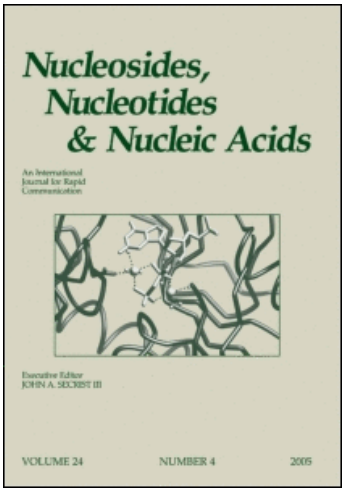

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PURIFICATION OF 5'-O-TRITYL-ON OLIGORIBONUCLEOTIDES. INVESTIGATION OF PHOSPHATE MIGRATION DURING PURIFICATION AND DETRITYLATION.

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ABSTRACT: Synthetic oligoribonucleotides (RNA) are efficiently prepared with 2'-*O*-tert-butyldimethylsilyl nucleoside 3'-*O*-phosphoramidites with labile base-protection; A^{dmf} or A^{pac}, G^{dmf}, C^{ibu}, U. After cleavage from the polystyrene support, the exocyclic amine protecting groups are removed with conc. NH_4OH : ethanol / 3:1 by heating at 55 °C for 3-5 h. The 2'-O- silyl protecting groups are removed with tetra-n-butylammonium fluoride in THF or more conveniently with neat triethylamine trihydrofluoride. To gain the advantages of increased capacity on reverse phase HPLC and the convenience of cartridge based purification (OPC, Oligonucleotide Purification Cartridge), the 5' trityl was left on the RNA as the final protecting group to be removed. The mild conditions which are effective for trityl removal are shown to preserve 3'-5' phosphate linkage integrity in RNA. The absence of phosphate migration is demonstrated by model studies, utilizing N^4-isobutyryl-5'-*O*-DMT-3'-*O*-TBDMS-2'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite) as a control monomer and digestion by 3'-5' selective P1 nuclease and alkaline phosphatase and HPLC analysis. Oligoribonucleotides were analyzed by MicroGel capillary electrophoresis, anion-exchange HPLC, and the enzymatic digest/HPLC method.

INTRODUCTION

The recent discoveries of catalytic activity[1,2] and potential antisense therapeutic applications[3] of RNA have stimulated refinements in chemical synthesis of oligoribonucleotides. The increasing demands of oligoribonucleic acids in biomedical and structural studies are difficult to satisfy by RNA polymerase assisted transcription method, which affords small quantities of RNA and only allows the incorporation of natural bases.[4] Chemical synthesis of RNA affords large quantities and the insertion of modified bases, sugars or phosphates at any site in the sequence.[5-11] The preferred method of solid phase automated synthesis of RNA employs ribonucleoside phosphoramidites.[12-16] The progress in RNA synthesis is slow compared to DNA due to the presence of the additional

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Dedicated to Professor Yoshihisa Mizuno on his 75th birthday

2'-hydroxyl group in the sugar moiety. The 2'-OH protecting group influences the rate of internucleotide coupling reaction and the stability of the partially deprotected RNA during post-synthesis processing. Although acid labile modified acetal groups[17-19] have been used, the tert-butyldimethylsilyl (TBDMS) group[16] has become the commercial standard. The TBDMS group is stable during acidic detritylation in each cycle and the alkaline conditions used to cleave RNA from the support and to deprotect exocyclic amine groups of the base.[20, 21] Usually tetra-n-butylammonium fluoride or triethylamine trihydrofluoride[22, 23] is used to remove 2'-O-tert-butyldimethylsilyl group. Removal of excess salts after desilylation is a time consuming step in post-synthesis processing. This problem can be solved by desalting followed by detritylation of trityl-on RNA on oligonucleotide purification cartridge (OPC). To our knowledge only one report has been published on cartridge based desalting and detritylation of RNA.[24]

We report here synthesis of oligoribonucleotides at 1 μmol scale on polystyrene support followed by desalting and detritylation on an oligonucleotide purification cartridge (OPC™). Enzymatic digestion and HPLC analysis of oligoribonucleotides is used to detect the presence of 2'-5' phosphate linkages which may form during post-synthesis processing. By validating the integrity of 3'-5' linkages and the lack of phosphate migration during normal detritylation conditions, large capacity trityl-on RNA purification by reverse phase HPLC is feasible.

