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Use of a Novel 5′-Regioselective Phosphitylating Reagent for One-Pot Synthesis of Nucleoside 5′-Triphosphates from Unprotected Nucleosides

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Abstract

The 5′-triphosphates are the building blocks for the enzymatic synthesis of DNAs and RNAs. This unit presents a protocol for the convenient synthesis of 2′-deoxyribo- and ribo-nucleoside 5′-triphosphates (dNTPs and NTPs) containing all the natural bases and the modified bases. This one-pot synthesis can also be applied to prepare the triphosphate analogs that contain sulfur or selenium atoms replacing the non-bridging oxygen atoms of the alpha phosphates of the triphosphates. These S- or Se-modified dNTPs and NTPs can be used to prepare diastereomerically-pure phosphorothioate nucleic acids (PS-NAs) or phosphoroselenoate nucleic acids (PSe-NAs, i.e., one type of selenium-derivatized nucleic acids: SeNA). Even without extensive purification, the synthesized dNTPs or NTPs are of high quality and can be directly used in DNA polymerization or RNA transcription. Synthesis and purification of the 5′-triphosphates, analysis and confirmation of natural and sulfur-or selenium-modified nucleic acids are described in this protocol unit.

Keywords

nucleoside 5′-triphosphates; nucleic acid modification; sulfur modification; selenium modification; nucleoside phosphorothioates; nucleoside phosphoroselenoates; diastereomers; DNA and RNA polymerases; X-ray crystallography

INTRODUCTION

The protocol described here is based on work published previously (Caton-Williams et al., 2011; Lin, et al., 2011; Caton-Williams et al., 2012) for the convenient synthesis of 2′-deoxyribo- and ribo-nucleoside 5′-triphosphates containing all the natural bases, the modified bases, and the S- or Se-modifications at the alpha phosphate positions. Nucleoside 5′-triphosphates (dNTPs and NTPs) play crucial roles in the synthesis of nucleic acids (DNA and RNA), and are involved in many biological regulations and pathways (Storz, 2002; Golubeva et al., 2008; Soutourina et al., 2011). In order to synthesize the natural and modified DNAs and RNAs enzymatically, the appropriate dNTPs and NTPs need to be prepared first.

Because of the presence of multiple functionalities (hydroxyl and amino groups) in the structure of the starting nucleosides, the nucleosides usually need to be protected. Protection and deprotection of these functionalities are achieved by several synthetic steps. To avoid

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the lengthy synthetic processes, Huang's research laboratory has developed a straightforward approach to conveniently synthesize the series of native and modified dNTPs and NTPs without the need for protecting the starting nucleosides. Basic Protocol 1 (Caton-Williams et al., 2011) describes the one-pot synthesis of the native 5′-triphosphates **8*O*** and **9*O*** (Figures 1 and 2). This protocol can also be applied to synthesize the 5′-triphosphates containing the modifications on the sugar and base moieties. The purification, analysis and confirmation of the native 5′-triphosphates are illustrated in Figures 3 and 4.

Basic Protocol 2 (Caton-Williams et al., 2012) describes the synthesis of the nucleoside 5′-(α-P-thio)triphosphates **8*S*** and **9*S*** (Figure 5) where a non-bridging oxygen atom of the alpha phosphate is replaced by a sulfur atom. Because of this modification at the alpha phosphate, diastereomers are formed. Typical HPLC profiles of diastereomers are illustrated in Figures 6 and 7. Basic Protocol 3 is a slight modification of the published article (Lin et al., 2011) and describes the synthesis of the nucleoside 5′-(α-P-seleno) triphosphates, **8*Se*** and **9*Se*** (Figure 8), where a non-bridging oxygen atom at the alpha phosphate is replaced by a selenium atom, resulting in diastereomeric mixtures, similar to the products of Basic Protocol 2. An HPLC profile demonstrating diastereomeric mixtures is shown in Figure 9. In all three scenarios, the facile synthesis is achieved by first reacting 2-chloro-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite, **1**) with pyrophosphate (**2**) to generate a phosphitylating reagent *in situ*, which offers high regioselectivity at the 5′-hydroxyl group of the unprotected nucleoside. Following oxidation and hydrolysis, each 5′-triphosphate is synthesized by a one-pot synthesis.

The products from Basic Protocol 1 are the building blocks for the synthesis of natural nucleic acids, whereas Basic Protocol 2 and Basic Protocol 3 products can be used to synthesize diastereomerically-pure phosphorothioate and phosphoroselenoate nucleic acids, respectively. Even without the HPLC or ion-exchange purification, the precipitated crude 5′-triphosphates are of sufficient quality for direct DNA polymerization and RNA transcription. The one-pot synthesis developed by our laboratory is convenient. Since the reaction conditions are mild and the synthesis is cost-effective, this novel strategy can be used to synthesize the triphosphates with various modifications.

CAUTION: Carry out all reactions in a well-ventilated fume hood, and wear lab coat, gloves and protective glasses. All reactions should first be performed on a small scale.

BASIC PROTOCOL 1: ONE-POT SYNTHESIS OF NATIVE NUCLEOSIDE 5′-TRIPHOSPHATES (dNTP AND NTP)

5′-dNTP or 5′-NTP is synthesized as depicted in Figure 1. This protocol describes the preparation of deoxy- and ribo-nucleoside 5′-triphosphates without any protecting groups on the starting materials. The first step of the synthesis involves the reaction of **1** with **2** in the presence of DMF and tributylamine to form an intermediate **3** *in situ* (Figure 1), which selectively phosphitylates the 5′-hydroxyl group of the unprotected nucleoside (A, C, G T, or U). After subsequent displacement of the phenolic group, the nucleoside cyclic phosphite (**6** or **7**; the key intermediate) is formed. This intermediate can then be converted to the native or alpha-modified 5′-triphosphate using the appropriate oxidizing agent to afford the desires nucleoside 5′-triphosphates (**8** or **9;** see Figure 2). Iodine/water solution is the commonly used oxidizing reagent for preparing native 5′-dNTPs and 5′-NTPs.

Materials

2′-Deoxyadenosine monohydrate (Sigma-Aldrich)

2′-Deoxycytidine monohydrochloride (ChemGenes Corporation)

2′-Deoxyguanosine monohydrate (ChemGenes Corporation)

2′-Thymidine 99% (Alfa Aesar)

Adenosine (Sigma-Aldrich)

Cytidine (ChemGenes Corporation)

Guanosine (ChemGenes Corporation)

Uridine (ChemGenes corporation)

Tributylammonium pyrophosphate (Sigma-Aldrich)

2-Chloro-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite, Sigma-Aldrich)

Anhydrous N,N-dimethylformamide (DMF, Sigma-Aldrich)

Tributylamine (TBA, Sigma-Aldrich)

Argon gas (Dried)

Methanol (MeOH)

Dichloromethane (methylene chloride, CH_2Cl_2)

Iodine solution (Glen Research)

Water (deionized)

Isopropanol (*ip*-OH)

Ammonium hydroxide (NH_4OH)

Sodium chloride (3 M, NaCl)

200 PROOF pure ethanol (KOPTEC)

2′-Deoxyadenosine 5′-triphosphate (dATP, Epicentre)

2′-Deoxycytidine 5′-triphosphate (dCTP, Epicentre)

2′-Deoxyguanosine 5′-triphosphate (dGTP, Epicentre)

2′-Thymidine 5′-triphosphate (TTP, Epicentre)

Adenosine 5′-triphosphate (ATP, Epicentre)

Cytidine 5′-triphosphate (CTP, Epicentre)

Guanosine 5′-triphosphate (GTP, Epicentre)

Uridine 5′-triphosphate (UTP, Epicentre)

Oven

15-, 10- and 5-mL round-bottom flasks

8 × 1.5-mm magnetic stir bar

Septa

Parafilm

High vacuum / vacuum pump

Balloons

1 ml syringes (Norm Ject)

Needles IM 1½ 23_GTW (Becton Dickinson)

Magnetic stir plate

Silica-coated thin-layer chromatography (TLC) plate with fluorescent indicator

Kieselgel $60F_{254}$ (Dynamic Adsorbents Inc. and Sorbent Tech.)

UV lamp

3 ml syringes

Disposable glass pipette (9″)

15- or 50-mL tube (Falcon)

Marker

−80 or −20° C Freezer

UV spectrophotometer

HPLC System

Centrifuge

Additional reagents and equipment for thin-layer chromatography (TLC: *APPENDIX 3D*)

NOTE: Dry all glassware in an oven and perform all reactions in an argon atmosphere. An argon atmosphere can be carried out through the use of a rubber septum that seals the flask, and an argon-filled balloon is inserted into the septum. Liquid chemicals are added using a syringe inserted through a rubber septum. A slightly positive pressure is maintained in the system to provide an anhydrous and oxygen-free atmosphere.

