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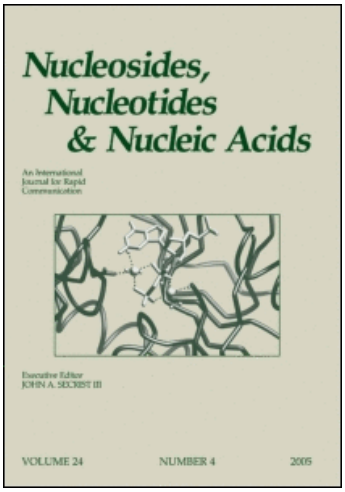

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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To cite this Article Kierzek, Ryszard , Steiger, Michelle A. , Spinelli, Sherry L. , Turner, Douglas H. and Phizicky, Eric M.(2000) 'The Chemical Synthesis of Oligoribonucleotides with Selectively Placed 2'-*O*-Phosphates', Nucleosides, Nucleotides and Nucleic Acids, 19: 5, 917 — 933
To link to this Article: DOI: 10.1080/15257770008033032
URL: http://dx.doi.org/10.1080/15257770008033032

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(5&6), 917–933 (2000)

THE CHEMICAL SYNTHESIS OF OLIGORIBONUCLEOTIDES WITH SELECTIVELY PLACED 2'-*O*-PHOSPHATES.

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ABSTRACT.

A phosphoramidite, solid support method for the chemical synthesis of oligoribonucleotides containing 2'-*O*-phosphate at a selected position is presented. Synthesis of these oligoribonucleotides is based on uridine- and adenosine-(2'-*O*-phosphate)-3'-phosphoramidites, and a new condition for removal of 2'-O-phosphate protecting groups, which does not cleave internucleotide bonds. The structure of oligoribonucleotides with 2'-*O*-phosphate has been proven by enzymatic digestions and dephosphorylation by yeast 2'-phosphotransferase.

Modified RNA and DNA play important roles in many cellular processes including transcription, translation and RNA processing[1]. One such modification is a 2'-*O*-phosphate on ribose. This modification is found as an intermediate of tRNA splicing in yeast, as a consequence of the ligation reaction[2-4]. The intron is excised from pre-tRNA by endonuclease, leaving a 2',3'-cyclic phosphate and a 5'-hydroxyl group on the 5' and 3' exons, respectively. Then tRNA ligase opens the 2',3' cyclic phosphate to a 2'-phosphate, phosphorylates the 5'-hydroxyl, and joins the exons, forming a (3'-5') phosphodiester bond and a 2'-*O*-phosphate.

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Similar ligases, which join RNA molecules to generate a 2'-*O*-phosphate at the junction, have been observed in wheat germ, *Chlamydomonas* and humans[5-7]; it is thus likely that RNAs with a 2'-*O*-phosphate occur widely in eukaryotes. A similar unique site containing a 2'-*O*-phosphate adjacent to a (3'-5') phosphodiester bond has also been found by Kiberstis et al[8] in the circular viral RNA of *Solanum modiflorum* mottle virus (SNMV). The authors speculate that this unique site marks a site of circular ligation from the linear form, and that this feature may be present in many viruses, virusoids and viroids.

The 2'-*O*-phosphate of ligated tRNA, which is one base 3' of the anticodon, is removed by a 2'-phosphotransferase (Tpt1p) both in vitro and in vivo[4,9]. Tpt1p from *Saccharomyces cerevisiae* catalyzes this final step of tRNA splicing by transferring the 2'-*O*-phosphate from ligated tRNA to a molecule of nicotinamide adenine dinucleotide (NAD) to produce ADP-ribose cyclic phosphate (Appr>p)[9]. A similar protein acts on the 2'-*O*-phosphate of ligated tRNA in Xenopus oocytes[9] and HeLa extracts[10].

The manner in which yeast Tpt1p finds its substrate, 2'-phosphorylated tRNA, is unknown. There are three possible mechanisms by which Tpt1p could find its substrate. First, Tpt1p may obtain its substrate from tRNA ligase, the enzyme that produces the 2'-phosphorylated tRNA. This would be an example of substrate channeling. Second, Tpt1p may interact with another protein or factor that recognizes the substrate tRNA. Finally, Tpt1p by itself may bind 2'-phosphorylated tRNA with high affinity and selectivity. We are investigating the final possibility by examining the interaction between Tpt1p and 2'-phosphorylated tRNA. To define the portion of the 2'-phosphorylated tRNA that Tpt1p recognizes, we have made synthetic oligoribonucleotides with a 2'-*O*-phosphate and sequences modeled after the anticodon stem-loop region of 2'-phosphorylated $tRNA^{Tyr}$.