RESULTS AND DISCUSSION

The solid support matrix plays an important role towards efficient synthesis of oligoribonucleotides. As ribonucleoside phosphoramidites are very sensitive to water, the support should be hydrophobic so as to not retain water. The traces of water retained from oxidation should be easily removed by acetonitrile washing before the phosphoramidite monomers are delivered to the column. We have reported the use of highly cross-linked non-swelling polystyrene with 1000 Å pores and 50-70 μm particle size as the solid support for the synthesis of oligoribonucleotides.[25] Because of the hydrophobic nature of polystyrene the residual water retained from oxidation step is easily removed by acetonitrile wash and gives better results than CPG support. The 2'-hydroxyl group of the support bound ribonucleoside is protected with acetyl instead of tert-butyldimethylsilyl to accelerate cleavage of the RNA from the support.[26]

The RNA sequences shown in FIG 1 were selected for phosphate-migration model study. The 3'-5' phosphate linkage containing C base was replaced by 2'-5' phosphate linkage by using N^4-isobutyryl-5'-*O*-DMT-3'-*O*-TBDMS-2'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (FIG 2) and is indicated by underlined C.

Oligoribonucleotide **1**: 5'-UGA CAG UCC UGU UU-3'
Oligoribonucleotide **2**: 5'-UGA CAG UC<u>C</u> UGU UU-3'
Oligoribonucleotide **3**: 5'-UGA CAG U<u>C</u>C UGU UU-3'
Oligoribonucleotide **4**: 5'-UGA CAG U<u>CC</u> UGU UU-3'
Oligoribonucleotide **5**: 5'-UGA <u>C</u>AG U<u>CC</u> UGU UU-3'

FIG 1

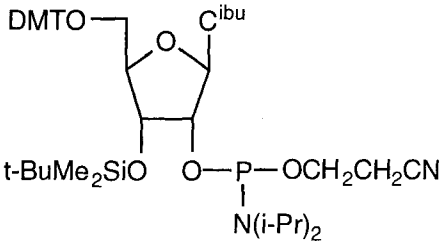


FIG 2

The oligoribonucleotides were synthesized in 1 μmol scale using Applied Biosystems ribonucleoside phosphoramidites (A^{dmf} or A^{pac}, G^{dmf}, C^{ibu}, U) and 1 μmol RNA synthesis column. The oligoribonucleotides were sequentially synthesized 5' trityl-on and trityl-off. After the synthesis was complete, the oligoribonucleotides were cleaved from the support in 2 h at room temperature on the synthesizer with a 3:1 conc. ammonium hydroxide:ethanol. The exocyclic amine protecting groups (A^{dmf} or A^{pac}, G^{dmf}, C^{ibu}) were removed by heating at 55 °C for 3-5 h.

First we examined desilylation of RNA with alternative reagents, such as, tetra-n-butylammonium fluoride (n-Bu4NF) and triethylamine trihydrofluoride ($Et_3N(HF)_3$). Tetra-n-butylammonium fluoride can be used for both trityl-on and trityl-off oligoribonucleotides whereas $Et_3N(HF)_3$ can only be used for trityl-off oligoribonucleotides because its acidic nature causes inadvertent loss of 5'-DMT. Desilylation using either reagent was carried out for 24 h at room temperature. The trityl-on oligoribonucleotides desilylated by n-Bu4NF were desalted and detritylated on OPC whereas trityl-off oligoribonucleotides desilylated with n-Bu4NF were desalted by size exclusion chromatography on Sephadex G-25 as described in the experimental section. Oligoribonucleotides desilylated with $Et_3N(HF)_3$ were desalted by precipitation from

n-butanol. Both desilylating reagents gave comparable results, but Et3N(HF)3 is preferred for large scale reaction because desalting is simplified. The oligoribonucleotides desalted by either OPC or G-25 or precipitation from n-butanol were analyzed by anion exchange HPLC. The OPC desalted oligoribonucleotides appeared to be more pure than those desalted by either Sephadex or precipitation from n-butanol (FIG 3). The oligoribonucleotides, **1-5**, were analyzed by anion exchange HPLC and MicroGel capillary electrophoresis[27] and it was not possible to differentiate oligoribonucleotides **1-5** (traces are not shown).

