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Solid-Phase Synthesis of RNA Analogs Containing Phosphorodithioate Linkages

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Abstract

The oligoribonucleotide phosphorodithioate modification (PS2-RNA) uses two sulfur atoms to replace two non-bridging oxygen atoms at an internucleotide phosphodiester backbone linkage. Like a natural phosphodiester RNA backbone linkage, a PS2-modified backbone linkage is achiral at phosphorus. PS2-RNAs are highly stable to nucleases and several *in vitro* assays have demonstrated their biological activity. For example, PS2-RNAs silenced mRNA *in vitro*, and bound to protein targets in the form of PS2-aptamers (thioaptamers). Thus, the interest in and promise of PS2-RNAs has drawn attention to synthesizing, isolating, and characterizing these compounds. RNA-thiophosphoramidite monomers are commercially available from AM Biotechnologies and this unit describes an effective methodology for solid-phase synthesis, deprotection, and purification of RNAs having PS2 internucleotide linkages.

Keywords

Solid-Phase Synthesis; phosphorodithioate oligoribonucleotide; PS2-RNA; sulfur-modified oligonucleotide; thiophosphoramidite

INTRODUCTION

The substitution of both non-bridging phosphate oxygen atoms in an oligodeoxynucleotide (DNA) or oligoribonucleotide (RNA) phosphodiester linkage with sulfur atoms gives rise to a phosphorodithioate (PS2) internucleotide linkage (Figure 1), which like natural phosphodiester linkages, is achiral at phosphorus (Marshall and Caruthers, 1993; Wiesler and Caruthers, 1996; Yang, 2016; Yang and Gorenstein, 2004; Yang and Mierzejewski, 2010; Yang et al., 2006). The PS2 substitution is a very attractive RNA analog because it closely mimics natural RNA, and should have other biochemical and biophysical properties similar to RNA with natural phosphodiester linkages. In addition, it has been shown that dinucleoside phosphorodithioates (PS2-dimers) have high nuclease resistance (Petersen and Nielsen, 1990). Moreover, a hammerhead ribozyme with a single PS2 substitution at the cleavage site was evaluated its cleaving rate to the expected product (Derrick et al., 2000). Further, PS2-dimers have shown great resistance to alkaline degradation when compared to natural RNA derivatives (Petersen and Nielsen, 1990). We have recently demonstrated that siRNAs containing several PS2 linkages in certain positions enhance gene silencing activity *in vitro* (Wu et al., 2014; Yang et al., 2012) and remarkably enhance binding affinity toward proteins (Abeydeera et al., 2016; Pallan et al., 2014). Over the last decade, the reported chemistries for preparation of PS2-RNA include the thiophosphoramidite solution-phase

synthesis of PS2-dimers (Petersen and Nielsen, 1990), the H-phosphonothioate solid-phase syntheses of a 12-mer RNA containing a single PS2 substitution (Derrick et al., 2000), a 15-mer RNA bearing exclusively PS2 substitutions (Greef et al., 1996), and thiophosphoramidite automated solid-phase synthesis of PS2-RNA via either 2,4-dichlorobenzyl sulfur protection group (Li et al., 2012) or β -(benzoylmercapto)ethyl protection group (Sierant et al., 2011; Yang et al., 2012). This unit describes the automated solid-phase synthesis of RNA containing achiral PS2 internucleotide linkages (Figure 1). The protocols described herein include synthesis of PS2-RNA on a DNA/RNA synthesizer using β -(benzoylmercapto)ethyl protected thiophosphoramidites (see Basic Protocol 1) wherein the 2'-hydroxy group is protected with *t*-butyldimethylsilyl (TBDMS) group. The protocols also describe methods for deprotection and purification of PS2-RNAs (see Basic Protocol 2).

The RNA-thiophosphoramidite building blocks (Figure 2) described by Yang's lab (Yang et al., 2012) are commercially available from AM Biotechnologies. A typical cycle (Figure 3) for the solid-phase synthesis of a PS2 linkage differs from a standard cycle for the synthesis of normal phosphate linkages. Each addition of a PS2-nucleotide using a RNA-thiophosphoramidite requires four standard steps: detritylation, coupling, sulfurization, and capping. However, RNA-thiophosphoramidite monomers require longer coupling and sulfurization times compared to normal phosphoramidite coupling inclusive of sulfurization, which forms phosphoromonothioate (PS)-RNA products containing chiral centers at phosphorus (Sierchala et al., 1996; Yang et al., 1998).