Two methods for chemical synthesis of dimers and trimers with 2'-*O*-phosphate have been reported[11,12]. However, neither method allows synthesis of long oligoribonucleotides. Sekine described the synthesis of a protected adenosine, 2'-*O*-(bis-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityl-6*N*-benzoyl-adenosine, which could be applied to the preparation of a phosphoramidite and used to synthesize long oligoribonucleotides on a solid support[11]. Here, we

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report optimization of the synthesis of this derivative and synthesis of a similarly protected derivative of uridine since a substrate with 2'-*O*-phosphorylated uridine occurs naturally in yeast tRNA processing. Conversion of both protected nucleosides into the corresponding 3'-phosphoramidites allows synthesis of long oligoribonucleotides containing a 2'-*O*-phosphate (Figure 1). We also describe a new condition that allows deprotection of the 2'-*O*-phosphate protecting group without cleavage of phosphodiester bonds. Using this method, several oligoribonucleotides with three to eighteen nucleotides have been prepared. The structures of these oligomers were confirmed by enzymatic digestion and by assaying activity with 2'-phosphotransferase.

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EXPERIMENTAL SECTION.

General Materials and Methods.

The 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityl-6*N*-benzoyladenosine (**6**) was synthesized according to the procedure published by Sekine[11] modified as indicated in Results and Discussion and converted into 3'-phosphoramidite (**7**) as described in Experimental Section. The synthesis of oligoribonucleotides was performed on an Applied Biosystem 392 solid-phase synthesizer using the manufacturer's suggested protocol for RNA synthesis. TLC was performed on silica gel 250F glass plates (Baker) using the following eluents: chloroform/methanol 9:1 v/v (system A), acetone/hexanes/triethylamine 45:45:10 v/v/v (system B), dichloromethane/hexanes/triethylamine 45:45:10 v/v/v (system C), 2-propanol/ammonia/water 7:1:2 v/v/v (system D) and 1-propanol/ammonia/water 55:35:10 v/v/v (system E), saturated ammonium sulfate/water/2-propanol 80:18:2 v/v/v (system F). Silica gel 500F glass plates (Baker) were used for preparative purification of oligomers. For analysis of the enzymatic digestion of trimers, TLC-cellulose plates (Machery-Nagel) were developed in saturated ammonium sulfate/water/2-propanol 80:18:2 v/v/v (system F) and the polyethylenimine-cellulose plates (EM Science) were developed in 2.6 M sodium formate (pH 3.5) (system G). ^{1}H NMR spectra were acquired on General Electric QE-300 and Varian Unity Inova spectrometers and

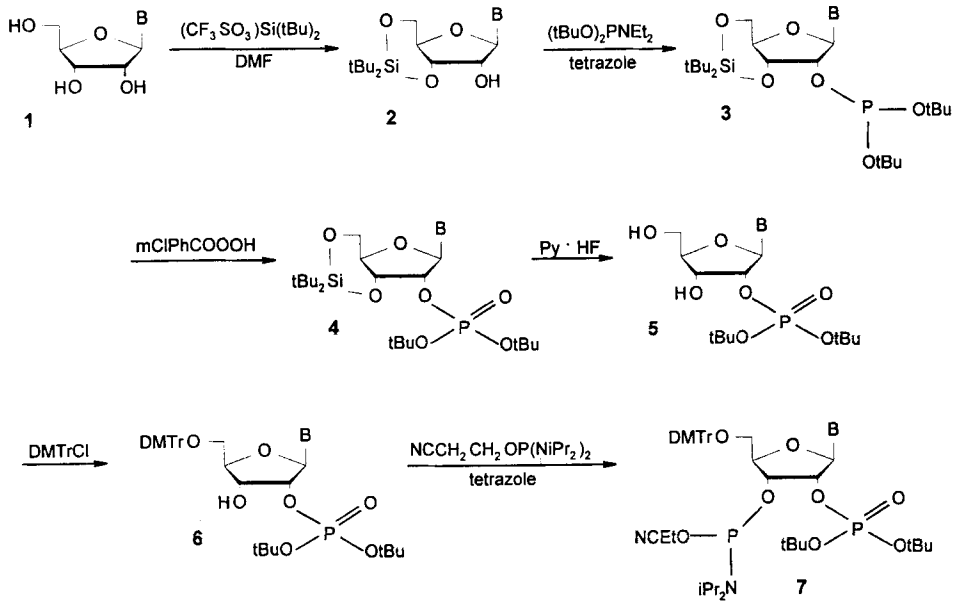

FIGURE 1. General scheme for the chemical synthesis of 2'-*O*-(di-tert-butoxyphosphoryl)-3'-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethoxy)phosphinyl]-5'-*O*-dimethoxytrityl-6*N*-benzoyladenosine and 2'-*O*-(di-tert-butoxyphosphoryl)-3'-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethoxy)phosphinyl]-5'-*O*-dimethoxytrityl-uridine.