Synthesis of native nucleoside 5′-triphosphates (dNTPs and NTPs)

1 To a 10- or 15-mL oven-dried, round-bottom flask, add a dried 8 × 1.5-mm magnetic stir bar.

2 Weigh 20 mg of starting nucleoside (0.07 to 0.08 mmol) directly into the flask.

For the deoxynucleoside as starting nucleoside: 2′-deoxyadenosine ***4a****, 2′-deoxycytidine* ***4c****, 2″-deoxyguanosine* ***4g****, thymidine* ***4t****. For the ribonucleoside as starting nucleoside: adenosine* ***5a****, cytidine* ***5c****, guanosine* ***5g***, uridine ***5u.***

NOTE: dissolve adenosine in a mixed solvent of 0.16 mL DMF and 0.06 mL DMSO, and guanosine in a mixed solvent of 0.11 mL DMF and 0.12 mL DMSO).

3 To another 10-mL oven-dried round-bottom flask, add a dried 8 × 1.5-mm magnetic stir bar and directly weigh 73 mg of tributylammonium pyrophosphate **2** (0.16 mol, 2 eq.) into the flask.

4 Seal each flask with a cream rubber septum and wrap with parafilm.

5 Place each flask on high vacuum to dry for over 2 h at room temperature.

6 Prepare argon filled balloons by using parafilm to wrapping a deflated balloon to the top end of a 1mL syringe.

7 Inflate two balloons with argon and insert into each septum of the flasks.

8 Quickly weigh 20 mg of 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one **1** (0.1 mmol, 1.2 eq.) into a 5- or 10-mL oven-dried round-bottom flask containing a 8 × 1.5-mm magnetic stir bar.

9 Insert an argon filled balloon to the septum and purge for 30 seconds.

10 Insert another inflated balloon into the cap of the anhydrous DMF bottle and purge. Be careful not to contaminate the DMF solvent during transfer.

11 Using a 1 mL syringe equipped with a 1½ ″ needle, transfer 0.2 mL purged DMF to the flask containing **1.**

12 Place the flask on a magnetic stirring plate and stir to dissolve.

13 Using a 1 mL syringe equipped with a 1 ½ ″ needle, transfer 0.2 mL purged DMF to the flask containing dried **2** (step **3**). Stir to dissolve, keeping the solution under an argon atmosphere.

14 Using another clean syringe, add 0.3 mL (approximately 18 eq) of dried TBA to the flask containing the dissolved pyrophosphate **2**.

15 Using the same syringe, transfer the solution in step **14** to the septum of the flask containing **1** and let the reaction stir for 30 min.

16 Remove the nucleoside from the high vacuum and insert an argon filled balloon on the septum of the flask.

17 Use a syringe to inject 0.2 mL dry DMF to dissolve the nucleoside.

18 Slowly (for 5 min) inject the reaction solution in step15 (mixture of **1** and **2)** into the dissolved nucleoside and let the reaction stir vigorously for 2 h.

19 Monitor the formation of the intermediate **6** (or 7) by TLC (using standard silica gel plates, Dynamic Abs., *APENDIX 3D*) and eluent: 10% MeOH in CH_2Cl_2 for the intermediates of compound ***4a*** and ***4t***; 15% MeOH in CH_2Cl_2 for the intermediates compound ***5a*** and ***5u;*** and 20% MeOH in CH_2Cl_2 for the intermediates of compound ***4c, 4g, 5c*** and 5***g*** (to monitor the consumption of the nucleoside)

> The nucleoside cyclic phosphite intermediates **6(7)** are visualized by UV lamp (254 nm) as fluorescent spots [Rf = 0.20, 0.13, 0.20, and 0.3, respectively for the 2′-deoxynucleosides (A, T, C, G) and 0.19, 0.12, 0.16, 0.12, respectively for the ribonucleosides(A, U, C, G)].
>
> NOTE: under these reaction conditions the starting nucleosides are approximately 70 % completed according to TLC observations.

20 Using a syringe, inject 1 mL iodine solution(0.02 M) to the reaction containing the nucleoside reaction until a permanent brown color is maintained, similar to that of the iodine solution.

21 Let the reaction continue with stirring for another 30 min. If the reaction solution becomes colorless, add drop wisely more iodine solution to main the brown color during this time. The oxidized products are not normally monitored by TLC in this step, since it is not stable. After this oxidation step, using a 3 mL syringe with a 1 ½ ″ needle, inject 2 volumes of water (twice the reaction volume) and let the cyclic phosphate hydrolyze for 1.5 h under stirring

22 Monitor the formation of the product by TLC using standard silica gel plates (Sorbent Tech., *APENDIX 3D*) and the eluent: 5:3:2 (*v/v/v*) *iso-Propanol*/ NH_4OH/water.

The product is visualized by UV lamp (254 nm) (Rf = 0.47, 0.37, 0.35, 0.42, respectively for the deoxynucleoside 5′-triphosphates **(8O; A, T, C, G)** and 0.35, 0.28, 0.23 and 0.30 for the ribonucleosides 5′-triphosphates **(9O; A, U, C, G**).

Perform Ethanol Precipitation

23 After the reaction is completed, use a disposable glass pipette (9″) to transfer the resulting solutions into two 15-mL tubes, or one 50-mL tube. Use a marker to label each tube.

24 For a 1 mL of reaction solution, add 0.1 mL of 3 M NaCl to each tube and shake, followed by the addition of 3 mL ethanol. Note the volumes and use this information to calculate the amount of NaCl and ethanol to use for your volume measured.

25 Place the two tubes at either −80 °C or −20 °C for 1 h and centrifuged at approximately 3000 rpm for 30 min.

[NOTE: A refrigerated centrifuge (4°C) gives better results than a non-refrigerated centrifuge. A refrigerated centrifuge (−10 °C) is preferred.]

26 Using a glass pipette, transfer the supernatant to another tube and air dry (for 30 min) the white residues by slanting on a 5- or 10-degree incline to remove excess ethanol.

27 Re-dissolve the dried pellet in 200 μL deionized water and repeat step 25–27 to reprecipitate the triphosphate.

28 Determine the concentration of the triphosphate sample using a UV-vis Spectrophotometer.

29 The crude 5′-triphosphate (dNTP or NTP) is now ready for use in DNA polymerization or RNA transcription.

Conduct purification of nucleoside 5′-Triphosphate

30 For product with high purity, centrifuge the solution of the crude product to remove any solid particles and use the supernatant to conduct HPLC purification.

31 Purify the crude nucleoside 5′-triphosphate on reversed-phase HPLC (RP-HPLC) using the following recommended conditions:

Column: 21.2 × 250–mm Welchrom C18 (or Ultisil C18)

Buffer A: 20 mM TEAA buffer, pH 7.1

Buffer B: 50% CH_3CN in buffer A

Gradient: 0% to 40% buffer B over 20 min

Flow rate: 6 mL/min

Detection wavelength: 260 nm.

32 Collect the nucleoside 5′-triphosphate fractions and lyophilize them.

33 Dissolve the residue in 200 μL deionized water and precipitate it with NaCl (20 μL, 3 M)/ethanol (660 μL) to afford the 5′-triphosphate as the sodium salt.

A typical profile of RP-HPLC analysis of crude thymidine 5′-triphosphate is shown in Figure **3.**

34 After the RP-HPLC purification, the 5′-triphosphates are characterized by ^{1}H-NMR, ^{31}P-NMR and high-resolution mass spectrometry (HR-MS). (Expected data is shown for the 2′-deoxynucleoside 5′-triphosphates.)

8aO (7.3 mg, 19% yield) as the triethylammonium salt, followed by re-precipitation with NaCl/ethanol to afford the sodium salt. ^{1}H NMR (400 MHz, D_2O): δ 8.50 (s, 1H, H-2), 8.27 (s, 1H, H-8), 6.54 (t, J = 6.5 Hz, 1H, H-1′), 4.41 (m, 1H, H-3′), 4.31 (m, 1H, H-4′), 3.88 (m 1H, H-5′), 3.24 (m 1H, H-5′), 2.84 (m, 1H, H-2′), 2.61 (m, 1H, H-2′); ^{31}P NMR (162 MHz, D_2O): δ −22.79 (t, J_β = 19.60 Hz, 1P, β-P), −11.21 (d, J_α = 19.60 Hz, 1P, α-P), −9.49 (d, J_γ = 19.60 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 259 nm; HR-MS (ESI): molecular formula $C_{10}H_{16}N_5O_{12}P_3$; $[M\text{-}H^+]^-$: 489.9938 (calculated: 489.9936).