BASIC PROTOCOL 1. SOLID-PHASE ASSEMBLY OF PROTECTED RNA-THIOPHOSPHORAMIDITES

The solid-phase synthesis of PS2-RNAs can be accomplished manually, but the use of an automated DNA/RNA synthesizer is certainly preferred. This protocol describes the setup and step-by-step instructions for solid-phase synthesis of PS2-RNAs on the Expedite 8909 DNA/RNA synthesizer equipped with a workstation (PerSeptive Biosystems), but can easily be adapted to other automated DNA/RNA synthesizers. All syntheses used to develop this protocol were performed on a 1 μ mole scale. The synthesis is performed on standard controlled-pore glass (CPG) solid support RNA synthesis columns with long-chain alkylamino (LCAA) linker. A major difference between standard RNA synthesis and PS2 linkage synthesis is the coupling time. An RNA-thiophosphoramidite requires ca. 12-min coupling step on the Expedite Synthesizer using 5-(bis-3,5-trifluoromethylphenyl)-1H-tetrazole (Activator 42) as activator (Table 1), whereas standard RNAs require ca. 4-min coupling using Activator 42. Another difference is that the sulfurization step is accomplished prior to capping and the sulfurization time has to be extended to 320 sec compared to about 120 sec for standard PS-ODN synthesis using 3-ethoxy-1,2,4-dithiazolidine-5-one (EDITH) as the sulfurization reagent.

Materials

RNA-thiophosphoramidites (AM Biotechnologies):

1. Bz-rA-thiophosphoramidite

2. Ac-rC-thiophosphoramidite
3. Ac-rG-thiophosphoramidite
4. rU-thiophosphoramidite

Normal RNA phosphoramidites (Glen Research):

1. Bz-A-CE phosphoramidite
2. Ac-C-CE phosphoramidite
3. Ac-G-CE phosphoramidite
4. U-CE phosphoramidite

XX-RNA-CPG column (Glen Research)

Helium gas, anhydrous

Diluent:

1. acetonitrile, anhydrous (Glen Research)
2. 10% anhydrous dichloromethane in anhydrous acetonitrile

Activator: 0.25 M activator-42 (Sigma-Aldrich)

Cap Mix A: tetrahydrofuran/acetic anhydride (THF/AC₂O) (Glen Research)

Cap Mix B: 10% 1-methylimidazole in tetrahydrofuran/Pyridine (Glen Research)

Oxidizing solution: 0.02 M I₂ in THF/H₂O/pyridine (Glen Research)

Deblocking mix: 3% trichloroacetic acid in dichloromethane (Glen Research)

Sulfurization reagent: 3-ethoxy-1,2,4-dithiazolidine-5-one (EDITH), Concentration, 0.2 M (163 mg in 20 mL acetonitrile), molecular weight: 163.22, Carbosynth Limited, UK.

Synthesizer vials with caps

Vacuum desiccators

Expedite 8909, Perseptive Biosystem with trityl monitor

Synthesize PS2-RNAs using RNA-thiophosphoramidites

1. Calculate the amount of the RNA-thiophosphoramidites required for the synthesis of PS2-linkages.

The typical concentration of RNA-thiophosphoramidites used with the Expedite Synthesizer is 0.15 M. Note that the volume of RNA-thiophosphoramidite solution delivered to the column during each coupling step is about 250 μ L. However, an excess of 700 μ L of 0.15 M RNA-thiophosphoramidite solution should be included when preparing each RNA-thiophosphoramidite solution in order to completely purge the lines of the synthesizer.

2. Weight out the calculated amount of each RNA-thiophosphoramidite into the appropriate synthesizer vial and leave the vial to dry in a high vacuum desiccator overnight.
3. Flush the desiccator with dry argon gas before opening, and carefully remove the pre-weighted RNA-thiophosphoramidites.
4. Edit the synthetic cycle for RNA-thiophosphoramidite based on the activator of choice.