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referenced to an external standard of TMS. ^{31}P NMR spectra were acquired on a Bruker AMX-400 spectrometer, referenced to external 1% phosphoric acid and locked in reference to CD_3CN (MSD isotopes). HPLC was performed on a Hewlett Packard series 1100 apparatus with reverse-phase C-8 column (Beckman octyl ultrasphere, 4.6 x 150 mm) using a linear gradient of buffer B (1% buffer B/min) at 1 mL/min flow rate. Buffer A was 10 mM phosphate (pH 7) and buffer B a mixture of buffer A with methanol (1:1 v/v).

Synthesis of 2'-*O*-(di-tert-butoxyphosphoryl)-3'-*O*-[(*N*,*N*-diisopropylamino)-(2-cyanoethoxy)phosphinyl]-5'-*O*-dimethoxytrityl-(*N*-protected) nucleoside.

The 1 mmol of 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityl-6*N*-

benzoyladenosine or protected derivative of uridine and 1.2 mmol of tetrazole were dried under vacuum for several hours. The protected nucleoside and tetrazole were dissolved in 10 mL of anhydrous acetonitrile and 1.5 mmol of 2-cyanoethoxy-di(*N,N*-diisopropylamino)phosphine was added to the stirred solution. Shortly thereafter diisopropylammonium tetrazolide started to precipitate. The reaction mixture was stirred for 2 h at room temperature and silica gel TLC analysis (system B and C) demonstrated completion of reaction. The reaction mixture was worked-up with saturated aqueous sodium bicarbonate (50 mL) and extracted with chloroform containing 2% triethylamine (3 x 50 mL). The organic layers were combined, dried with anhydrous sodium sulfate, and evaporated. The residue was purified by silica gel column chromatography. The column was prepared in a mixture of hexanes/dichloromethane/triethylamine (75:25:2 v/v/v). The amount of dichloromethane was increased by 10% for every 100 mL of eluent. Products were eluted with a mixture of solvents containing ca. 60-70% of dichloromethane. The phosphoramidite was lyophilized from benzene. The yield: 0.91 mmol (91%) for adenosine derivative and 0.82 mmol (82%) for uridine derivative.

*Protected adenosine-(2'-O-phosphate)-3'-phosphoramidite (**7**, B = 6-benzoyladenosine) (for mixture of two diastereoisomers):* TLC - 0.66 (system B), 0.51 and 0.57 (system C); ^{31}P NMR (CD_3CN, 162 MHz) δ (ppm): 157.04, 156.24, -4.09, -3.95; ^{1}H NMR (CD_3CN, 300 MHz) δ (ppm): 1.05-1.40 (30H, m, iPr, tBu), 3.73 (6H, s, OCH_3), 6.27 and 6.29 (1H, 2d, J=6Hz, H-1'), 6.75-6.90 (4H, m, ArH), 7.20-7.65 (15H, m, ArH), 8.29 and 8.30 (1H, 2s, H-2), 8.51 and 8.54 (1H, 2s, H-8).

*Protected uridine-(2'-O-phosphate)-3'-phosphoramidite (**7**, B = uracil) (for higher mobility diastereoisomer):* TLC - 0.65 (system B), 0.44 (system C); ^{31}P NMR (CD_3CN, 162 MHz) δ (ppm): 157.43 and -3.77; ^{1}H NMR (CD_3CN, 300 MHz) δ (ppm): 1.10-1.25 (12H, m, iPr,), 1.36, 1.40, 1.42 (18H, 3s, tBu), 3.76 (6H, s, OCH_3), 5.37 (1H, 2d, J=4.5Hz, H-5), 6.11(1H, d, J=4Hz, H-1'), 6.80-6.95 (4H, m, ArH), 7.30-7.50 (9H, m, ArH), 7.62 (1H, d, J=4.5Hz, H-6).

*Protected uridine-(2'-O-phosphate)-3'-phosphoramidite (**7**, B = uracil) (for lower mobility diastereoisomer):* TLC - 0.62 (system B), 0.35 (system C); ^{31}P NMR

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(CD_3CN, 162 MHz) δ (ppm): 156.85 and -3.89; ^1H NMR (CD_3CN, 300 MHz) δ (ppm): 1.03 and 1.16 (12H, 2d, J=7Hz, iPr,), 1.43 (18H, s, tBu), 3.75 (6H, s, OCH_3), 5.38 (1H, 2d, J=4.5Hz, H-5), 6.09(1H, d, J=4Hz, H-1'), 6.80-6.95 (4H, m, ArH), 7.25-7.45 (9H, m, ArH), 7.58 (1H, d, J=4.5Hz, H-6).

Deprotection and purification of oligoribonucleotides.