RNA is known to undergo phosphate migration (2'-5') and/or chain cleavage during deprotection of 2'-OH in both basic and acidic conditions (scheme I).[19, 28-33] This problem is solved by protecting 2'-OH with tert-butyldimethysilyl group which can be removed by tetra-n-butylammonium fluoride without any phosphate migration or chain cleavage.[20, 21] After our desilylation experiment with Et3N(HF)3 was complete Strömberg et al.[23] reported that Et3N(HF)3 did not cause any detectable phosphate migration of uridine dimer. Migration studies of oligoribonucleotide desilylated with Et3N(HF)3 have not been reported. For full biological activity of oligoribonucleotides, it is necessary that the structural integrity remains intact, including the 3'-5' phosphate linkage. Enzymatic digestion is an efficient and simple method to identify structural modifications in oligoribonucleotides.

Damha et al.[34] has recently reported that 2'-5' phosphate linkages are resistant to P1 nuclease and that 2'-5' phosphate containing fragments can be detected by digesting the oligoribonucleotides with nuclease P1 and alkaline phosphatase. Here, oligoribonucleotides **1-5** were treated with the enzymes at 37 °C for 2 h. The digest mixtures were analyzed by reverse phase HPLC. Since nuclease P1 hydrolyzes 3'-5' but not 2'-5' phosphodiester linkages in RNA, the HPLC analysis should show the 2'-5' linked nucleotidyl units in addition to natural ribonucleosides if there was any phosphate migration. HPLC analysis of the digest mixture of oligoribonucleotide **1** desilylated with Et3N(HF)3 only showed ribonucleosides A, G, C and U and gave the expected base composition after correcting for the relative extinction coefficients (FIG 4). The absence of additional detectable peaks in the chromatogram strongly suggests that no 2'-5' phosphate migration occurred during desilylation with Et3N(HF)3. The HPLC analysis of the digest mixture of oligoribonucleotide **1** desilylated with n-Bu4NF and desalted on Sephadex column also did not show any 2'-5' linked nucleotidyl fragments, but only A, G, C and U. Digest mixtures of oligoribonucleotides **1-5** desalted and detritylated on OPC were analyzed. The HPLC of the digest mixture of oligoribonucleotide **1** showed the correct base composition of A, G, C and U. The absence of additional peaks suggests that 2'-5' phosphate migration and/or base modification did not occur during desalting and detritylation on OPC cartridge. The