Activator-42: Table 1.

5. Edit the sequence to be synthesized.
6. Dissolve the RNA-thiophosphoramidites in anhydrous acetonitrile, except for Bz-A and AC-G thiophosphoramidites that are dissolved in anhydrous acetonitrile containing 10% anhydrous dichloromethane (DCM) (v/v) to a final concentration of *ca.* 0.15 M.

Use an argon-dried syringe to transfer the anhydrous acetonitrile or acetonitrile containing 10% anhydrous DCM through the rubber septum of each capped synthesizer vial to ensure the reagents are protected from humidity. Do not open the RNA-thiophosphoramidite solution vials until they are ready to be installed directly on the synthesizer. In addition, unlike normal RNA phosphoramidites, the Bz-A and Ac-G RNA-thiophosphoramidites are not completely soluble in anhydrous acetonitrile diluent. Rather, 10% anhydrous DCM (v/v) in acetonitrile is an ideal solvent for the Bz-A and Ac-G thiophosphoramidites for a final concentration of 0.15 M. For convenience, 10% anhydrous DCM (v/v) in acetonitrile can be used for all four RNA-thiophosphoramidites.

7. Thoroughly wash the RNA-thiophosphoramidite line with anhydrous acetonitrile prior to installing the RNA-thiophosphoramidite solution vial.
8. Purge the lines of the synthesizer with all the solutions and solvents.
9. Install the appropriate nucleoside column.
10. Start synthesis, choosing the “DMT-Off” option.
11. Check the coupling efficiency periodically using the trityl monitor.
12. Remove the column from the synthesizer when the synthesis is complete, then dry the column with argon.
13. Deprotect and purify the synthesized PS2-RNAs (see Basic Protocol 2).

BASIC PROTOCOL 2. DEPROTECTION AND PURIFICATION OF PS2-RNAs

This protocol describes the steps necessary to remove the protecting groups from the heterocyclic bases and phosphotriesters and/or phosphorodithiotriesters, which also cleaves the PS2-ODNs from their solid support. In addition, removal of the 2'-O-TBDMS protection group is described. The procedure for quantifying isolated PS2-RNAs is further described.

PS2-RNAs can be successfully analyzed and purified by HPLC using an anion-exchange column or by denaturing gel electrophoresis. Desalting PS2-RNAs after chromatographic or gel isolation using one of several methods completes the purification procedure. Common desalting methods include RP-HPLC, size-exclusion chromatography or reverse-phase SepPak C18 cartridge desalting.

Materials

Fully protected PS2-RNAs attached to the solid support of a synthesis column (see Basic Protocol 1)

Anhydrous ethanol (Sigma-Aldrich)

Acetonitrile (HPLC grade, TEDIA)

Trizma hydrochloride (Sigma-Aldrich)

EDTA (Sigma-Aldrich)

Sodium hydroxide (Sigma-Aldrich)

>28% Ammonium hydroxide or concentrated ammonium hydroxide (Sigma-Aldrich)

41% Methyl amine (Fluka, Cat. # 65580)

Triethylamine (TEA) (Sigma-Aldrich)

DL-Dithiothreitol (Sigma-Aldrich)

Triethylamine trihydrofluoride 98% (Sigma-Aldrich, Cat. # 344648)

N,N-Dimethylformamide (DMF) (Sigma-Aldrich)

Ammonium acetate (HPLC grade, Fluka)

Diethylpyrocarbonate (DEPC) (Sigma-Aldrich)

Sterile syringe filter, 0.2 μ m cellulose acetate (VWR, Cat.#28145-477)

DIONEX DNAPac PA-100 4 $\times$ 250 mm analytical column (Product No. 043010)

A divinyl benzene/polystyrene copolymer reverse-phase column (Hamilton PRP-1): Part No. 79426, Flow=2 ml/min or Part No. 79425, Flow=1 mL/min

Mono Q column (GE Healthcare Life Sciences)

Amicon ultra-15 centrifugal filter units, 3000 Da cut-off (Millipore Sigma)

Deprotection of base-labile protection groups of PS2-RNAs

- 1 Dry the synthesis column with argon gas.
- 2 Transfer the fully protected ODNs attached to the solid support of the synthesis column into a 4.0 mL sealable vial.
- 3 Add 1.0 mL of concentrated ammonia:ethanol (3:1, v:v) mix containing 20 mM DTT.
- 4 Seal the vial and incubate the vial at 55 °C for 15–16 hr.

