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Synthesis and enzymatic characterization of sugar-modified nucleoside triphosphate analogs

Stella Diafa, Damien Evéquoz, Christian J. Leumann, and Marcel Hollenstein*

Abstract

Chemical modification of nucleic acids can be achieved by the enzymatic polymerization of modified nucleoside triphosphates (dN*TPs). This approach obviates some of the requirements and drawbacks imposed by the more traditional solid-phase synthesis of oligonucleotides.

Here, we describe the protocol that is necessary to synthesize dN*TPs and evaluate their substrate acceptance by polymerases for their subsequent use in various applications including selection experiments to identify aptamers. The protocol is exemplified for a sugar-constrained nucleoside analog, 7ˈ,5ˈ-bc-TTP.

Key words nucleoside triphosphate, primer extension reactions, DNA polymerases, XNA, solid-phase synthesis, bicyclo-DNA.

1. Introduction

Initially, interests in the chemical modification of nucleic acids were spurred by the need for more resistant and potent therapeutic and antiviral agents.(Goyenvalle et al. 2015; Khvorova and Watts 2017; Taskova et al. 2017) Since then, chemical functionalization of nucleic acids has advanced as a key step in a number of applications including the development of potent and serum resistant aptamers and DNAzymes,(Diafa and Hollenstein 2015; Herdewijn and Marliere 2009) questioning the origin of life,(Jia et al. 2016) and the creation of novel biomaterials.(Chen and Romesberg 2017) Traditionally, chemical modifications were incorporated into DNA or RNA by solid-phase synthesis of oligonucleotides using activated phosphoramidite building blocks. While larger amounts of oligonucleotides, decorated with a number of functional groups are accessible, this method presents some limitations: i) only rather short sequences (≤ 100 nucleotides) can be synthesized, ii) some sensitive functional groups are not compatible with the rather harsh conditions that are required particularly for the deprotection and oxidation steps, and iii) there is no direct replication of the oligonucleotides or a facile creation of multiple modified sequences at once which thus precludes its use in evolution experiments.(Loakes and Holliger 2009) As a strongly developing alternative, nucleic acids can be functionalized through the polymerase-mediated incorporation of modified nucleoside triphosphates (dN*TPs).(Hocek 2014; Hollenstein 2012) Application of this methodology has permitted to introduce an impressive variety of functional groups ranging from small amino acid-like residues(Hollenstein et al. 2009; Jäger et al. 2005; Sakthivel and Barbas 1998) or electrochemical tags(Balintová et al. 2015; Hocek and Fojta 2011) to entire oligonucleotides(Baccaro et al. 2012; Verga et al. 2015) and even

polymerases.(Welter et al. 2016) The only prerequisite for the enzymatic synthesis of modified DNA is that the dN*TPs are recognized as a substrate by the polymerase.

In this report, we describe the entire protocol ―from synthesis to biochemical characterization― required for the evaluation of new dN*TPs in order to endow nucleic acids with additional modifications and properties. The protocol is exemplified with the modified triphosphate 7′,5′-bc-TTP,(Diafa et al. 2017; Evequoz and Leumann 2017) which is a member of the general bicyclo-DNA family of conformationally constrained nucleosides,(Leumann 2002) but can be broadly applied to any modified analog.

2. Materials

Use ultrapure water of 18 MΩ-cm resistivity for the preparation of all buffers as well as for the reactions. All anhydrous organic solvents used for reactions were obtained by filtration through activated aluminum oxide or by storage over activated molecular sieves (4 Å).

2.1 Synthesis of a bicyclo-DNA triphosphate

1. Two round bottom flasks (5 mL volume), one round bottom flask (25 mL volume) two magnetic stir bars (1 cm in length), two septa, two balloons, syringes, needles, two Falcon™ tubes of 15 ml volume.

2. $Na_2S_2O_3$: 10% solution in water.

3. $NaClO_4$: 2% solution in acetone.