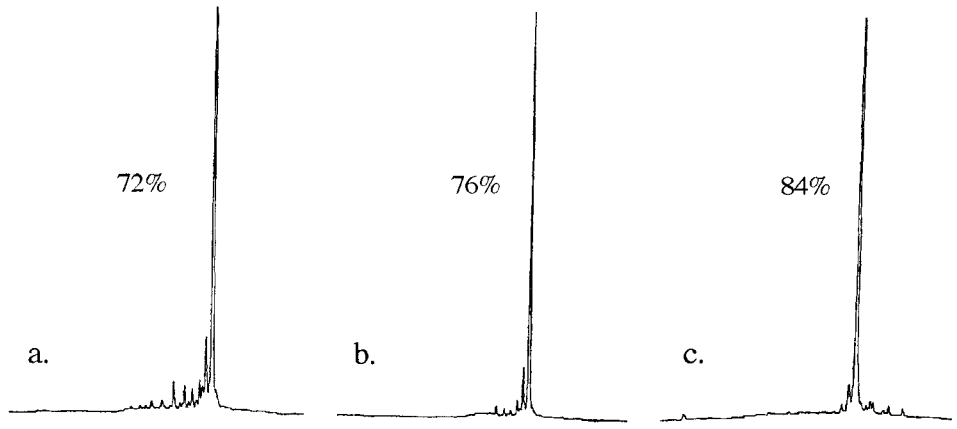
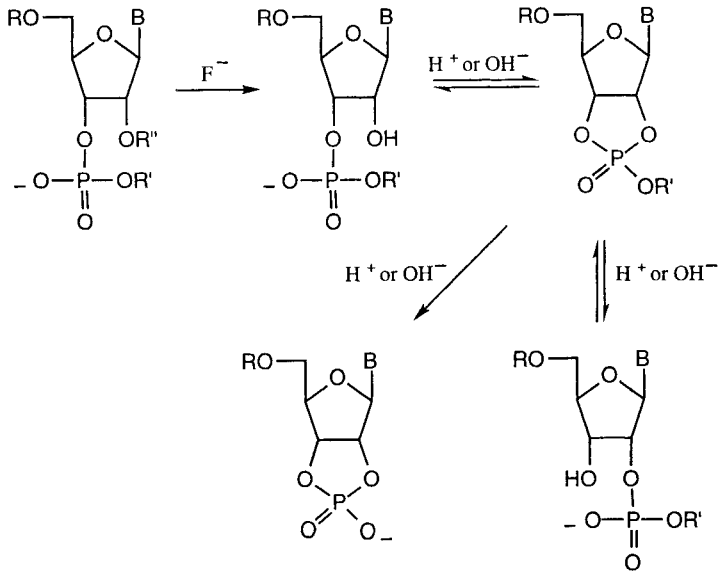


FIG 3. Anion-exchange HPLC of oligoribonucleotide **1** (a 1 μmol scale trityl-on synthesis was divided into one trityl-on and two trityl-off portions): a. Desilylated with n-Bu4NF and desalted on Sephadex G-25 (trityl-off **1**); b. Desilylated with $Et_3N(HF)_3$ and precipitated from n-BuOH (trityl-off **1**); c. Desilylated with n-Bu4NF, and desalted and detritylated on OPC (trityl-on **1**). 72%, 76% and 84% in chromatograms a, b and c respectively refer to the percentage of the full length product.