Alternatively, a solution of AMA was prepared by mixing equal volumes of 41% methyl amine and ammonium hydroxide (1:1, v:v) containing 20 mM DTT. 1.0 mL of AMA solution is used instead of 1.0 mL of ammonia:ethanol on step 3. The vial is sealed and then incubated at 65°C for 15 min.

- 5 Once the deprotection is complete, cool the vial into room temperature.
- 6 Add 1 mL of de-ionized water into the vial, then transfer the supernatant into a 15 mL conical tube, wash the solid support with 0.1% diethylpyrocarbonate (DEPC)-treated water (4 X 1 mL), combine the wash supernatant into the 15-mL conical tube.
- 7 Place the 15 mL conical tube into -70°C for 1 hr.
- 8 Lyophilize the ODN sample to dryness.

2'-O-TBDMS protection group removal

- 9 Upon removal from the lyophilizer, the sample is placed in a stream of argon gas to avoid moisture. A 115 μL aliquot of DMF is added to each sample and the sample is then heated for 5min at 65°C to dissolve the oligo. 60 μL of TEA is added to each solution and mixed gently. 75 μL of is added to each solution and the tube is then sealed tightly and placed at 65°C for 2.5 hours. After incubation, the sample is allowed to cool to room temperature before proceeding to step 10.

Quantitation of the PS2-RNAs

(All the water used below are DEPC-treated!)

- 10 Add 750 μL of DEPC-treated water to the vial of step 9 to quench the reaction. Quickly filter the solution via 0.22 μm sterile syringe filter, then wash the filter by using 1 mL of DEPC-treated water.
- 11 Remove a 10- μL aliquot and dilute this aliquot to 1 mL with deionized water in a UV cuvette.
- 12 Measure the absorption of the sample at 260 nm. Calculate the amount of the crude PS2-RNA obtained in A_{260} units. The total $\text{OD}_{260} = 100 \times A_{260}$
- 13 Calculate the number of milligrams from the OD_{260} using the following conversion factors

For single stranded PS2-RNA, 1 OD_{260} is ca. 40 μg .

Analysis of crude PS2-RNA by denaturing PAGE

- 14 Prepare a 24% denaturing polyacrylamide gel on a 0.75-mm-thick analytical gel plate for gel electrophoresis.
- 15 Prepare samples of PS2-RNAs to be analyzed (e.g., about 150–200 ng per well). Dissolve each sample in 10 μL of loading buffer.

- 16 Load the samples into wells and run them next to loading buffer alone, which is placed in either the first or last lane of the gel. Connect the gel apparatus to the power supply and start running at 200 V until the dye has fully diffused into the gel. Increase the voltage to 300 V and maintain at this setting until the faster running dye marker has traveled $\frac{3}{4}$ down the length of the gel.
- 17 Turn off the current and dismantle the gel apparatus. Put the gel in plastic wrap. Place a fluorescing TLC Merck silica plate under the gel and examine by illumination with a hand-held 254-nm UV lamp. If necessary, photograph the gel using a camera with a UV filter.

Typically, crude PS2-RNAs consist of one intense band on the gel. The presence of multiple bands below the desired product usually indicates poor coupling efficiency.

Purification of PS2-RNAs by denaturing PAGE

- 18 Prepare a 20% to 15% denaturing polyacrylamide gel on a 1.5-mm-thick gel plate for preparative analysis.