2.2 Reverse Phase High Pressure Liquid Chromatography (RP-HPLC)

1. Stock solution: 1 M triethylammonium bicarbonate (TEAB), pH = 7.5: Add 139 mL of Et_3N in a graduated 1 L cylinder and fill to the top with water. Transfer the solution to a 1 L

glass bottle containing a magnetic stir bar and bubble dry ice through it until the pH of the solution drops to 7.5 (this requires at least 4 hours of stirring). Store the bottle at 4°C (see Note 1).

2. Elution Buffer A: 50 mM TEAB. In a 2 L graduated cylinder, add 100 mL of TEAB from the stock solution and 1.9 L of H_2O. Filter the solution under vacuum and transfer it to a 2.5 L bottle containing a magnetic stir bar. Store the bottle at 4°C.

3. Elution Buffer B: 50 mM TEAB in ACN/H_2O 1:1. In a 2 L graduated cylinder, add 100 mL of TEAB from the stock solution, 900 mL H_2O and 1 L acetonitrile. Filter the solution under vacuum and transfer it to a 2.5 L bottle equipped with a magnetic stir bar. Store the buffer at 4°C.

4. Type of column: core-shell, C18 columns are ideal choices for the purification of nucleoside triphosphates, particularly the LUNA semi-preparative RP-HPLC column (5 μm, C18, 100 Å) from Phenomenex.

5. Type of HPLC running system: Åkta pure or purifier systems are adequate but a Waters modular HPLC system is a valid alternative.

2.3 Anion Exchange High Pressure Liquid Chromatography (AE-HPLC)

1. Elution buffer B (for the purification of triphosphates): 2 M ammonium acetate (AA) in H_2O: In a 2.5 L bottle cooled on ice, add 0.5 L of H_2O and then 275 mL of ammonium hydroxide (28% solution in H_2O). Then carefully add 230 mL of glacial acetic acid and then adjust the pH to 8 with glacial acetic acid. Store the bottle at 4°C.

2. Elution buffer A (for the purification of triphosphates): 20 mM AA in H_2O: In a 2 L graduated cylinder, add 20 mL of AA from elution buffer B and 1.98 L of H_2O. Filter the solution under vacuum and transfer it to a 2.5 L bottle containing a magnetic stir bar. Store the bottle at 4°C.

3. Elution buffer A (for the purification of oligonucleotides): 25 mM Trizma® base (Tris(hydroxymethyl)aminomethane), pH = 8: add 3.029 g of Trizma® base and 950 mL of H_2O in a graduated 1 L cylinder. Adjust the pH to 8.0 with HCl 1 M or NaOH 1 M. Add H_2O to obtain a 1 L solution. Filter the buffers through a glass microfiber filter.

4. Elution buffer B (for the purification of oligonucleotides): 25 mM Trizma® base, 1.25 M NaCl, pH = 8: add 3.029 g of Trizma® base, 73.05 g NaCl and 950 mL of H_2O in a graduated 1 L cylinder. Adjust the pH to 8.0 with HCl 1 M or NaOH 1 M. Add H_2O to obtain a 1 L solution. Filter the buffers through a glass microfiber filter.

5. Type of column: semi-preparative DNAPac 100 or 200 columns (4 μm, 9.0 × 250 mm) containing quaternary amine functionalized beads and equipped with guard columns (4.0 x 50 mm) both from Thermo Fisher Scientific.

6. Sep-Pak® C18 Cartridge for desalting.

2.4 Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

1. 20% Acrylamide solution, 1 L: In a 1 L bottle equipped with a lid and a stir magnet, add 420 g urea, 100 ml of 10x TBE (Tris borate EDTA buffer), 90 mL of H_2O and 500 mL of 40% w/v Acrylamide (Bis solution 19:1). Stir at rt for 1h until all the urea has been dissolved. Store the solution at 4°C.

2. Diluent solution, 1 L: In a 1 L bottle equipped with a lid and a stir magnet, add 420 g urea, 590 mL H_2O and 100 mL of 10x TBE buffer. Stir at rt for 1h until the urea has been dissolved. Store the bottle at 4°C.