8cO (18.8 mg, 46% yield) as the triethylammonium salt, followed by re-precipitation with NaCl/ethanol to afford the sodium salt. ^{1}H-NMR (400 MHz, D_2O): δ 7.98 (d, J = 7.6 Hz, 1H, H-6), 6.34 (t, J = 6.0 Hz, lH, H-1′), 6.14 (d, J = 7.6 Hz, 1H, H-5), 4.63 (br, 1H, H-3′), 4.21 (m, 2H, H-4′ & 5′), 3.65 (m, 1H, H-5′), 2.39 (m, 1H, H-2′), 2.33 (m, 1H, H-2′); ^{31}P-NMR (162 MHz, D_2O): δ −22.65 (t, J_β = 19.60 Hz, 1P, β-P), −10.85 (d, J_α = 19.60 Hz, 1P, α-P), −10.16 (d, J_γ = 19.60 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 271 nm; HR-MS (ESI): molecular formula $C_9H_{16}N_3O_{13}P_3$; $[M\text{-}H^+]^-$: 465.9814 (calculated: 465.9817).

8gO (11.5 mg, 30% yield) as the triethylammonium salt, followed by re-precipitation with NaCl/ethanol to afford the sodium salt. ^{1}H-NMR (400 MHz, D_2O): δ 8.12 (s, 1H, H-8), 6.33 (t, J = 6.8 Hz, lH, H-1′), 4.71 (m, 1H, H-3′), 4.21 (m, 3H, H-4′& 5′), 2.84 (m, 1H, H-2′), 2.52 (m, 1H, H-2′); ^{31}P-NMR (162 MHz, D_2O): δ -22.73 (t, J_β = 19.44 Hz, 1P, β-P), −11.10 (d, J_α = 19.44 Hz, 1P, α-P), −9.50 (d, J_γ = 19.44 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 252 nm; HR-MS (ESI): molecular formula $C_{10}H_{16}N_5O_{13}P_3$; $[M\text{-}H^+]^-$: 505.9893 and calculated: 505.9885.

8tO (15 mg, 39% yield) as the triethylammonium salt, followed by re-precipitation with NaCl/ethanol to afford the sodium salt. ^{1}H-NMR (400 MHz, D_2O): δ 7.75 (s, 1H, H-6), 6.35 (t, J = 6.8 Hz, 1H, H-1′), 4.69 (m, 1H, H-3′), 4.22 (m, 3H, H-4′& -5′), 2.38(m, 2H, H-2′), 1.94 (s, 3H, Me-5); ^{31}P-NMR (162 MHz, D_2O): δ −21.22 (t, J_β = 18.6 Hz, 1P, β-P), −10.50 (d, J_α = 18.6 Hz, 1P, α-P), −6.65 (d, J_γ = 18.6 Hz, 1P, γ-P); UV (H_2O): λ_{max} = 267 nm; HR-MS (ESI): molecular formula $C_{10}H_{17}N_2O_{14}P_3$; $[M\text{-}H^+]^-$: 480.9831 (calculated: 480.9820).

BASIC PROTOCOL 2: ONE-POT SYNTHESIS OF NUCLEOSIDE 5′-(ALPHA-P-THIO) TRIPHOSPHATES

Basic protocol 1 describes the synthesis of native 5′ triphosphates (Figures 1 and 2). In Basic protocol 2, this synthetic strategy is applied to the preparation of nucleoside 5′-(alpha-P-thio)triphosphates (5′-dNTPαS and 5′-NTPαS). In this protocol, the oxidation step utilizes a sulfurizing agent as the oxidant instead of iodine in step 3 (Figure 1). Sulfurization of the nucleoside cyclic phosphite **6** (**7**) is performed using 3-[(dimethylaminomethylidene)amino]-3H-1,2,4,dithiazol-e-3-thione (sulfurizing Reagent II), followed by hydrolysis to afford the crude dNTPαS and NTPαS analogs as mixtures of S_p and R_p diastereomers (Figure 6), with yields up to 60%. When using this synthetic strategy,

only one diastereomer of dGTPαS and GTPαS is observed, and can be confirmed by comparing with commercially available compounds as the standards (Figure 7).

Materials: (see Basic Protocol 1)

Additional Material

Acetonitrile, anhydrous 99.8% (Sigma-Aldrich)

Pyridine (Sigma-Aldrich)

3-[(Dimethylaminomethylidene)amino]-3H-1,2,4,dithiazol-e-3-thione (Sulfurizing Reagent II) from Glen Research

2′-Deoxyguanosine 5′-(α-P-thio)triphosphates (dGTPαS, TriLinks Inc.)

Guanosine 5′-(α-P-thio)triphosphates (GTPαS, TriLinks Inc.)

Synthesis of nucleoside 5′-(alpha-P-thio) triphosphates

1 For the synthesis of the deoxynucleoside 5′-(alpha-P-thio) triphosphates and the ribonucleoside 5′-(alpha-P-thio) triphosphates, follow the synthesis in Basic Protocol 1 from step 1 to step 19.

2 Weigh 33 mg of Sulfurizing Reagent II (0.16 mmol, 2 eq) directly into a 5-mL round-bottom flask.

3 Seal the flask with a septum, wrap it with parafilm and dry it on high vacuum for over 2 h.

4 Insert an argon filled balloon on the septum.

5 Using a 1 mL syringe with a 1 ½″ needle, transfer 0.3 mL of dried pyridine to Sulfurizing Reagent II.

6 Use another 1 mL syringe and add 0.2 mL of anhydrous acetonitrile to the flask. Stir to dissolve (a yellow solution results).

7 Using the same syringe, slowly transfer this reaction mixture to the flask containing the cyclic phosphite (step 19 of Basic Protocol 1).

8 Let the reaction continue with stirring on a magnetic stir plate for 2 h. NOTE: The cyclic phosphate is not normally monitored by TLC in this step, since it is unstable.

9 Add two volumes of water (twice the reaction volume) to the reaction flask and let stirring continue for another 2 h to afford the desired 5′-triphosphate **8*S*** or **9*S***.

10 Monitor the formation of the product by TLC (APENDIX 3D) using the eluent: 5:3:2 (*v/v/v*) *ip*-OH/NH_4OH/water.

The products are visualized by UV lamp (254 nm) [Rf = 0.58, 0.45, 0.43, and 0.52, respectively for the deoxynucleoside 5′-triphosphates **(8S; A, T, C, G)** and 0.47, 0.33, 0.32, and 0.40 for the ribonucleosides 5′-triphosphates **(9S; A, T, C, G)**].

Perform Ethanol Precipitation

After the reaction is completed, use a disposable glass pipette to transfer the resulting solutions into two argon-purged 15-mL tubes, or one 50-mL tube. Use a marker to label each tube.

Measure the volume and calculate the amounts of 3 M NaCl (10% of the volume) and ethanol (3 fold of the volume) for the precipitation. NOTE: purge the NaCl and ethanol with argon for over 5 min prior to use.

11 Place the two tubes at either −80 °C or −20 °C for 1 h and centrifuged at approximately 3000 rpm for 30 min.

A refrigerated centrifuge (−10 °C) is preferred.

12 Using a glass pipette, transfer the supernatants to another tube and air dry the yellow residues by slanting on a 5- or 10-degree incline to remove excess of ethanol for over 30 min.

13 Re-dissolve the dried pellet in 1 mL deionized water and determine the concentration with a UV-vis Spectrophotometer.

14 The crude product is now ready for use in DNA polymerization or RNA transcription.

15 Keep the product under argon in −80 °C for extended storage.

Conduct purification of nucleoside 5′-(alpha-P-thio) triphosphates

16 Ensure to centrifuge the crude sample prior to the HPLC purification.

17 For a high purity compound, purify the crude 5′-triphosphate on reversed-phase HPLC using the following recommended conditions:

Column: 4.6 × 250 mm Welchrom C18 (or Ultisil C18)

Buffer A: 20 mM TEAA buffer, pH 6.5

Buffer B: 50% CH_3CN in buffer A

Gradient: 0% to 20% buffer B over 25 min

Flow rate: 1 mL/min

Detection wavelength: 260 nm.

18 Collect the individual HPLC peaks of both diastereomers of the nucleoside 5′-triphosphate and evaporate the solvent by lyophilization.

A typical HPLC profile of uridine 5′-(alpha-P-thio)triphosphate is shown in Figure 6.

19 Only one diastereomer of dGTPαS or GTPαS is obtained with this protocol (Figure 7).

20 Further analyses of the 5′-triphosphates can be performed and compared with the commercially available native dNTPs.

21 Confirm the integrity of all synthesized nucleoside 5′-(alpha-P-thio) triphosphates by HR-MS analysis (Table 1).

BASIC PROTOCOL 3: SYNTHESIS OF NUCLEOSIDE 5′-(αLPHA-P-SELENO) TRIPHOSPHATE

Basic Protocol 1 describes the general procedure for the synthesis of nucleoside 5′-(α-P-seleno)triphosphates (Figure 1). Since oxygen, sulfur and selenium are in the same family of the periodic table, a suitable selenizing reagent can be used to introduce selenium (Basic Protocol 3; Figure 8) replacing a non-bridging oxygen atom at the alpha phosphate of the

triphosphate. BTSe (commercially available at SeNA Research, Inc.), a selenium-introducing oxidant, can be used to introduce the selenium functionality during the oxidative step. The synthesis yields of the nucleoside 5′-(α-P-seleno)triphosphates are generally greater than 30%. The 5′-triphosphate products of Basic Protocol 3 are diastereomeric mixtures, similar to the products in Basic Protocol 2.

