 0.75 M solution of ethylthio-1*H*-tetrazole as the activator (FIG. 8). The desilylation was performed with triethylamine trihydrofluoride and the crude RNA precipitated with 1-butanol. This was then purified by anion exchange HPLC and the product precipitated with 1-propanol. Catalytic activity was determined at 37 °C by combining substrate with ribozyme and analyzing the products by gel electrophoresis. The results show cleavage levels of the enzymatic RHIV to be 16% compared to the chemically synthesized RCHIV to be 25%. These are roughly comparable and show full biological activity of the chemically synthesized ribozyme. In summary, we have developed a highly efficient method for the isolation and purification of chemically synthesized oligoribonucleotides.

EXPERIMENTAL

Tetrabutylammonium fluoride (TBAF, Bu4NF, 1M solution in THF), triethylamine trihydrofluoride {Et3N(HF)3}, 1-propanol, 1-butanol (HPLC grade), G-25 Sephadex™, sodium acetate and lithium perchlorate were purchased from Aldrich. *t*-Butyl hydroperoxide (3M solution in isooctane) was purchased from Fluka. RNA phosphoramidites bearing the same protecting groups as the supports and other ancillary reagents for automated syntheses were obtained from Applied Biosystems. Ammonium hydroxide (28-30%) was purchased from Baker.

GENERAL PROCEDURES

The oligoribonucleotides were synthesized on Applied Biosystems Model 390Z and 394 DNA/RNA synthesizers. Syntheses were carried out at either 0.2 or 1 μmole scales on the 394. Syntheses on the Model 390Z synthesizer were performed at 25 μmole scale. Removal of the exocyclic amine protecting groups was complete within 4-5 hours in ammonium hydroxide : ethanol (3:1) at 55 °C. The silyl protecting groups were removed with either a 1M solution of tetrabutylammonium fluoride in THF or with neat

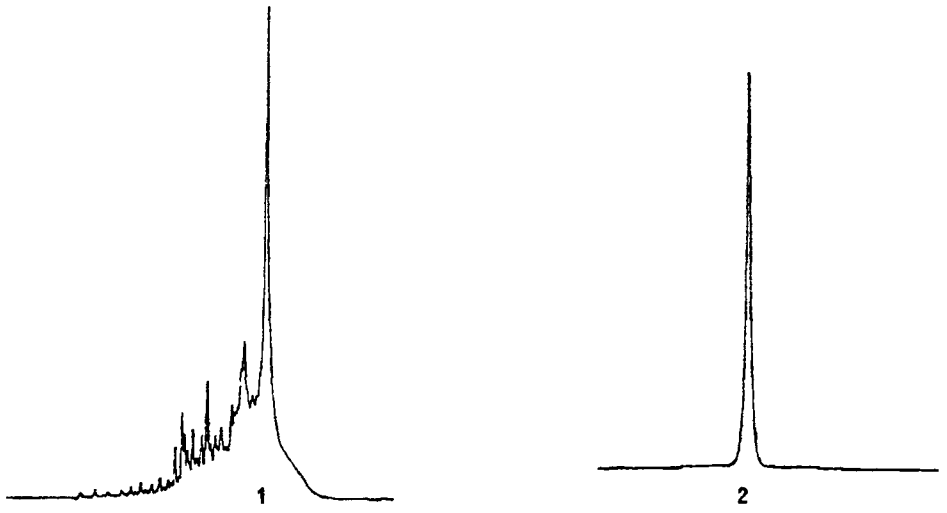


FIG.8. Anion-exchange HPLCs (at 50 °C) of the crude (1) and purified (2) 52 mer RNA (sequence 4) synthesized at 1 μmole scale. Purification was achieved with anion-exchange HPLC at 50 °C and RNA precipitated with propanol.

triethylamine trihydrofluoride (20-24 hours, room temperature). Desalting was performed on a G-25 Sephadex column when TBAF was used. The oligoribonucleotides (synthesized trityl-off) were analyzed by anion-exchange HPLC (NucleoPac PA-100 column, 250 x 4 mm, Dionex Corporation) and by MicroGel capillary electrophoresis (Applied Biosystems Model 270A). Some of the oligoribonucleotides were purified by anion-exchange HPLC (NucleoPac PA-100, 250 x 9 mm, Dionex Corporation). A Hitachi column oven (CTO-6A) was used in conjuction with ion-exchange HPLC at 50 °C to analyze and purify oligonucleotides that deviated from predictable size dependent elution patterns due to strong secondary structure. Negative ion-spray mass spectra were run on a PE Sciex instrument.

Desilylation and desalting when using tetrabutylammonium fluoride

The ammonia / ethanol solution containing the crude oligoribonucleotide (trityl off) was dried after heating for 4-5 hours at 55 °C. To this solution was added a 1M solution of tetrabutylammonium fluoride

in THF {15 µl /per ODU (40µg)}. The mixture was vortexed thoroughly, stirred at ambient temperature for 20-24 hours, quenched with an equal volume of sterile deionized water, and concentrated to approximately one half using a vacuum centrifuge. This RNA sample was dissolved in a minimum amount of deionized water and loaded onto a glass column (0.7 x 20 cm) packed with G-25 Sephadex swollen in sterile deionized water. After the sample had descended to the bed level, about 10 mL of deionized water was added. Fractions (1 ml) were collected in sterile tubes and assayed on a UV spectrometer at 260 nm to determine which tubes contain the oligoribonucleotide. Appropriate fractions containing RNA were pooled and evaporated to dryness. The dried RNA was then dissolved in water before analysis/ purification.