A lower percentage of acrylamide may be used for purifying PS2-RNAs if the crude PS2-RNA migrates as one main band in analytical compositions. When multiple bands corresponding to failed sequences are present in the gel, a high percentage of acrylamide solution should be used.
- 19 Prepare and dry about ~ 20 units of A_{260} of the PS2-RNA sample. Dissolve the sample in about 100 μ L loading buffer.
- 20 Load the sample on the gel and run the gel as described above.
- 21 Cut out the segment of gel containing the band of interest using a razor blade.
- 22 Transfer the excised band to a 15-mL conical plastic tube with screw cap and crush with a spatula. Add 10 mL DEPC-treated water and leave the tube on a platform shaker overnight to extract the PS2-RNA from the gel.
- 23 Filter the supernatant through a 0.22- μ m sterile syringe filter and lyophilize the sample.
- 24 Desalt the sample using SepPak C18 cartridges. Wash a cartridge first with 10 mL DEPC-treated water, then 10 mL 80 acetonitrile in DEPC-treated water, and finally with 10 mL DEPC-treated water.
- 25 Load ca. 20 units of A_{260} of PS2-RNA per cartridge, wash the cartridge with 10 mL DEPC-treated water, then elute the pure PS2-RNA with 5 mL of 50% acetonitrile in water. Quantitate the amount of the PS2-RNA obtained.
- 26 Add 5 mL of DEPC-treated water to the elution and lyophilize the PS2-RNA.

Analysis and purification of PS2-RNA by anion-exchange HPLC or Mono-Q FPLC and desalting by RP-HPLC

- 27 Prepare about 0.5 ~ 1.0 OD₂₆₀ of crude PS2-RNA to be analyzed via DNAPac-100 (Dionex) column.
- Typical injection volume for analysis is 10 to 50 µL, limited by the sample loop size of the injector. Sample loops as large as 2 mL are available for purification injections.
- 28 Program the gradient system to start with 100% mobile phase A (1 mM EDTA, 25 mM Tris-HCl, pH 8), increasing the percentage of mobile phase B (1 mM EDTA, 25 mM Tris-HCl, 1 M NaCl, pH 8) with time. Ensure that sufficient mobile phase is used to keep the intake covered during run.
- 29 Equilibrate the HPLC system with the starting mobile phase composition until a flat baseline is achieved at the desired detection wavelength of 260 nm.
- 30 Load ca. 0.5 ~ 1.0 OD₂₆₀ crude PS2-RNA sample to the column. Wash 5 min with mobile phase A, then apply a linear gradient of 0% to 50% mobile phase B in 40 min to elute the PS2-RNAs. Wash and re-equilibrate the column.
- The desired fraction should elute close to the end of the applied gradient. A high percent of mobile phase B should be used if the RNA contains a high percentage of PS2 linkages.
- 31 Optimize the condition for the next run. A suitable chromatographic gradient depends on the number of PS2 linkages incorporated (Yang et al., 2002).

Purify PS2-RNA by Mono-Q column on a FPLC system

- 32 Mono-Q is a strong anion exchanger pre-packed with MonoBeads in a Tricorn column. This column is an excellent choice for small-scale optimization of purification of PS2-RNA when high purity is required (Abeydeera et al., 2016; Yang et al., 2002; Yang et al., 2012). In addition, Mono-Q columns offer high-resolution separations with greater loading capacity.
- 33 About 20 OD₂₆₀ of crude PS2-RNA can be loaded on a Mono-Q 5/5 column. By comparison, about 50 OD₂₆₀ of crude PS2-RNA can be loaded on a Mono-Q 10/10 column. Gradient and buffer system can be found in author's article (Yang et al., 2002).
- 34 A divinyl benzene/polystyrene copolymer reverse-phase column (Hamilton PRP-1) offers a high-capacity method for desalting of PS2-RNAs. In particular, PS2-RNAs are purified by anion exchange column.
- Alternatively, desalting of PS2-RNA can be performed by Amicon ultra-15 centrifugal filter unit.*