Materials: (see Basic Protocol 1)

Additional Material

Dioxane (Sigma-Aldrich)

Triethylamine (Sigma-Aldrich)

3H-1,2-Benzothiaselenol-3-one (BTSe, SeNA Research, Inc.)

Synthesis of nucleoside 5′-(alpha-P-seleno) triphosphates

1 For the synthesis of the 2′-deoxynucleoside 5′-(alpha-P-seleno) triphosphates and the ribonucleoside 5′-(alpha-P-seleno) triphosphates, follow the synthesis in Basic Protocol 1 from step 1 to step 19.

2 Weight 35 mg of 3H-1,2-benzothiaselenol-3-one (BTSe, 0.16 mmol, 2 eq.) directly into a 5- or 10-mL round-bottom flask.

3 Seal the flask with a septum, wrap it with parafilm and dry it on high vacuum for over 2 h.

4 Insert an argon filled balloon on the septum.

5 Using a 1 mL syringe equipped with a 1 ½″ needle, transfer 0.3 mL of dioxane to dissolve BTSe.

6 Use another 1 mL syringe and add 0.2 mL of dried triethylamine (TEA) to the flask and stir to dissolve (a yellow solution or suspension results).

7 Using the same syringe, slowly transfer this reaction mixture to the flask containing the cyclic phosphite (step 19 of Basic Protocol 1).

8 Let the reaction continue with stirring on a magnetic stir plate for 2 h. NOTE: The cyclic phosphate is not normally monitored by TLC in this step, since it is unstable.

9 Add two volumes of water (twice the reaction volume) to the reaction flask and let stirring continue for another 2 h to afford the desired 5′-triphosphates ***8Se*** or ***9Se***.

10 Monitor the formation of the product by TLC (*APENDIX 3D*) using the eluent: 5:3:2 (*v/v/v*) *ip*-OH/NH_4OH/water.

The product is visualized by UV lamp (254 nm) as a fluorescent spot [Rf = 0.67, 0.53, 0.52, and 0.58, respectively, for the deoxynucleoside 5′-triphosphates **(8Se; A, T, C, G)** and 0.54, 0.40, 0.38 and 0.48 for the ribonucleosides 5′-triphosphates **(9Se; A, T, C, G)**].

Perform Ethanol Precipitation

11 After the reaction is completed, use a disposable glass pipette (9″) to transfer the resulting solutions into two argon purged 15-mL tubes,or one 50-mL tube. Use a marker to label each tube.

12 Measure the volume and calculate the amounts of 3 M NaCl (10% of the volume) and ethanol (3 fold of the volume) for the precipitation.

NOTE: purge the NaCl and ethanol with argon for over 10 min prior to use.

13 Place the two tubes at either −80 °C or −20 °C for 1 h and centrifuged at approximately 3000 rpm for 30 min.

A refrigerated centrifuge (−10 °C) is preferred.

14 Using a glass pipette, transfer the supernatant to another tube and air dry the yellow residue by slanting at a 5- or 10 degree incline to remove excess of ethanol for over 30 min.

22 Re-dissolve the dried pellet in 0.6 mL deionized water and determine the concentration with a UV-vis Spectrophotometer.

23 The crude product is now ready for use in DNA polymerization or RNA transcription.

24 Keep the product under argon in −80 °C for extended storage.

Conduct purification of nucleoside 5′-(alpha-P-seleno) triphosphates

25 Ensure to centrifuge the crude sample prior to the HPLC purification or analysis.

26 For a high purity compound, purify the crude 5′-triphosphate on reversed-phase HPLC using the following recommended conditions:

Column: 4.6 × 250 mm Welchrom C18 (or Ultisil C18)

Buffer A: 20 mM TEAA buffer, pH 6.5

Buffer B: 50% CH_3CN in buffer A

Gradient: 0% to 30% buffer B over 30 min

Flow rate: 6 mL/min

Detection wavelength: 260 nm.

23 Collect the individual HPLC peaks of both diastereomers of the nucleoside 5′-triphosphate and evaporate the solvent by lyophilization.

A typical HPLC profile of guanosine 5′-(alpha-P-seleno) triphosphates is shown in Figure 9.

24 Further analyses of the 5′-triphosphates can be performed and compared with the commercially available native dNTPs and NTPs.

25 After the RP-HPLC purification, the integrity of all synthesized nucleoside 5′-(alpha-P-seleno)triphosphates is confirmed by ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR and HR-MS. (The expected data is shown for the ribo-nucleoside 5′-(alpha-P-seleno)triphosphates; Tables 2–5.)

COMMENTARY

Background information

Nucleoside 5′-triphosphates (NTPs and dNTPs) play key roles in biochemistry, molecular biology and medicine (Eckstein, 1985; Bogdanov et al., 2010). They are the essential building blocks to the synthesis of nucleic acids (DNA and RNA) both *in vivo* and *in vitro* and depend on template, primer and polymerases to perform their functions. To further understand their roles played in nucleic acids and protein regulations *in vitro* and *in vivo* and

their biological and medicinal significances, there are urgent needs to synthesize their analogs. The native and modified nucleoside triphosphates are often prepared *via* chemical synthesis of phosphorylated derivatives and analogs. They are very challenging to synthesize chemically and isolate in high purity (>99%). Burgess and Cook (review article) have discussed the general practical strategies and problems associated with preparing, isolating, characterizing, and storing of 5′-triphosphates (Burgess and Cook, 2000).

The first chemical synthesis of nucleoside 5′-triphosphate was achieved over six decades ago (Baddiley, 1949). Currently, there are numerous strategies (Zou et al., 2005; Horhota, et al., 2006; Sun, et al., 2008; Warnecke and Meier, 2009; Schultheisz et al., 2010; Jansen, 2011) developed to synthesize nucleoside 5′-triphosphates, but no one is universal. This scenario is still challenging largely due to the multiple functionalities (i.e., 5′-, 2′-, and 3′-sugar hydroxyl groups and nucleobase amino groups) of the natural nucleosides as well as the modified nucleosides containing other functionalities. Therefore, from the starting nucleosides to the final 5′-triphosphate products, the functional groups need to be introduced and removed, causing longer synthesis steps (Burgess and Cook, 2000; Wu et al., 2004; Sun et al., 2008). Protection of these groups is necessary in order to minimize the formation of by-products and region-isomers, and to ensure good yields and minimum by-products. The major region-isomers and by-products in the 5′-triphosphate synthesis are the 3′- and 2′-triphosphates, in addition to the mono-, di and oligo-phosphates, which are difficult to remove during the purification process. Despite many synthetic strategies that have been developed, such as the “one-pot, three-step” method developed by chemists (Yoshikawa et al., 1967; Ludwig and Eckstein 1989; He et al., 1998; Zou et al., 2005; Cheek, 2008), there is still a pressing need to develop convenient strategies for synthesizing nucleoside 5′-triphosphates from unprotected nucleosides with high 5′-regioselectivity.

To address the concern of regioselectivity, thereby avoiding the protection and deprotection steps, and simplifying the triphosphate synthesis, Huang’s research group has generated a mild and selective phosphitylating reagent to distinguish these functionalities, showing greater selectivity toward the 5′-hydroxyl group of the starting nucleoside. This mild reagent is attained through the reaction of the highly reactive salicyl phosphorochloridite and pyrophosphate, affording a weak, bulky phosphitylating agent, when compared to salicyl phosphorochloridite (Caton-Williams et al., 2011). This bulky phosphitylating reagent can offer a better selectivity to distinguish the primary 5′-OH group from the secondary 2′- and 3′-OH groups. Furthermore, at reduced temperature (0 or −10 °C), the 5′-selectivity is further increased, but the reaction requires a longer time to consume the nucleosides (Gillerman and Fischer, 2010). After oxidation and hydrolysis, the nucleoside 5′-triphosphates are synthesized.

The strategy developed to synthesize native nucleoside 5′-triphosphates can be extended to the one-pot synthesis of the modified triphosphates starting from nucleoside derivatives, such as ethanodeoxyadenosine (a base modified deoxyadenosine) (Caton-Williams et al., 2011) and the S or Se-alpha phosphate. With the appropriate oxidizing reagent, either a sulfur or a selenium atom can be substituted for a non-bridging oxygen atom at the alpha-phosphate, resulting in S_p and R_p diastereomers of modified 5′-triphosphates (Ludwig and Eckstein 1989; He, et al., 1998; Lin and Shaw, 2000; Carrasco and Huang, 2004; Carrasco, et al., 2005; Brandt, et al., 2006).