The support containing oligoribonucleotides was treated with a mixture of ethanol and concentrated aqueous ammonia (1:3 v/v) for 16 h at 55°C. The solid support was filtered off and filtrate was evaporated to dryness. The residue was coevaporated with pyridine and a pyridine solution of triethylammonium fluoride was added (50 fold for every tert-butyldimethylsilyl) and left for 48 h at 55°C. The pyridine was evaporated, the residue dissolved in 1 mL of water and extracted with ethyl ether (3 x 2 ml). The aqueous layer was diluted to 10 mL with 10 mM ammonium acetate and passed through a sep-pak column. The fraction eluted with a mixture of water and acetonitrile (7:3 v/v) was evaporated. The residue was dissolved in 0.5 mL of 0.01 M HCl (pH 2); the pH value was adjusted to 2 with 0.1 M HCl and the sample was incubated 3 h at 37°C. The reaction mixture was neutralized with diluted ammonia and purified on silica gel TLC plates in system D or E (dependent on length of oligomer) or by polyacrylamide gel electrophoresis (PAGE). The spot containing product was scratched-out and oligomer was eluted with water and passed through a sep-pak column again.

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Analysis of AU^pU.

AU^pU was 5' end labeled with [γ - ^{32}P] ATP and polynucleotide kinase, and the kinase product was purified from a silica TLC plate developed in system E (symbol p in superscript marks position of the 2'-*O*-phosphate). The p^*AU^pU was treated with 53 nM 2'-phosphotransferase (expressed in and partially purified from *E. Coli* as a His_6-tagged protein) and 1 mM NAD for 30 min at 30°C as described previously[13]. Reaction mixtures (10 μL) were then treated with either 10 μg ribonuclease A, 1μg nuclease P1, or 0.1 units calf intestinal alkaline phosphatase, as indicated, for 30 min at 37°C, and analyzed on polyethylenimine-cellulose plates developed in system G.

Analysis of GUAA^{p}AUCU and ACAAGACUGUAA^{p}AUCUUG.

The octamer and the octadecamer were 5' end labeled and treated with 2'-phosphotransferase (2.1 nM) and NAD as described above. Portions of these reactions were treated with 50 mM sodium carbonate, pH 9.1 at 95°C for 5 min to produce the base hydrolysis ladders, stopped on ice with the addition of 40 mM Tris, pH 7.5 and 0.08 mM EDTA, and analyzed on 20% polyacrylamide gels containing 8.3 M urea and TBE (88 mM Tris-borate, 1 mM EDTA, pH 8.3).

RESULTS AND DISCUSSION.

The synthesis of 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-(dimethoxytrityl)-6*N*-benzoyladenosine (**5**) has been described by Sekine[11]. However, many steps of the synthesis proceed in milder conditions than originally described. For example, phosphorylation of the 2'-hydroxyl group of 3',5'-*O*-(di-tert-butylsilanediyl)-6*N*-benzoyladenosine (**2**) proceeds quantitatively with 5 equivalents of di-tert-butoxy(*N,N*-diethylamino)phosphine[14] in the presence of 5 equivalents of tetrazole instead of 9 and 13.5 equivalents of phosphine and tetrazole, respectively. After oxidation with m-chloroperbenzoic acid, the reaction mixture was purified by silica gel column chromatography using a mixture of hexanes, chloroform and triethylamine as eluent. Product (**4**) was obtained in 75% yield (based on 6*N*-benzoyladenosine). The presence of triethylamine during work-up and purification of derivatives containing 2'-*O*-(di-tert-butoxyphosphate) prevents significant formation of the diester derivative due to deprotection of the 2'-*O*-phosphate protecting group. Moreover, triethylamine prevents this side reaction more effectively than pyridine. The use of hexanes for purification allows easy removal of the large excess of phosphine (5 equivalents) used for phosphorylation. We found that removal of the 3',5'-silyl protecting group by the literature procedure gives some amounts of charged side products[11]. However, 4 equivalents of pyridinium fluoride in pyridine (10 min at room temperature) selectively and quantitatively removed the di-tert-butylsilyl group. Pyridinium fluoride was prepared by coevaporation of an aqueous solution of hydrofluoric acid and pyridine and it was dissolved in pyridine to give

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a 0.5 M solution. After desilylation and protection of 5'-hydroxyl with dimethoxytrityl chloride, 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityl-6*N*-benzoyladenosine (**6**) was obtained in 64% yield. The derivative of uridine was prepared in a similar manner with similar yield. Both derivatives, 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityl-6*N*-benzoyladenosine (**6**, B = 6-benzoyladenine) and 2'-*O*-(di-tert-butoxyphosphoryl)-5'-*O*-dimethoxytrityluridine (**6**, B = uracil), were quantitatively converted over a period of 2 h into the corresponding 3'-phosphoramidites (**7**) using 1.5 equivalent of 2-cyanoethoxy-bis(*N,N*-diisopropylamino)phosphine in the presence of 1.2 equivalents of tetrazole. After purification on a silica gel column, the 3'-phosphoramidites were obtained in 80-90% yield.