COMMENTARY

Background Information

RNA aptamers (Keefe et al., 2010; Osborne and Ellington, 1997; Tuerk and Gold, 1990), siRNAs (Behlke, 2006; Elbashir et al., 2001; Pushparaj et al., 2008), and miRNAs (Melo and Esteller, 2011; Small and Olson, 2011) show tremendous potential as therapeutics against viral infections, cancer, genetic disorders, and neurological diseases (Bouchard et al., 2010; Soutschek et al., 2004). Beyond the therapeutic potential, aptamers are of high value as tools for biological research, such as target validation (Blank and Blind, 2005; Jain, 2004; Pendergrast et al., 2005), and as biosensors in diagnostics (Germer et al., 2013; Zhou et al., 2014). It has been widely acknowledged that the phosphate backbone of RNA is highly susceptible to nuclease degradation in biological systems. Insufficient metabolic stability and poor pharmacokinetic properties render unmodified RNAs unsuitable as drug candidates (Bramsen et al., 2009). Much research has been focused on chemically modifying RNAs at the sugar and/or phosphate moieties (Deleavey and Damha, 2012) to overcome these stability and pharmacokinetic issues. The commonly-used phosphoromonothioate (PS)-modification affords only limited protection against hydrolysis by nucleases (Vortler and Eckstein, 2000) and results in a mixture of diastereomeric RNAs that raises the potential for variable biophysical properties and variable batch-to-batch quality (Lebedev and Wickstrom, 1996; Verma and Eckstein, 1998; Yang et al., 2012). While stereocontrolled synthesis of P-chiral PS-RNAs represents one possible solution to this problem (Stec and Wilk, 1994; Wada et al., 2004), another lies in the synthesis of modifications that are achiral at phosphorus such as PS2-RNAs (Yang et al., 2012). Recently, PS2-RNAs were investigated as potential RNAi compounds and exhibited the ability to silence β -secretase mRNA associated with Alzheimer disease (Yang et al., 2012). The author and others have found that PS2-RNAs bind proteins with a higher affinity than their phosphodiester counterparts and PS analogues as well (Abeydeera et al., 2016; Pallan et al., 2014), suggesting that PS2-RNAs may have additional utility in the form of sulfur-modified phosphate ester aptamers (thioaptamers) (Fennewald et al., 2007; Ferguson et al., 2006; Hecht et al., 2010; Wang et al., 2006; Yang et al., 1999; Yang and Gorenstein, 2004; Yang et al., 2011) in therapeutic and diagnostic applications (Marshall and Caruthers, 1993; Yang and Gorenstein, 2004b; Yang et al., 2011). In particular, the author and his colleagues have developed PS2-RNA aptamers against two cellular proteins, VEGF and thrombin, that demonstrate pM K_D of binding affinity (Abeydeera et al., 2016). The VEGF PS2-RNA aptamer has been shown to specifically bind cancer cells (Abeydeera et al., 2016). The biological interest and promise of PS2-RNA has inspired us to develop a novel strategy for synthesizing, isolating, characterizing and purifying these compounds (Sierant et al., 2011; Yang et al., 2012). Some of these strategies include an innovative method for synthesizing RNA-thiophosphoramidites using Caruthers' protecting group (Wiesler and Caruthers, 1996). Another describes an effective activator (Activator-42) for RNA-thiophosphoramidite coupling (Figure 4), as well as an effective method for isolating high quality PS2-RNAs from a crude synthetic mixture containing PS-RNA and normal phosphate ester backbone RNA impurities by using a strong anion-exchange Mono-Q column (Yang et al., 2002) (Figure 5). Finally, we have also developed a strategy to characterize PS2 linkage(s) using ^{31}P -NMR spectroscopy (Figure 6).

Critical Parameters and Troubleshooting

The solvents such as acetonitrile used to dissolve RNA-thiophosphoramidite monomers as well as the activator must be kept rigorously anhydrous because the synthesis method described is extremely sensitive to water. In addition, the author has observed that base deprotection can cause a small percentage of PS impurities, which can be reduced through the addition of 20 mM dithiothreitol (DTT) (X. Yang, unpublished results). Moreover, EDITH is the reagent of choice for sulfurizing the thiophosphite intermediate.

Anticipated Results

Synthesis of RNAs containing several PS2 linkages with yields of 60 to 100 A260 units of crude material for a 20-mer at a 1- μ mol scale synthesis, and isolated yields of ~30 to 50 A260 units should be routinely achievable using the commercially available RNA-thiophosphoramidites in conjunction with the methods presented. Coupling with activator-42, sulfurizing with EDITH, and base deprotecting with DDT minimize PS impurities. These considerations, together with the optimized mono-Q ion-exchange HPLC chromatographic procedures (Yang et al., 2002), should enable the facile isolation of PS2-RNAs with high purity (Figure 6). In the course of learning how to synthesize and purify RNAs containing high percentages of PS2 linkages, the author observed that they are retained for a longer time on both anion exchange and reverse-phase columns than their unmodified and PS counterparts, which indicates a high degree of hydrophobicity (Pallan et al., 2014).