3. Running buffer, 10x TBE, 5 L: In a 10 L beaker with a stir magnet, add 540 g of Trizma® base, 275 g of boric acid, 200 mL of EDTA (0.5 M) solution and 4.5 L H_2O. Stir at rt until the buffer is homogeneous. Transfer the buffer to a 5 L container and store it at rt.

4. APS (Ammonium persulfate): 10% solution in H_2O (see Note 8).

5. TMED (Tetramethylethylenediamine).

2.5 ^{32}P-labeling of DNA primers

Appropriate care should be taken when working with radioactive material. The labeling should be performed in certified labs designated for this purpose.

1. ^{32}P-γ-ATP (270 μCi, Hartmann Analytic)

2. T4-PNK enzyme (polynucleotide kinase supplied by New England BioLabs®)

3. Buffer A (provided with the enzyme)

4. Spin column (Amicon® Ultra 0.5 mL by Sigma-Aldrich)

2.6 Primer Extension Reactions

1. Annealed oligonucleotides: In a 1.5 mL Eppendorf tube add 10 pmol of unlabeled primer, 10 pmol of template and 1 pmol of the radiolabeled primer. Mix gently, heat the oligonucleotides at 95°C for two minutes and let them slowly cool down to rt. This solution will be diluted in the reaction mixture to give a final concentration of 500 nM (see Note 10).

2. Loading buffer: 70% formamide, 50 mM EDTA, 0.1% bromophenol, 0.1% xylene cyanol.

3. dNTPs, 1 mM solutions

4. Therminator™ polymerase (New England BioLabs)

5. 10x buffer (provided with the enzyme)

6. $MnCl_2$, 100 mM solution

2.7 Solid phase synthesis of an oligonucleotide containing bicyclo-DNA residues

1. *Pharmacia-Gene-Assembler-Plus* DNA synthesizer, columns for the solid support, natural DNA phosphoramidites (dT, dC^{4bz}, dG^{2DMF}, dA^{6Bz}), modified bc-DNA phosphoramidite,{Evequoz, 2017 #9} solid support (dmf-dG-Q-CPG 500), bottles and vials for the synthesizer, 1.5 mL Eppendorf tubes, grease. All the glassware should be dried

overnight in an oven and carefully purged with argon prior to use. All the solutions should be stored under an argon atmosphere.

2. Molecular sieves: wash approximately 150 g of molecular sieves (beads of ~2 mm diameter, 4 Å pore diameter) with 500 mL of hexane, filtrate off the solvent and activate the molecular sieves by heating to 200°C overnight under vacuum (<1 mbar). Store the molecular sieves under argon.

3. Dried acetonitrile: fill a 1 L bottle with anhydrous acetonitrile. Add approximately 50 g of the previously activated molecular sieves and store the solution for a least one night before any further use.

4. Dried dichloroethane: fill a 500mL bottle with dichloroethane. Add approximately 25 g of the previously activated molecular sieves and store the solution for a least one night before any further use.

5. Detritylation solution: 3% dichloroacetic acid in dichloroethane.

6. Capping solution A: prepare a 0.5 M solution of 4-(dimethylamino)pyridine in dry acetonitrile.

7. Capping solution B: prepare a solution of acetonitrile, 2,4,6-trimethylpyridine and acetic anhydride in a 5:3:2 ratio.

8. Oxidation solution: prepare a 0.01 M solution of iodine in 2,4,6-trimethylpyridine/acetonitrile/water: Per 100 mL of solution, add 255 mg of iodine, 5.95 mL of 2,4,6-trimethylpyridine and 64.35 mL of acetonitrile. Assure the complete dissolution of iodine and then add 29.70 mL of H_2O.

9. DNA phosphoramidite solutions: 0.1 M phosphoramidite in acetonitrile: in 10 mL vials, add the DNA phosphoramidite and transfer with a syringe purged with argon the previously prepared dried acetonitrile solution. Add 4 beads of activated molecular sieves.