The two diastereoisomers of the 3'-phosphoramidite of adenosine (**7**, B = 6-benzoyladenine) gives four ^{31}P resonances by NMR: 157.04 ppm and 156.24 ppm for trivalent phosphorus and -3.95 ppm and -4.09 ppm for pentavalent phosphorus. Two resonances for the 2'-*O*-(di-tert-butoxylphosphoryl) group can be explained by the presence of the chiral phosphorus on the 3'-phosphoramidite moiety. In 1H NMR spectra it has been observed that the presence of a chiral center (on carbon or phosphorus) leads to a different chemical shift for the same protons in each diastereoisomer[15,16]. Several oligoribonucleotides were synthesized by the phosphoramidite[17] method on solid support with 2'-hydroxyl protected by a tert-butyldimethylsilyl group[18]. The coupling yield of phosphoramidite with 2'-protected phosphate was at least 93%, as determined by high performance liquid chromatography (HPLC) analysis of crude reaction mixtures of the dimer A^pA. The following sequences of the oligoribonucleotides were synthesized: AA^pA, pAA^pA, AU^pU, pAU^pU, AAA^p, AAA^pp, AAA^pOCH_3, $UA^pU^{(5Br)}$, $GUAA^pAUCU$ and $ACAAGACUGUAA^pAUCUUG$. The trimers are fragments of the anticodon loop of $tRNA^{Tyr}$ or simple variants derived from this sequence[19]. The octadecamer $ACAAGACUGUAA^pAUCUUG$ corresponds to the entire anticodon stem and loop of $tRNA^{Tyr}$, whereas $GUAA^pAUCU$ is a fragment thereof.

For deprotection, oligoribonucleotides were treated with a mixture of ethanol and aqueous ammonia[20] followed by a pyridine solution of

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triethylammonium fluoride[15]. Initially, the last step in deprotection was treatment with a mixture of trifluoroacetic and acetic acids (1:4 v/v) for 4 h at room temperature to remove the tert-butyl groups used for protection of the 2'-*O*-phosphate[11,21]. Sekine used this condition to remove the tert-butyl protection of 2'-*O*-phosphate in dimers and trimers with a 2'-*O*-phosphate at every nucleoside[11]. We were interested in synthesis of oligoribonucleotides containing only one 2'-*O*-phosphate per oligomer. Analysis of the reaction mixture after removal of the tert-butyl group showed significant amounts of (3'-5') phosphodiester bond cleavage. Moreover, we observed that the first of the two t-butyl protecting group was removed during ammonia treatment (see below for details) Model studies of the dimer - adenylyl-(3'-5')-adenosine, demonstrated that treatment with a mixture of trifluoroacetic and acetic acids (1:4 v/v) for 4 h at room temperature results in ca. 50% cleavage of the internucleotide bond. HPLC analysis of products of this hydrolysis indicated a mixture of adenosine-2'- and 3'-*O*-phosphate, adenosine, adenine and one unidentified product (data not shown). Thus this deprotection method was not suitable for synthesis of long oligoribonucleotides. For this reason, a new and milder acidic condition was developed. Selective and complete removal of tert-butyl groups was achieved with 0.01M HCl (pH 2) at 37°C (Figure 2). Thin layer chromatography (TLC) and HPLC showed that the reaction was complete within 2-4 h. At 37°C, no products of (3'-5') phosphodiester bond cleavage were found after 24 h of incubation. At room temperature, the deprotection reaction was complete after 24 h. These results were consistent with Reese's reports that the internucleotide bond of adenylyl-(3'-5')-adenosine is stable in 0.01 M HCl (pH 2) at room temperature for at least 96 h[22].

The stability of the phosphodiester bond during removal of the tert-butyl from the 2'-*O*-phosphate was also confirmed by polyacrylamide gel electrophoresis (PAGE). In Figure 3, the comparison of lanes a (crude reaction mixture octadecamer - ACAAGACUGUAA^{p}AUCUUG containing tert-butyl protecting group) and c (TLC purified octamer - GUAA^{p}AUCU containing tert-butyl protecting group) with lanes b and d (fully deprotected octadecamer and octamer, respectively) indicates the stability of the internucleotide bond during