It was reported that polymerases recognize the S_p diastereomer and not the R_p (Romaniuk and Eckstein, 1982; Eckstein and Gindl, 1983), therefore HPLC separation of the diastereomeric mixture is not necessary for the enzymatic synthesis of modified nucleic acids (PS-NA and PSe-NA). This is an important step toward therapeutic applications of the modified nucleic acids (Mori et al., 1989; Levin, 1999; Juliano et al., 2008; Lin et al., 2009,

Gan, et al., 2011) and X-ray structural determination by using the Se-derivatized nucleic acids (SeNA; Buzin et al., 2004; Carrasco et al., 2004; Salon et al., 2007; Caton-Williams and Huang, 2008; Salon et al., 2008; Sheng et al., 2008; Hassan et al., 2009; Lin et al., 2011).

Application of the natural and modified nucleoside 5′-triphosphates—The strategy developed by Huang's research group to synthesize natural and modified nucleoside 5′-triphosphates is straightforward and convenient. Such synthesis will contribute tremendously to the fields of biochemistry, molecular and cellular biology, as well as medicine. It is quite useful for researchers interested in small scale synthesis of 5′-triphosphates to perform various molecular biology applications, such as polymerase chain reaction (PCR), real-time PCR, cDNA synthesis, primer extension, nick translation, DNA sequencing, DNA labeling, and RNA transcription.

The sulfur- and selenium-derivatized nucleoside 5′-triphosphates possess unique properties. Nucleoside 5′-(α-P-thio)triphosphates are the building blocks for the enzymatic synthesis of phosphorothioate nucleic acids (PS-NAs). Because of their bioavailability and nuclease resistance properties, PS-NAs have shown to be promising as potential therapeutics in antisense DNA, siRNA and microRNA to selectively inhibit gene expression by mRNA inactivation (Kunkel et al, 1981; Levin, 1999; Juliano et al., 2008).

Phosphorothioate modification has been used in combination with other modifications, such as boranophosphates (Summerton et al., 1997; Lorenz et al., 1998; Krishna and Caruthers, 2011) to further increase their usefulness as active components of drugs and mechanistic probes. Phosphoroselenoate modification can be introduced into DNA and RNA through enzymatic synthesis, utilizing nucleoside 5′-(α-P-seleno)triphosphates to prepare SeNA. Although phosphoroselenoate nucleic acids have not been well studied in therapeutic applications as its sulfur counterpart, there is great potential in therapeutics. Phosphoroselenoate modification, however, has great potential in X-ray crystallography, contributing enormously to the phase problem determination in structure study of non-coding RNAs and protein-RNA complexes, as well as DNAs (Ferre-D'Amare et al. 1998; Ke and Doudna, 2004; Keel et al., 2007; Ferre-D'Amare, 2010; Koldobskaya et al., 2011).

Such a convenient synthetic strategy for 5′-triphosphates will contribute greatly to fundamental and applied research in cellular and molecular biology.

Critical parameters and troubleshooting

Small-scale reaction should be carried out first. It is essential that all glassware, starting materials and reagents are thoroughly dried. During the synthesis process, it is important to keep the reaction environment dry and free of oxygen. During the ethanol precipitation step, it is important to purge the ethanol thoroughly with argon prior to use. To ensure consumption of the starting nucleoside and formation of the nucleoside cyclic phosphite intermediate, the reaction could be extended to more than 5 hr prior to oxidation and hydrolysis. For the extended storage, the sulfur- and selenium-modified 5′-triphosphates should be kept under argon at −80 °C.

Anticipated results

The 5′-triphosphates synthesized according to Basic Protocols 1, 2 and 3 are of high quality. Even without HPLC and ion-exchange purification, they can be directly used as substrates for DNA polymerization and RNA transcription. Because of the replacement of the non-bridging oxygen atom at the alpha-phosphate with either sulfur or selenium, a chiral center is resulted. The modifications creating this chirality have increased the difficulty in

chemically synthesizing diastereomerically-pure DNAs and RNAs. The chemically synthesized PS-NAs or PSe-NAs are diastereomeric mixtures as the current chemical synthesis is unable to fully control the diastereomer formation at each phosphorus center. On the other hand, DNA polymerization and RNA transcription enable diastereomer-pure synthesis of phosphate-modified nucleic acids. Herein, DNA polymerase (Klenow) (Eckstein, 1979; Brody et al, 1982) and RNA polymerase (T7 RNA) (Burgers and Eckstein, 1978; Ueda et al., 1991)accept only the S_p diastereomers of dNTPαS (dNTPαSe) and NTPαS (NTPαSe) analogs, respectively. Since the R_p diastereomers are neither substrates nor inhibitors, fortunately, the S- or Se-modified triphosphates can be directly used to conveniently synthesize diastereomerically-pure sulfur- and selenium-derivatized nucleic acids with DNA (or RNA) polymerases, without HPLC or ion-exchange purification of the triphosphates. The expected results of the PS-DNAs and PS-RNAs utilizing crude dNTPαS and NTPαS are shown in Figure 10 and 11. This type of substitutions has been used extensively to study the conformational properties of nucleic acids and enzymatic cleavage of phosphodiester bonds (Burgers et al., 1979; Burgers and Eckstein, 1979; Frey, 1982; Eckstein, 1985).

Time Considerations

The synthesis of the nucleoside 5′-triphosphate (the native or the modified ones) is easy and convenient and can be achieved in one day. Fortunately, both the sulfurizing and selenizing reagents are commercially available.

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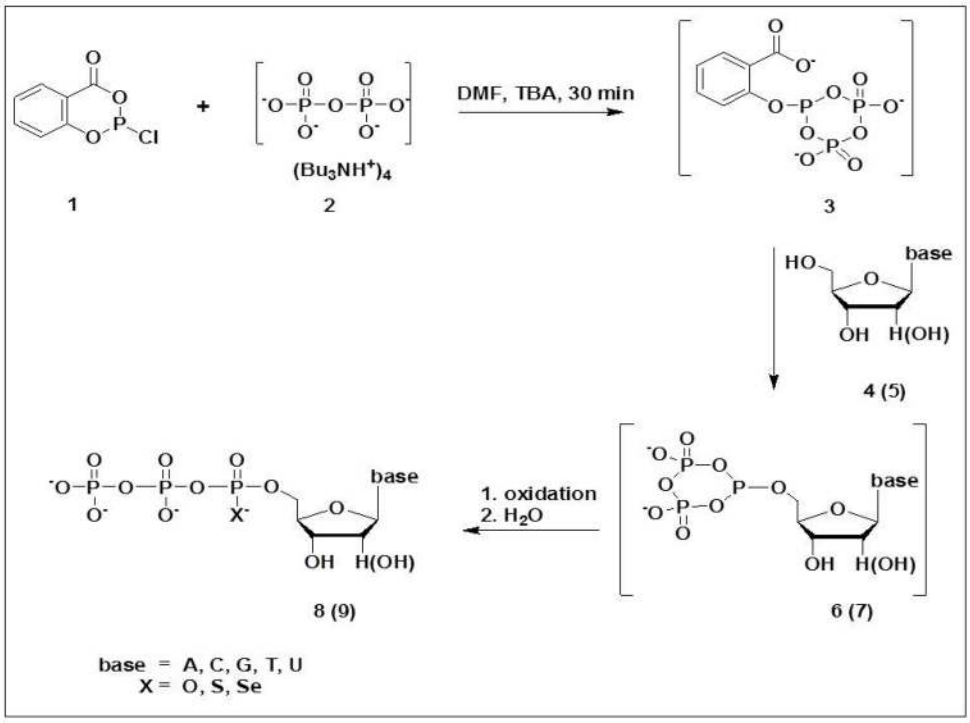

Figure 1.
General scheme of synthesizing natural and sulfur- or selenium-modified 5′-triphosphates (Basic Protocol 1–3).