Desilylation and desalting when using triethylamine trihydrofluoride

The ammonia / ethanol solution containing the crude oligoribonucleotide (trityl off) was dried after heating for 4-5 hours at 55 °C. To this solution was added neat triethylamine trihydrofluoride {10 µl /per ODU (40µg)}. The mixture was vortexed thoroughly, stirred at ambient temperature for 20-24 hours, and quenched with sterile deionized water (2 µl/ODU). To the above solution was added 1-butanol (100 µl/ODU). It was mixed and chilled at -20 °C or lower for about 45 minutes (or in dry ice/ acetone for 20 minutes). The tube was centrifuged at 3000 r.p.m. for 5 minutes and the butanol decanted to collect the precipitated RNA. It was then dissolved in water prior to analysis.

Gradient System used for Anion-Exchange HPLC Analyses

Start Time (Min)	%B at start time
0	0
40	70

Mobile phase : **Solvent A**: 20 mM $LiClO_4$ + 20 mM NaOAc in H_2O: CH_3CN (9:1) (pH 6.5 with dil. AcOH)
Solvent B: 600 mM $LiClO_4$ + 20 mM NaOAc in H_2O: CH_3CN (9:1) (pH 6.5 with dil. AcOH)

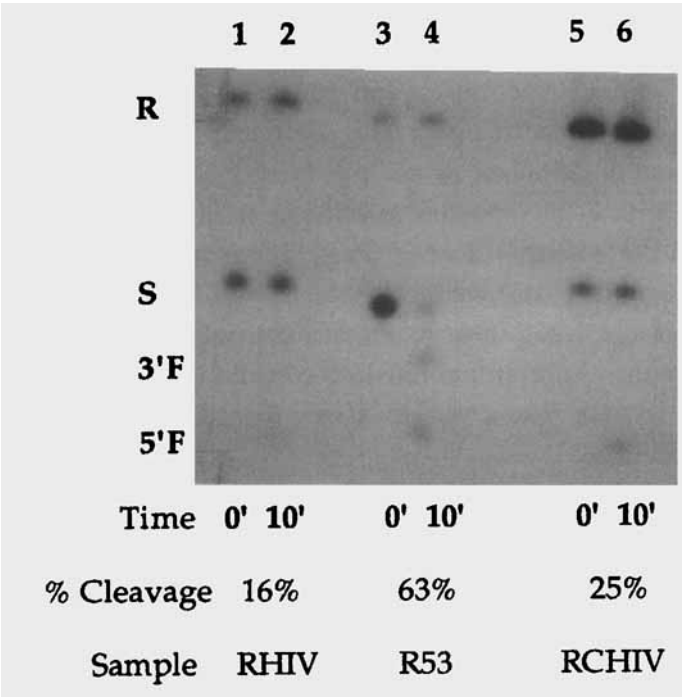


FIG.9. Cleavage of substrate by chemically synthesized HIV specific hairpin ribozyme (RCHIV). Lanes 1, 2: Enzymatically synthesized HIV specific hairpin ribozyme (RHIV) and corresponding substrate (sequence 5). Percent cleavage in lane 2 was 16%. Lanes 3, 4: Enzymatically synthesized native hairpin ribozyme (R 53) and corresponding substrate. Percent cleavage in lane 4 was 63%. Lanes 5, 6: Chemically synthesized and purified HIV specific hairpin ribozyme (RCHIV) and corresponding substrate. Percent cleavage in lane 6 was 25%. Each sample was allowed to cleave for 0 min (lanes 1,3,5) and 10 min (lanes 2,4,6). The abbreviations R, S, 3'F and 5'F represent ribozyme, substrate and the 3' and 5' cleavage fragments respectively.

Flow Rate :	1.0 mL/min
Cartridge :	250 x 4 mm (NucleoPac PA-100, analytical)
	250 x 9 mm (NucleoPac PA-100, semi-preparative)

Purification, precipitation and isolation of RNA

The crude oligoribonucleotides were purified on an anion exchange (NucleoPac PA-100, 250 x 9 mm) column using the above gradient. The tubes containing the product were pooled in a sterile tube. 1-Propanol (4 volumes)

was added, the solution mixed thoroughly and the tube kept at -20 °C for 4-6 hours. After centrifugation at 3000 r.p.m. for approximately 10 minutes, the supernatant was decanted off and the precipitated RNA pellet washed with propanol and dried.

Chemically synthesized ribozyme end labeled with 5' ^{32}P by the kinase reaction was purified by polyacrylamide gel electrophoresis, bands cut out and extracted. Cleavage of the labeled substrate was done in 12 mM $MgCl_2$, 40 mM Tris pH 7.5 and 2 mM spermidine for 10 min at 37 °C [48,49]. Substrate concentrations were 20 nM and ribozyme concentrations 8 nM.

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