Scheme I

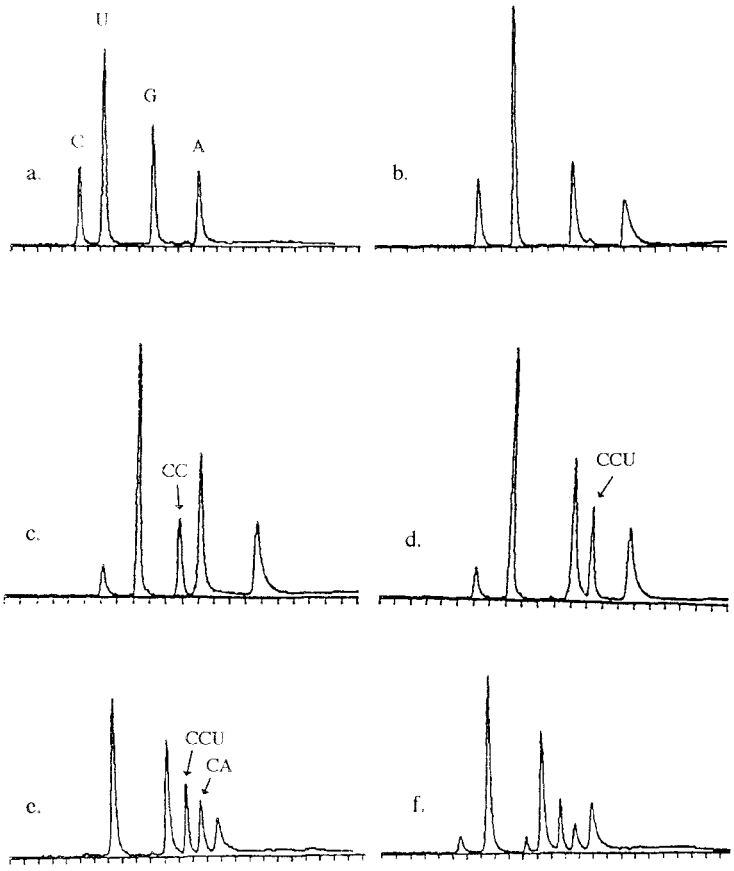


FIG 4. Reverse phase HPLC analysis of enzymatic digestion of oligoribonucleotides **1-5**: a. trityl-off **1** desilylated with Et3N(HF)3; trityl-on **1-5** were desilylated with n-Bu4NF, and desalted and detritylated on OPC; b. trityl-on **1**; c. trityl-on **3**; d. trityl-on **4**; e. trityl-on **5**; f. trityl-on **5** (digestion reaction run for overnight).

HPLC of the digest mixtures of oligoribonucleotide **2** and **3** showed peaks due to CU (2'-5') and CC (2'-5') dimer respectively as expected in addition to A, G, C and U. The appearence of CU (2'-5') and CC (2'-5') dimer peaks in HPLC analysis clearly indicates that presence of 2'-5' phosphate linkages can be easily detected by enzymatic digestion of RNA. The digest mixture of oligoribonucleotide **4** showed CCU trimer containing all 2'-5' phosphate linkage in addition to A, G, C and U. The digest mixture of oligoribonucleotide **5** did not show any C because all C nucleosides were attached by 2'-5' phosphate linkages and were not hydrolyzed by nuclease P1, and showed peaks for A, G, CA (2'-5'), CCU

(2'-5') and U residues. The presence of CU, CA and CCU fragments containing 2'-5' phosphate linkages was confirmed by separately synthesizing these fragments and co-injecting with the respective digest mixtures. The CC and CA fragments were further confirmed by co-injecting the digest mixtures with authentic samples.[35] When oligoribonucleotide **5** was digested for overnight it was found that some 2'-5' phosphate linkages were hydrolyzed. HPLC analysis of the digest mixture showed peaks for A, G, C, U, CA, CCU, CU and CC residues. Partial hydrolysis of 2'-5' phosphate containing fragments CA and CCU gave C, CU and CC units. A possible explanation for the hydrolysis of 2'-5' phosphate containing fragments, CA and CCU, is that some 2'-5' phosphate linkages might have migrated to more stable 3'-5' linkages under digest reaction condition and then hydrolyzed by nuclease P1 and alkaline phosphatase. Snake venom phosphodiesterase and bacterial alkaline phosphatase completely digested oligoribonucleotides **1-5** and analysis of the digest mixtures gave the requisite number of A, G, C and U. From this study it is concluded that desalting and detritylation on OPC do not induce 2'-5' phosphate migration in oligoribonucleotides .

In large scale preparation, oligoribonucleotides are preferably synthesized with 5'-trityl-on and purified after base and sugar deprotection by reverse phase HPLC. The trityl group is then removed by treatment with aqueous acetic acid. We planned to examine whether this acid treatment induces any 2'-5' phosphate migration. The oligoribonucleotide **1** was synthesized with 5' trityl-on and purified by reverse phase HPLC as described in the experimental section. The trityl group was removed by treatment with 80% acetic acid and the oligoribonucleotide was finally precipitated from ethanol. A sample of the oligoribonucleotide was then digested with nuclease P1 and alkaline phosphatase. The analysis of the digest mixture did not show the presence of 2'-5' phosphate linkages in the oligoribonucleotide.

In conclusion, oligoribonucleotides were synthesized in 1 μmol scale with Applied Biosystems ribonucleoside phosphoramidites in good yield and were cleaved from the support in 2 h using 3:1 ammonium hydroxide /ethanol. Desilylation with n-Bu_4NF or $Et_3N(HF)_3$ gave comparable results, but $Et_3N(HF)_3$ is more convenient for large scale. Trityl-on oligoribonucleotides can be conveniently desalted and detritylated on OPC without any phosphate migration or base modification. The presence of 2'-5' phosphate linkages was detected by a simple enzymatic digestion of oligoribonucleotides.