Time Considerations

The amount of time required for solid-phase synthesis of RNAs containing PS2-linkage(s) is generally longer than for unmodified RNAs due to extended coupling and sulfurization times required for the successful use of RNA-thiophosphoramidites. Deprotection, purification and characterization are achievable in the same amount of time as required for standard RNA synthesis.

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Figure 1.
Structures of phosphodiester and phosphorodithioate linkages.

Figure 2.
Structures of RNA-thiophosphoramidites.

Figure 3.
A cycle of solid-phase synthesis of PS2-RNA.

Figure 4.
The readings of DMT cation assay to determine the stepwise yield of the coupling reactions.

Figure 5.
FPLC chromatogram of 0.2 μ mole of crude synthetic 5' -
AAGUCGUGCU_{PS2}G_{PS2}CUUCAUGUdTdT-3'. Column: Mono Q 5/5. Buffer A: 25 mM
Tris-HCl, 1 mM EDTA, pH 8; buffer B: 25 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 8;
linear gradient: 0.0 –100.0 % B in 50 min; flow rate: 1 ml/min; UV detector wave length:
254 nm.

Figure 6.

^{31}P -NMR spectrum of 5'-AAGUCGUGCUG_{PS2}CUUCAUG_{PS2}UTT-3' that was purified on a Mono-Q column using chloride as eluting anion. The spectrum was recorded on a Varian Unity Plus spectrometer operating at 161 Hz. The sample contained ca. 30 OD A260 units dissolved in 500 μL of D₂O. Chemical shifts were referenced to external 85% phosphoric acid.

Table 1

Automated 1 μ mol Synthesis Cycle for the Synthesis of PS2-RNAs on the Expedite 8909 DNA\RNA Synthesizer via Activator-42 as activator

Function	Pulse #	Time(sec)	Description
<u>Detritylation of the support bound protected nucleotide:</u>			
Trityl monitor on/off	N/A	1	Trityl analysis monitor is turned on for data collection
Detritylation	10	0	Detritylation solution is rapidly delivered to column
Detritylation	50	49	Prolonged delivery of detritylation solution to column
Washing with wash A	40	0	Lines are flushed with anhydrous acetonitrile (wash A)
Trityl monitor on/off	N/A	1	Trityl analysis monitor is turned off
Washing with wash A	40	0	Lines are flushed with anhydrous acetonitrile (wash A)
<u>Coupling of thiophosphoramidite via activator-42 as activator (12 min)</u>			
Wash	5	0	Lines are flushed with acetonitrile (wash)
Activator	5	0	Lines are flushed with activator
Thioamidite + Activator	6	0	Rapid deliver the thioamidite and tetrazol solution to the column
Thioamidite + Activator	3	166	Coupling of the activated thioamidite
Activator	3	166	Coupling of the activated thioamidite
Wash	7	388	Coupling of the activated thioamidite
Wash	10	0	Lines are flushed with acetonitrile (wash)
<u>Sulfurization of the thiophosphite:</u>			
Washing with wash A	10	0	Lines are flushed with anhydrous
Aux	10	0	Sulfurization solution is rapidly delivered
Aux	10	160	Slowly deliver sulfurizing reagent to column
Wash	10	160	Sulfurization process
Wash	10	160	Sulfurization process
Washing with wash A	15	0	Lines washed with Wash A
<u>Capping of the un-reacted 5'-OH free of nucleotide on solid support:</u>			
Washing with wash A	15	0	Lines washed with Wash A
Cap A + Cap B	8	0	Equal volumes of Cap A and Cap B solutions are rapidly delivered to column
Cap A + Cap B	7	14	Slowly deliver the Cap A and Cap B to column
Wash	10	20	Slowly purge the remaining Cap A and Cap B to column
Washing with wash A	30	0	Lines washed with Wash A