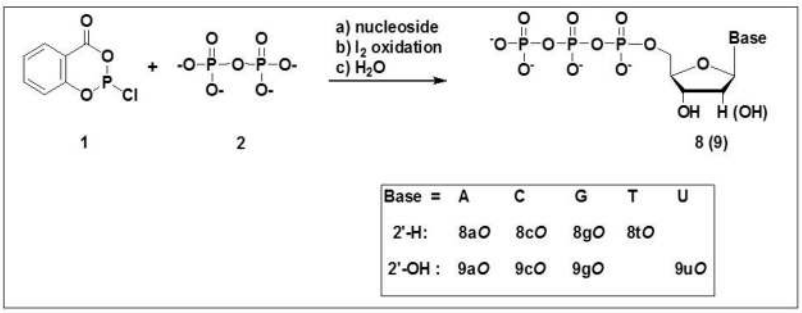

Figure 2.
One-pot synthesis of native nucleoside 5′-triphosphates utilizing iodine as the oxidizing agent

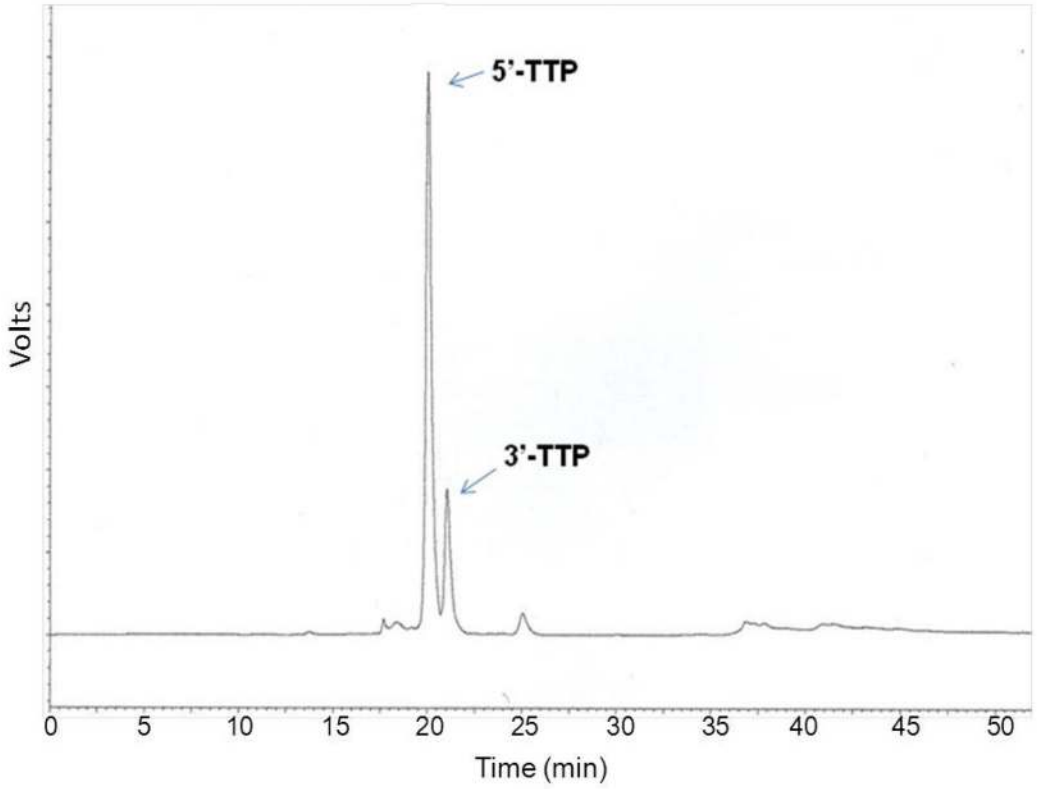

Figure 3.

HPLC profile of chemically synthesized thymidine 5′-triphosphate **8t*O*** (crude). The crude sample can be analyzed on a Welchrom C18 (or Ultisil C18) reversed phase column (4.6 × 250 mm) measured at 260 nm at a flow of 1.0 ml/min and with a linear gradient of 0 to 40% B in 20 min. Buffer A: 20 mM triethylammonium acetate (TEAA, pH 7.1) and buffer B: 50% acetonitrile in 20 mM TEAA (pH 7.1). Crude 5′-TTP and 3′-TTP after NaCl-ethanol precipitation (retention time: 19.4 and 20.7 min), respectively. (3′-TTP was compared and characterized with a 3′-TTP standard.)

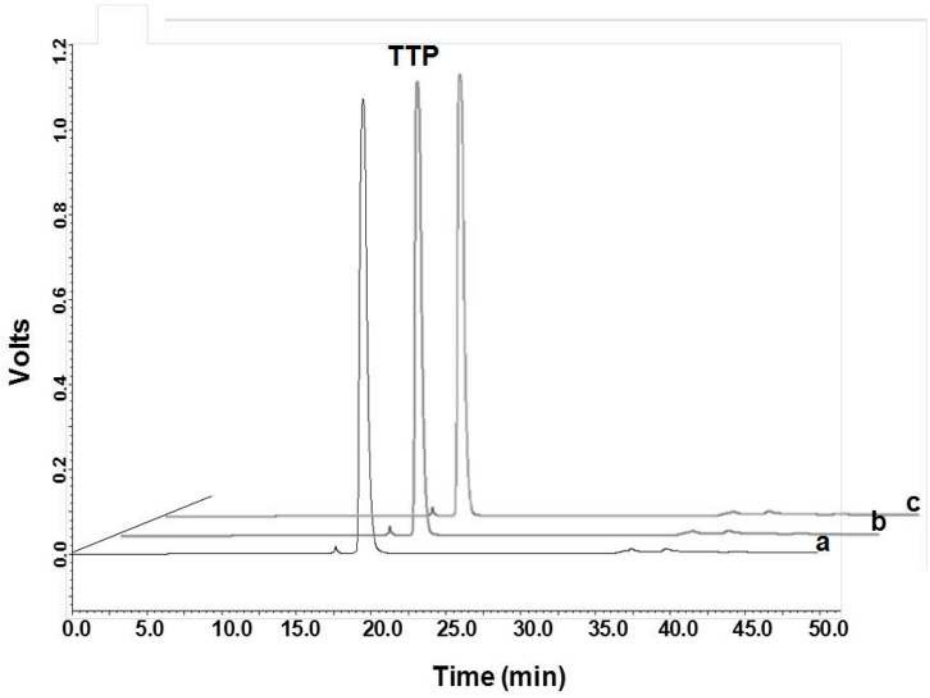

Figure 4.
RP-HPLC profiles of chemically synthesized and commercial 2′-deoxynucleoside 5′-triphosphates. a) Synthesized 5′-dTTP after NaCl-ethanol precipitation and RP-HPLC purification (retention time: 19.8 min); b) standard 5′-dTTP (retention time: 19.4 min); c) co-injection of a and b (retention time: 19.4 min).

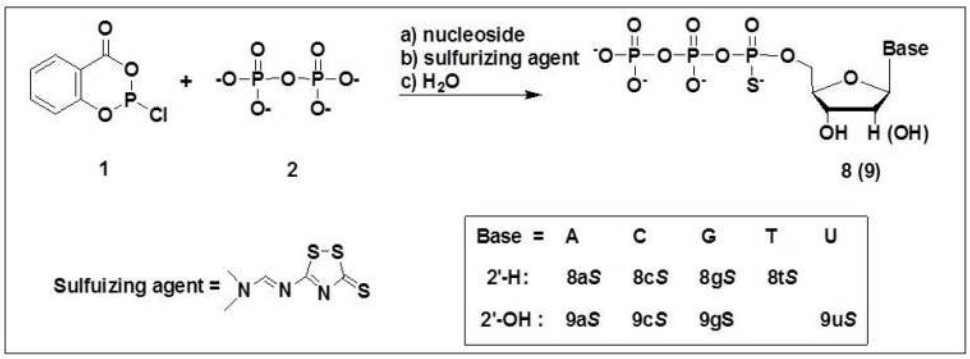

Figure 5.
One-pot synthesis of nucleoside 5′-(alpha-P-thio)triphosphates utilizing Sulfurizing Reagent II as the oxidizing agent.

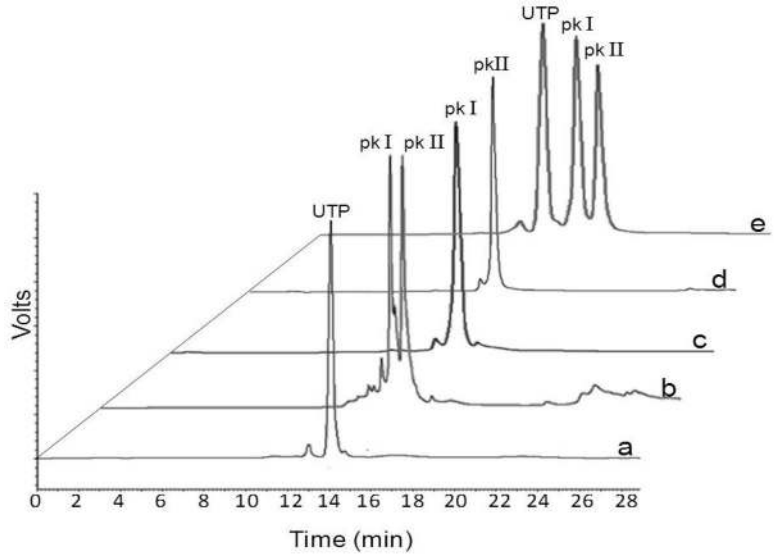

Figure 6.