EXPERIMENTAL

Ribonucleoside phosphoramidites, 1 μmol RNA synthesis column, oligonucleotide purification cartridge (OPC), and other ancillary reagents were obtained from Applied

Biosystems Division, Perkin Elmer Corporation. Ammonium hydroxide (28 %) was purchased from Baker. Tetra-n-butylammonium fluoride (n-Bu4NF, 1 M solution in THF), triethylamine trihydrofluoride (Et3N(HF)3), 1-butanol (HPLC grade), Sephadex G-25, sodium acetate and lithium perchlorate were purchased from Aldrich Chemical Company. Nuclease P1 (penicillium citrium) was obtained from Boehringer Mannheim. Alkaline phosphatase (calf intestinal muscosa) was purchased from Sigma Chemical Company. Snake venom phosphodiesterase (crotalus adamanteus venom) and alkaline phosphatase (E. coli) were purchased from Phamacia.

Oligoribonucleotide synthesis: Oligoribonucleotides shown in FIG 1 were synthesized on Applied Biosystems Model 394 DNA/RNA synthesizer at 1 μmol scale on highly cross-linked polystyrene support. The oligoribonucleotides **2-5** containing 2'-5' phosphate linkages were synthesized using N^4-isobutyryl-5'-*O*-DMT-3'-*O*-TBDMS-2'-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite) prepared by following literature procedures.[15, 36] The underlined bases contain 2'-5' phosphate linkages in the sequence. The oligoribonucleotides were autocleaved on the synthesizer using NH4OH:EtOH (3:1) in 2 h, and the exocyclic amine protecting groups were removed by heating at 55 °C for 3 h. Oligoribonucleotides were synthesized both trityl-on and -off.

Desilylation and desalting: The trityl-off oligoribonucleotides were desilylated by treating at room temperature for 24 h either with a 1M solution of tetra-n-butylammonium fluoride (n-Bu4NF) in THF (15 μL per OD unit) or with neat triethylamine trihydrofluoride (Et3N(HF)3, 10 μL per OD unit). Oligoribonucleotides desilylated by n-Bu4NF were desalted by size exclusion on Sephadex G-25: An equal volume of 0.1 M triethylammonium acetate was added to the reaction mixture and vortexed. The solution was evaporated to one half volume. A 0.7 X 20 cm Econo Column (Bio-Rad, P/N 737-0721) was loaded with a slurry of Sephadex G-25 in sterile water, allowing water to flow through the column until the Sephadex has settled. The column was packed approximately 15 cm. RNA solution was loaded on to the column and sterile water (10 mL) was added when sample solution descended to the Sephadex level. Fractions (1 mL) were collected in sterile tubes (RNA generally elutes in tubes 2-4). Fractions containing RNA were pooled and evaporated to dryness.

Oligoribonucleotides desilylated by Et3N(HF)3 were conveniently desalted by: Water (2 μL per OD unit) and n-butanol (100 μL per OD unit) were added to the reaction mixture and then vortexed. The solution was cooled for 10 min in dry ice and centrifuged for 10 min. The supernatant was discarded and the precipitated oligoribonucleotide was collected.

The trityl-on oligoribonucleotides desilylated by treating with n-Bu4NF were desalted and detritylated on OPC as described. After the desilylation reaction is complete, THF was removed by vacuum centrifugation and the concentrate was dissolved in 1 mL of 0.1M triethylammonium acetate (TEAA). The OPC was washed with 5 mL of acetonitrile followed by 5 mL of 2 M TEAA. The oligoribonucleotide solution was loaded onto the OPC, recycling it through twice (up to 40 OD unit of crude oligoribonucleotide could be loaded on a cartridge). The cartridge was washed with 10 mL of 0.1 M TEAA, followed by 5 mL of 8% acetonitrile in 0.1M TEAA and 10 mL of sterile water. Then 5 mL of 2% TFA was passed through the cartidge (approximately 1 mL solution was pushed through the cartridge and paused for 4 min, then flushed the remaining TFA solution through the cartridge). The column was washed with 10 mL of sterile water. RNA was eluted with 30% aqueous acetonitrile (2 mL) and collected in 1 mL fractions. Appropriate fractions were collected and volatiles were removed to give desalted RNA.