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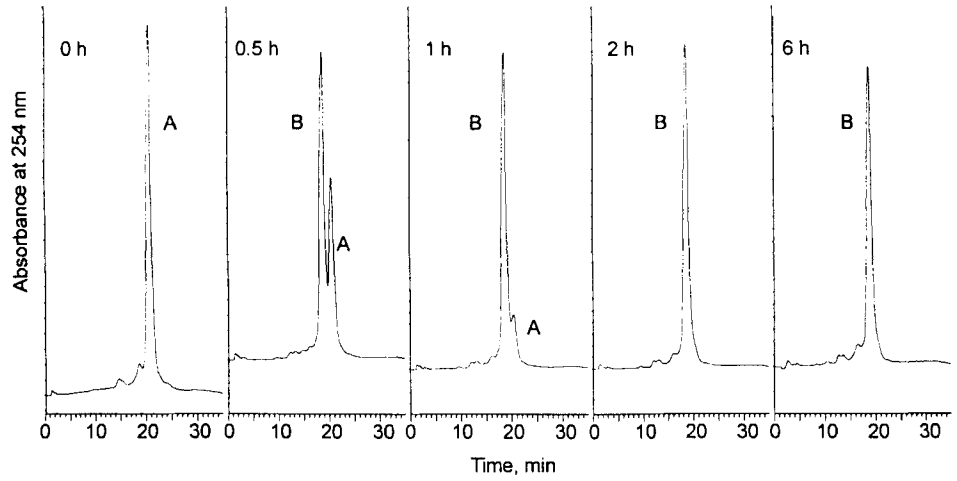

FIGURE 2. HPLC analysis of deprotection of the tert-butyl protecting group of 2'-*O*-phosphate of GUAA^{p}AUCU. The analysis was performed after 0, 0.5, 1, 2 and 6 h treatment with 0.01M HCl (pH 2) at 37°C. Peak A at 20.6 min corresponds to the octamer with a tert-butyl group on the 2'-*O*-phosphate, and peak B at 18.5 min corresponds to fully deprotected octamer.

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removal of the 2'-*O*-phosphate protecting group. It is interesting that the tert-butyl protected 2'-*O*-phosphate does not significantly affect polyacrylamide gel mobility of the octadecamer and octamer (Figure 3, lanes a-d). TLC analysis (system E) of both oligoribonucleotides indicates a difference of mobility of fully deprotected and tert-butyl 2'-*O*-phosphate protected oligomers.

Additional experiments demonstrated that the first of the two tert-butyl protecting groups on the 2'-*O*-phosphate was removed during ammonia treatment. Model studies showed that 2'-*O*-(tert-butoxyphosphoryl) diester is formed when 3',5'-di(dimethoxytrityl)-2'-*O*-(di-tert-butoxyphosphoryl)uridine is treated with a mixture of ethanol and aqueous ammonia (1:3 v/v, 16 h at 55°C). Treatment of the 3',5'-di(dimethoxytrityl)-2'-*O*-(tert-butoxyphosphoryl)uridine with 0.01 M HCl (pH 2) for 4 h at 37°C converts it quantitatively into uridine-2'-*O*-phosphate. Removal of one tert-butyl protecting group from 2'-*O*-phosphate was also proven by ^{1}H and ^{31}P NMR spectra of adenylyl-(2'-*O*-phosphate)-(3'-5')-

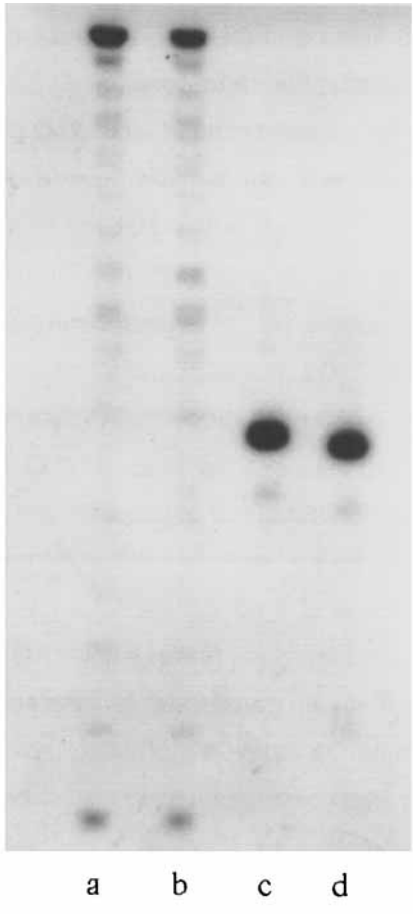

FIGURE 3. Polyacrylamide gel electrophoresis analysis (20% gel) of octadecamer -ACAAGACUGUAA^{p}AUCUUG and octamer - GUAA^{p}AUCU. Lanes a and c correspond to oligomers with a tert-butyl group on 2'-*O*-phosphate and b and d to the fully deprotected octadecamer and octamer, respectively. The octadecamer is a crude reaction mixture whereas the octamer was pre-purified on TLC silica gel before analysis. Removal of the tert-butyl group could also be observed by silica gel TLC. For example, the mobility (R_f) of octamer GUAA^{p}AUCU changed from 0.31 to 0.18 (system E) for tert-butyl protected and fully deprotected octamer, respectively.