RP-HPLC profiles of chemically synthesized uridine 5′-(α-P-thio)triphosphates **9S**. a) Commercial UTP, retention time 14.1 min; b) synthesized (crude) UTPαS, following NaCl-ethanol precipitation showing diastereomers, (retention times:17.5 and 18.3 min, respectively); c) HPLC purified diastereomer of UTPαS (pk I, S_p isomer) retention time: 15.6 min. d) HPLC purified diastereomer of UTPαS (pk II, R_p isomer) retention time: 16.6 min; d) co-injection of resolved UTP and UTPαS (pk I and pk II) retention times: 14.2, 15.4 and 16.3 min, respectively.

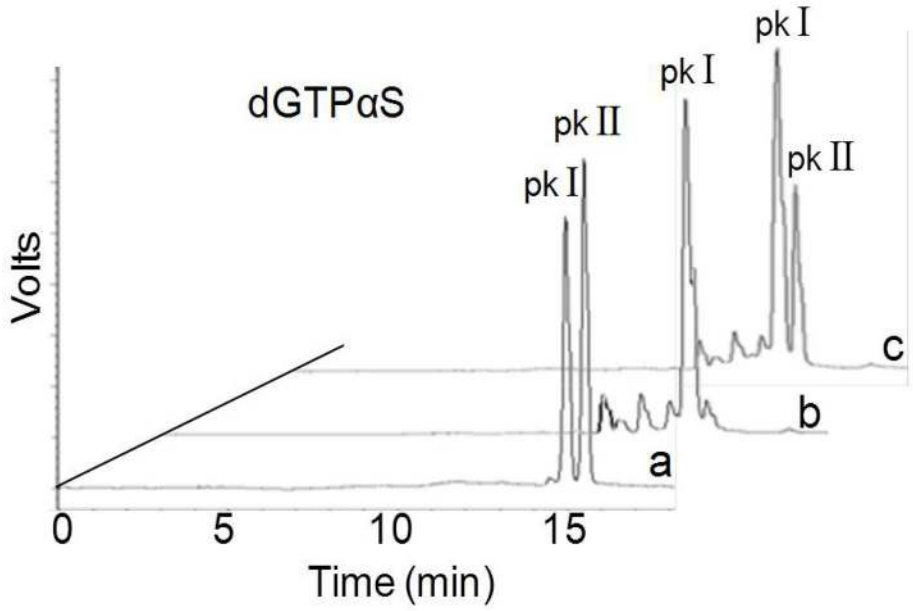

Figure 7.

High resolution HPLC profiles of commercial dGTPαS and synthesized dGTPαS. a) Commercial 5′-dGTPαS showing resolution of S_p and R_p diastereomers, (retention times: 14.9 and 15.5 min, respectively); b) synthesized 5′-dGTPαS, following NaCl-ethanol precipitation, showing only S_p isomer (retention times: 14.7 min); c) co-injection of commercial dGTPαS and synthesized 5′-dGTPαS showing enhanced peak I over peak II (retention times: 14.8 and 15.3 min, respectively).

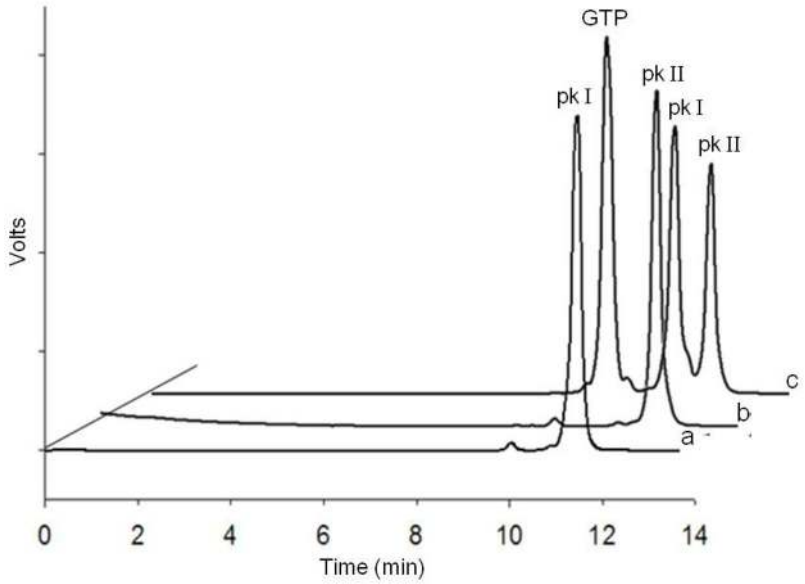

Figure 8.
One-pot synthesis of nucleoside 5′-(alpha-P-seleno)triphosphates utilizing BTSe as the oxidizing agent.

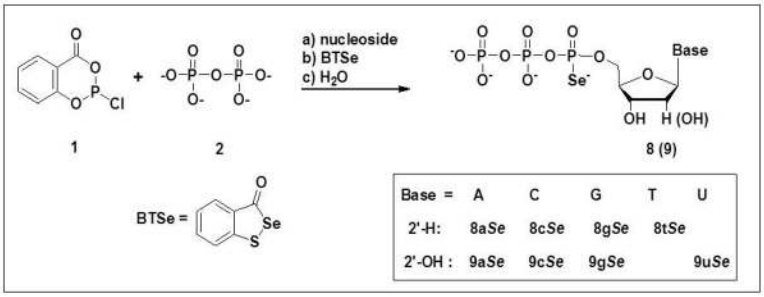

Figure 9.

RP-HPLC profiles of commercial GTP and chemically synthesized guanosine 5′-(α-P-seleno) triphosphates **9g*Se*** showing resolution of S_p and R_p diastereomers (pk I and pk II, respectively). a) Synthesized GTPαSe (pk I, S_p isomer; retention times: 11.5 min). b) Synthesized GTPαSe (pk II, R_p isomer; retention time: 12.1 min, and c) co-injection of GTP (retention time: 10.0 min) and GTPαSe (pk I and pk II).

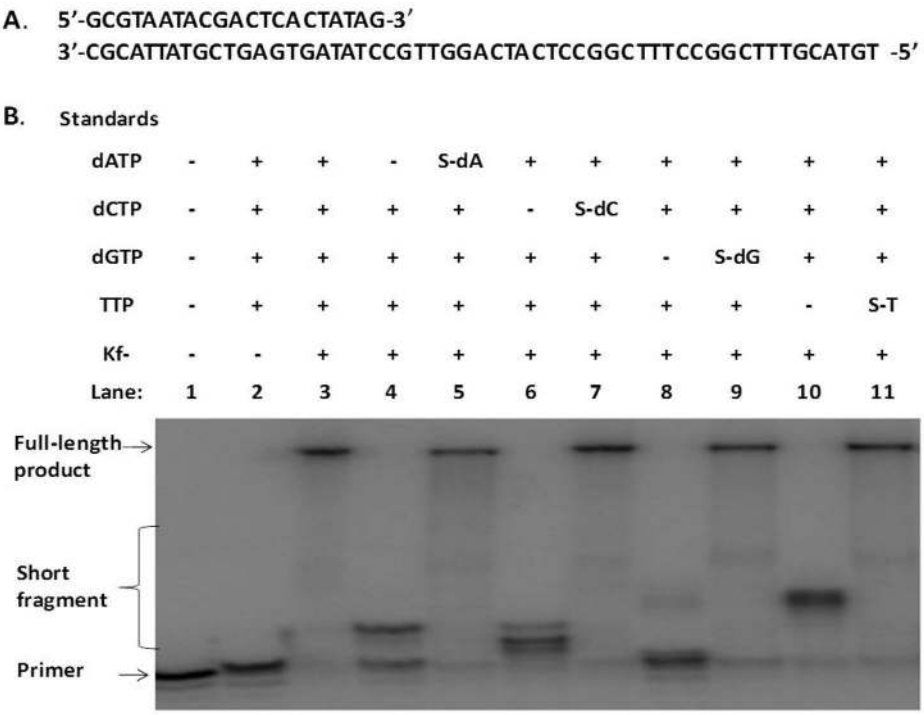

Figure 10.

A. Primer and template sequences to use in the polymerization experiment. **B**. DNA primer extension reaction using chemically synthesized dNTPαS and commercial dNTPs by Klenow fragment exo(-) (Kf-). Primer is 5′-end labeled using [γ-^{32}P]-ATP by polynucleotide kinase. Polymerization reactions are performed with primer (3.5 μM), template (5 μM), all dNTPs or dNTPαS (0.1 mM each, final concentration), and Kf- (0.015 μL per μL reac-tion) at 37 °C for 1 h. Reactions are analyzed by 19% polyacrylamide gel electrophoresis. Lane 1: 5′-^{32}P-labled primer; Lane 2: primer (P) and all dNTPs, but no Kf-; Lane 3 (positive control): P, template (T), all commercial dNTPs, and Kf-; Lanes: 4, 6, 8 and 10 (negative controls) with the omission of a dATP, dCTP, dGTP and TTP from Lane 3, respectively; Lanes: 5, 7, 9 and 11 were compensated with the synthesized (crude) dATPαS, dCTPαS, dGTPαS and TTPαS represented as S-dA, S-dC, S-dG and S-T (respectively) to the corresponding Lanes 4, 6, 8 and 10.