HPLC analysis of oligoribonucleotides: The oligoribonucleotides were analysed by anion exchange HPLC following a published method.[37] HPLC conditions: Nucleopac PA-100 column (250 X 4 mm, Dionex Corporation); solvent A: 20 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5); solvent B: 600 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5); flow rate, 1 mL/min; gradient: 0-70% B in 40 min; detector, 260 nm.

Purification of trityl-on oligoribonucleotides by reverse phase HPLC and detritylation by acetic acid: After the desilylation reaction was complete, THF was removed by vacuum centrifugation. The oligoribonucleotide was dissolved in 0.1M triethylammonium acetate (TEAA) and filtered. The trityl-on oligoribonucleotide was then purified on reverse phase HPLC using the following conditions: RP-18 column (4.6 X 220 mm, 7 μ, Perkin Elmer), solvent A, 0.1 M TEAA; solvent B, CH_3CN; gradient, 10-50% B in 25 min; flow rate, 1 mL/min. Appropriate fractions were combined and evaporated. The purified trityl-on oligoribonucleotide was treated with 80% acetic acid for 15 min at room temperature to remove trityl group. EtOH was added to the reaction mixture and evaporated to dryness and finally precipitated from EtOH.

Enzymatic digest with nuclease P1 and alkaline phosphatase: Lyophilized nuclease P1 was dissolved in 30 mM NH_4OAc (pH 5.3, 1 mg enzyme/mL or 300 units/mL). Alkaline phosphatase was obtained as a suspension in 3.2 M $(NH_4)_2SO_4$/0.1 mM $ZnCl_2$ (pH 7.0). Typically 0.4 A_{260} units of oligoribonucleotides were dissolved in 0.1 M Tris-HCl/1 mM $ZnCl_2$ (pH 7.2, 25 μL) in 0.5 mL micro-centrifuge tube, and

nuclease P1 (4 μL) and alkaline phosphatase (1.5 μL) solutions were added. After incubation at 37 °C for 2 h the samples were diluted with water (100 μL) and extracted with $CHCl_3$ (2 X 150 μL). The aqueous layer was dried and then dissolved in 60 μL of water. The digested samples were analyzed on reverse phase HPLC.

Enzymatic digest with snake venom phosphodiesterase and alkaline phosphatase: The snake venom phosphodiesterase was obtained as a powder which was dissolved in water (1 mg/mL). A digest cocktail (55 μL) for each sample was prepared by mixing the following reagents: water (44 μL), 1 M $MgCl_2$ (0.8 μL), 0.5 M Tris buffer (pH 7.5, 3.5 μL), alkaline phosphatase (4.0 μL) and snake venom phosphodiesterase (2.4 μL). Typically, 0.4 A_{260} of oligoribonucleotide was dissolved in digest cocktail and heated at 37 °C for 8 h. The digest mixture was worked up as described above and digested nucleoside mixture was analyzed on reverse phase column.

HPLC analysis of enzymatic digest: HPLC method: Applied Biosystems 400 solvent delivery system, ABI 783A programable detector, Perkin Elmer ISS200 autosampler, PE Nelson analytical data system, Applied Biosystems RP-18 reverse phase column(220 X 4.6 mm). The detector was set at 254 nm, 0.1 M triethyammonium acetate was solvent A and 80% acetonitrile in water was solvent B. The gradient was 0-25% B in 25 min followed by 25-40% B in 10 min with flow rate 1.0 mL/min. The order of elution of nucleosides were C, U, G and A. The nucleoside composition was calculated from peak areas and reported extinction coefficients.[34]

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