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uridine recorded after each step of deprotection. After ammonia treatment, a singlet containing only 9 protons (1.03 ppm, tert-butyl group) was observed by ^{1}H NMR. The phosphorus spectrum had peaks at -0.725 ppm and -4.225 ppm, corresponding to the internucleotide bond and 2'-*O*-phosphate, respectively. Subsequent treatment with triethylammonium fluoride does not change the ^{1}H and ^{31}P NMR spectra demonstrating the stability of the tert-butyl protecting group of 2'-*O*-phosphate during this step of deprotection. However, after treatment with 0.01 M HCl (pH 2) the ^{1}H resonance at 1.03 ppm disappears, and the ^{31}P resonances for the 2'-*O*-phosphate and internucleotide bond phosphorus move to -0.112 ppm and -0.658 ppm, respectively (data not shown).

All synthetic oligoribonucleotides with a 2'-*O*-phosphate serve as a substrate for yeast Tpt1p. Treatment of 2'-phosphorylated oligomers with Tpt1p and NAD causes a shift in the mobility of the oligomers on a TLC plate, corresponding to the removal of a phosphate from the RNA. Furthermore, we have shown that for several oligomers (for example: $AA^{p}A$, $pAA^{p}A$, octamer and octadecamer) the phosphate is transferred to labeled NAD, making labeled Appr>p. To further examine the structures of these molecules, we analyzed the products formed when the oligoribonucleotides were treated with various nucleases, as described below.

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For $p^{*}AU^{p}U$, mobility on TLC shifts after treatment with both Tpt1p and NAD (Figure 4, lanes a-d). The substrate $p^{*}AU^{p}U$ is resistant to ribonuclease A, as expected, since the internal uridine residue has a phosphate and not a hydroxyl group at 2'-position. However, treatment of $p^{*}AU^{p}U$ with NAD and Tpt1p removes the 2'-*O*-phosphate and produces a molecule that is susceptible to RNase A cleavage (Figure 4, lanes e-h). Nuclease P1 and calf intestinal phosphatase (CIP) digestions of $p^{*}AU^{p}U$ produce p*A and inorganic phosphate, respectively, independent of the prior $p^{*}AU^{p}U$ treatment. This series of treatments to $p^{*}AU^{p}U$ indicates that there is a phosphate at the 2'-position between the uridine residues, and this phosphate is removed by the Tpt1p with NAD.

The trimers with an internal 2'-*O*-phosphate were analyzed also without labeling, by treatments with RNase T2, nuclease P1 and snake venom

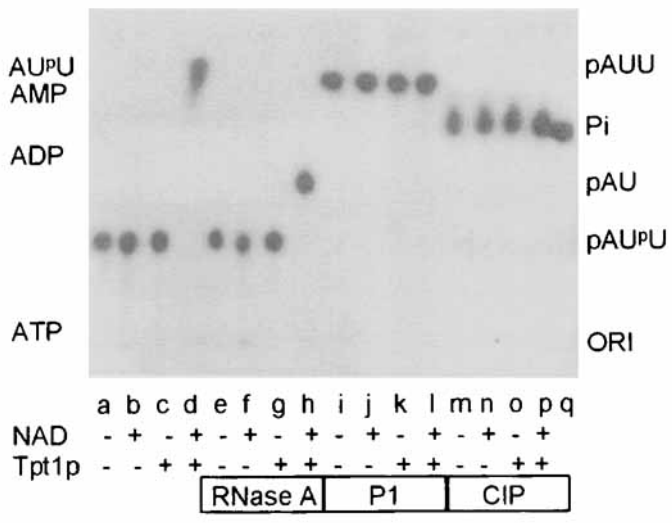

FIGURE 4. Analysis of AU^{p}U. Trimer was labeled at the 5' end as described in Materials and Methods and treated with NAD or Tpt1p or both as indicated. Reaction mixtures were then treated with ribonuclease A (lanes e-h), nuclease P1 (lanes i-l), or calf intestinal alkaline phosphatase (lanes m-p) and resolved on a polyethylenimine TLC plate in system G. Migration of unlabelled standards is indicated on the left; lane q is inorganic phosphate standard.

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phosphodiesterase. For AA^{p}A, nuclease P1 treatment results in the release of A and a molecule that has a TLC mobility consistent with pA^{p}A, while RNase T2 treatment produces Ap and a molecule with TLC mobility consistent with A^{p}A. As expected snake venom phosphodiesterase, can cleave across the phosphodiester bond bearing the 2'-*O*-phosphate, resulting in roughly equal amounts of A, pA^{p}, and pA. The identity of pA^{p} was confirmed because the TLC system used to resolve these products (system F), easily separates pA^{p} (terminal 2'-*O*-phosphate) from pAp (terminal 3'-*O*-phosphate), and because treatment of the venom products with nuclease P1 (which has a 3' phosphatase activity) does not change the mobility of pA^{p} (data not shown). Other trimer oligonucleotides (pAA^{p}A, AU^{p}U and pAU^{p}U) were analyzed in the same fashion, although a pU^{p} standard was not available for comparison.