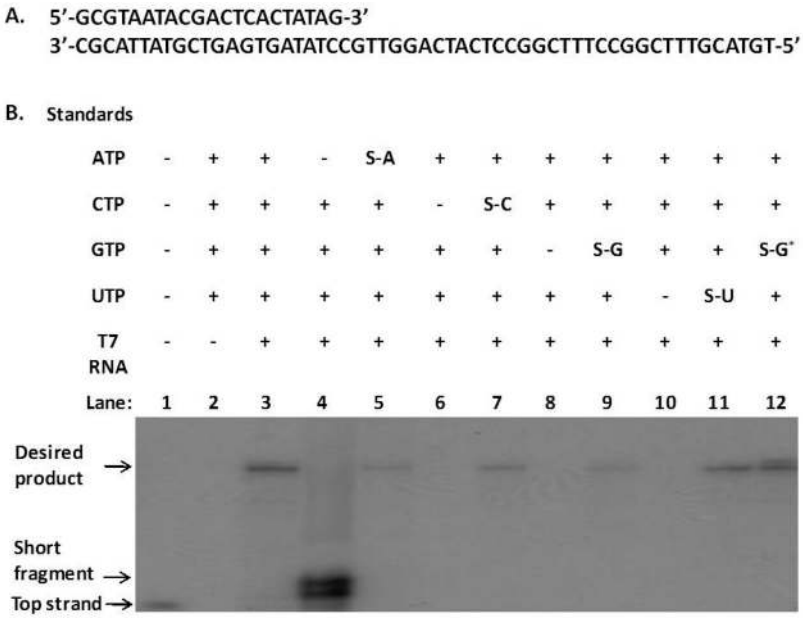

Figure 11.

Incorporation of chemically synthesized NTPαS and commercially available NTPs into RNA by T7 RNA polymerase. Transcribed RNAs are bodily-labeled by [α-^{32}P]-ATP during transcription. Transcription reactions are performed with the promoter strand (P) and template (T, 1.0 μM, each), NTPs (1.0 mM each), NTPαS (1.0 mM), and RNA polymerase (0.1 μL per μL of reaction) at 37 °C for 2 h. Reactions were analyzed on 19% polyacrylamide gel electrophoresis. Lane 1: promoter strand (P, 5′-^{32}P-labled as marker); Lane 2: P, T, [α-^{32}P]-ATP, and all NTPs without RNA polymerase; Lane 3: P, T, [α-^{32}P]-ATP, and all NTPs with the enzyme; Lane 4, 6, 8, and 10: equivalent to Lane 3 without ATP, CTP, GTP and UTP, respectively; Lane 5, 7, 9, and 11: equivalent to Lane 4, 6, 8, and 10 compensated with the synthetic NTPαSs (crude ATPαS, CTPαS, GTPαS and UTPαS, respectively). Lane 12: P, T, [α-^{32}P]-ATP, ATP, CTP, commercial GTPαS (s-G*) and UTP with T7 RNA polymerase.

Table 1

HR-MS (ESI) analysis of the synthesize nucleoside 5′-(alpha-P-thio)

Entry	Compounds	Molecular Formula	Calculated $[M-H^+]^-$	Measured $[M-H^+]^-$
8aS	dATPαS I	$C_{10}H_{16}N_5O_{11}P_3S$	505.9707	505.9716
	dATPαS II			505.9716
8cS	dCTPαS I	$C_9H_{16}N_3O_{12}P_3S$	481.9595	481.9583
	dCTPαS II			481.9570
8gS	dGTPαS I	$C_{10}H_{16}N_5O_{12}P_3S$	521.9656	521.9656
	*dGTPαS II			-
8tS	TTPαS I	$C_{10}H_{17}N_2O_{13}P_3S$	496.9591	496.9572
	TTPαS II			496.9572
9aS	ATPαS I	$C_{10}H_{16}N_5O_{12}P_3S$	521.9656	521.9657
	ATPαS II			521.9596
9cS	CTPαS I	$C_9H_{16}N_3O_{13}P_3S$	497.9544	497.9558
	CTPαS II			497.9533
9gS	GTPαS I	$C_{10}H_{16}N_5O_{13}P_3S$	537.9605	537.9620
	*GTPαS II			-
9uS	UTPαS I	$C_9H_{15}N_2O_{14}P_3S$	498.9384	498.9396
	UTPαS II			498.9398

triphosphates (dNTPαS)

NOTE: *dGTPαS II and *GTPαS II are not observed.

TABLE 2

HR-MS analysis of nucleoside 5′-(alpha-P-seleno)triphosphates

Entry	NTPαSe	Molecular Formula	Calculated $[M-H^+]^-$	Measured $[M-H^+]^-$
9aSe	ATPαSe I	$C_9H_{15}N_2O_{14}P_3Se$	546.8901	546.8848
	ATPαSe II			546.8818
9cSe	CTPαSe I	$C_9H_{16}N_3O_{13}P_3Se$	545.8983	545.8989
	CTPαSe II			545.8972
9gSe	GTPαSe I	$C_{10}H_{16}N_5O_{12}P_3Se$	569.9174	569.9082
	GTPαSe II			569.9077
9uSe	UTPαSe I	$C_{10}H_{16}N_5O_{13}P_3Se$	585.9044	585.9043
	UTPαSe II			585.9052

TABLE 3

^{1}H-NMR (400 MHz, D_2O): chemical shifts (ppm) of nucleoside 5′-(α-P-seleno) triphosphates.

NTPαSe	H1′	H2′	H3′	H4′	H5′	H8	H2	H6	H5
ATPαSe I	6.06	4.71	4.56	4.33	4.28 -4.18	8.64	8.16		
ATPαSe II	6.06	4.66	4.56	4.36	4.27 -4.19	8.55	8.16		
CTPαSe I	5.91	4.75	4.66	4.32	4.25 -4.22			8.01	6.08
CTPαSe II	5.91	4.75	4.66	4.33	4.26 -4.21			7.96	6.07
GTPαSe I	5.85	4.74	4.65	4.55	4.31 -4.20	8.12			
GTPαSe II	5.85	4.75	4.65	4.51	4.30 -4.21	8.19			
UTPαSe I	5.89	4.75	4.65	4.39	4.34 -4.24			8.05	6.01
UTPαSe II	5.88	4.75	4.65	4.40	4.35 -4.23			7.96	5.93

TABLE 4

^{13}C-NMR (D_2O): chemical shifts (ppm) of nucleoside 5′-(α-P-seleno) triphosphates.

NTPαSe	C4′	C1′	C3′	C2′	C5′	C4	C2	C6	C8	C5
ATPαSe I	86.7	83.8	74.3	70.4	65.1	149.1	152.8	155.6	140.4	118.6
ATPαSe II	86.7	83.8	74.3	70.5	65.8	149.1	152.8	155.6	140.1	118.5
CTPαSe I	89.0	82.5	74.2	69.4	64.4	166.2	157.8	142.0		96.8
CTPαSe II	88.9	82.5	73.9	69.3	65.1	166.0	157.3	141.8		96.6
GTPαSe I	86.7	83.8	73.7	70.7	65.4	151.8	154.0	158.9	138.0	116.1
GTPαSe II	86.7	83.7	73.8	70.6	65.8	151.7	153.9	158.7	137.7	115.8
UTPαSe I	88.1	83.1	73.7	69.6	64.6	166.3	151.9	142.1		102.7
UTPαSe II	88.2	83.2	73.8	69.6	65.5	166.3	151.9	143.0		102.8

TABLE 5

^{31}P-NMR (D_2O): chemical shifts (ppm) of nucleoside 5′-(α-P-seleno)triphosphates.

NTPαSe	αP	γP	βP	$J_{\alpha,\beta(Hz)}$	$J_{\beta,\gamma(Hz)}$
ATPαSe I	34.0	−6.9	−22.8	32.3	19.4
ATPαSe II	33.8	−6.2	−22.7	34.0	19.4
CTPαSe I	33.6	−9.1	−24.0	34.0	19.4
CTPαSe II	33.2	−7.1	−23.4	30.8	19.4
GTPαSe I	33.8	−9.5	−24.0	32.4	19.4
GTPαSe II	33.1	−6.3	−23.1	32.4	21.1
UTPαSe I	32.8	−6.5	−23.2	30.0	19.4
UTPαSe II	33.7	−5.6	−22.5	34.0	19.4