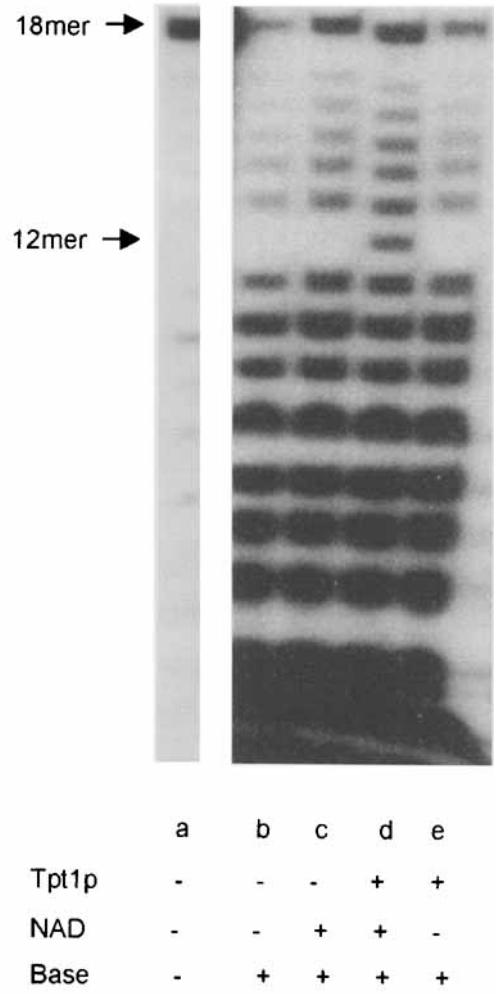

FIGURE 5. Analysis of ACAAGACUGUAA^{p}AUCUUG. The octadecamer was labeled at the 5' end and treated with NAD or Tpt1p as indicated. Reaction mixtures were then subjected to base hydrolysis and resolved on a denaturing 20% polyacrylamide gels.

Trimers with a terminal 2'-*O*-phosphate were similarly analyzed with RNase T2 and nuclease P1. As expected, treatment of AAA^{p} with RNase T2 produced Ap and A^{p} in approximately the expected ratio, and nuclease P1 treatment produced A, pA, pA^{p}. Likewise, AAA^{p}p and AAA^{p}pOCH$_3$ produced the

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expected products after RNase T2 treatment, based on comparison to the mobility of synthetic A^pp and A^ppOCH_3 standards (data not shown).

The octamer and octadecamer with an internal 2'-*O*-phosphate were analyzed by base hydrolysis. Base hydrolysis, like RNase A cleavage, requires a hydroxyl group at the 2'-position for cleavage of the RNA phosphodiester backbone at that residue. Thus, base hydrolysis of substrates bearing an internal 2'-*O*-phosphate will result in cleavage at every position except between the two nucleotides where there is a 2'-*O*-phosphate. As shown in Figure 5, the base hydrolysis ladder of the octadecamer does not have a product that is 12 nucleotides long, as expected since the 2'-*O*-phosphate is located between residues 12 and 13 from the 5' end (lane b). However, after the octadecamer is treated with Tpt1p and NAD, base hydrolysis readily yields a product that is 12 nucleotides long (Figure 5, lane d). This indicates that there is a blockage at the 2'-position between the expected nucleotides, and that this blockage is a phosphate. Similarly, the base hydrolysis ladder produced by the octamer does not have a product that is 4 nucleotides long, as expected (since the 2'-*O*-phosphate is between bases 4 and 5), unless it is treated with Tpt1p and NAD (data not shown). Partial sequence analysis of the octamer confirms also that the nucleotides in this RNA oligomer are correct (data not shown).

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The octadecamer adopts the expected hairpin structure in solution. This oligomer, ACAAGACUGUAA^{p}AUCUUG, corresponds to the anticodon stem and loop of $tRNA^{Tyr}$. UV-melting studies of this oligomer at 9 different strand concentrations ranging from 1.5×10^{-4} M to 3.6×10^{-6} M gave essentially identical values for melting temperature, ΔH°, and ΔS°. In the buffer used for dephosphorylation experiments (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM spermidine, 0.1 mM dithiothreitol), the thermodynamic parameters (ΔH° = -47.4 kcal/mol, ΔS° = -144.1 eu, ΔG°_{37} = -2.68 kcal/mol and Tm = 55.6°C) are very similar to those expected for melting in a buffer containing 1M NaCl, 20mM cacodylic buffer (pH 7) and 1mM EDTA[23].

ACKNOWLEDGMENTS:

Supported by NIH Grant GM22939 (D.H.T), NIH Grant RO1-GM52347 (E.M.P) and State Committee for Scientific Research Grant 3T09A 023 11 (R.K.).

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Received 10/20/99
Accepted 2/9/00

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