10. bc-DNA phosphoramidite solution: 0.1 M phosphoramidite in dichloroethane: in a 10 mL vial, add the bc-DNA phosphoramidite and transfer with a syringe purged with argon the previously prepared dried dichloroethane solution. Add 4 beads of activated molecular sieves.

11. Coupling agent solution: 0.25 M 5-(Ethylthio)-1*H*-tetrazole (ETT) in acetonitrile: In a 50 mL vial, add ETT and transfer with a syringe purged with argon the previously prepared dried acetonitrile solution. Add approximately 2.5g of molecular sieves per 100 mL.

3. Methods

3.1 Synthesis

The reaction is based on the protocol of Ludwig-Eckstein (Ludwig and Eckstein 1989). All the glassware should be dried overnight in an oven prior to usage. Use dry solvents for all steps of the reaction. Perform the reactions under inert atmosphere of Argon.

1. In a 5 mL round bottom flask co-evaporate 1 equivalent of the nucleoside (see Note 2) two times with 0.3 mL of anhydrous pyridine, then add a magnetic stir bar and let it dry overnight on a Schlenk line.

2. In a 5 mL round bottom flask containing a magnetic stir bar add 1.3 equivalent of bis-(tri-*n*-butylammonium) pyrophosphate (see Note 3) and let it dry overnight on a Schlenk line.

3. Dissolve the nucleoside in 0.2 mL pyridine. Add 0.4 mL of 1,4-dioxane and 1.1 equivalent of 2-chloro-1,3,2-benzodioxaphosphorin-4-one (see Note 4) and stir the yellow suspension for 45 min at rt.

4. Dissolve the pyrophosphate in 170 μL DMF and 58 μL of *n*-tri-butylamine (*n*-Bu_3N) and add the new solution to the suspension containing the nucleoside. Stir at rt for 1h.

5. Add 1.6 equivalent of I_2 as a solution in pyridine/H_2O (98:2) and stir for 30 min at rt.

6. Add a 10% $Na_2S_2O_3$ solution (0.5 mL) to quench the excess of I_2.

7. Evaporate the solvents in the flask under vacuum until dryness (see Note 5).

8. Dissolve the residue in 5 mL H_2O and transfer it to a 25 mL round bottom flask. Stir at rt for 30 min.

9. Add 10 mL NH_4OH (28-30%) and stir for 2h at rt.

10. Evaporate the solvents to dryness under vacuum.

11. Add 2 mL H_2O into the flask to dissolve the residue and split the content between two Falcon™ tubes. Fill the tubes with a 2% $NaClO_4$ solution in acetone, mix and centrifuge for 30 min at 13,000 rpm. Discard the organic layer, redissolve the yellow precipitate in the $NaClO_4$ solution and repeat the precipitation. Remove the organic phase, dissolve the residues in H_2O and combine them in one tube with a total volume of 4 mL. Store in the freezer until the end and during the purification by RP-HPLC or AE-HPLC.

3.2 Purification by RP-HPLC

1. Bring buffers A and B to rt and degas them for 20 min by stirring them under vacuum prior to their usage.

2. Carry out the purification of the triphosphate in aliquots of 1 mL on a C18 Semi-Prep Reverse Phase column equipped with a guard column.

3. Inject 200 µL of the crude solution per round and use the following gradient conditions for the purification: 0 to 8 min: 0% B; 8 to 48 min: 0-15% B; 48 to 50 min: 15-100% B (see Note 6).

4. Collect the fractions corresponding to the triphosphate peak (R_t = 43 min) in round bottom flasks and store them in the freezer until the end of the purification.

5. Freeze-dry all the collected aliquots and co-evaporate with H_2O two times to sublime the triethylammonium bicarbonate salt.

6. Confirm the presence of the triphosphate in the sample by MALDI-MS (see Note 7).

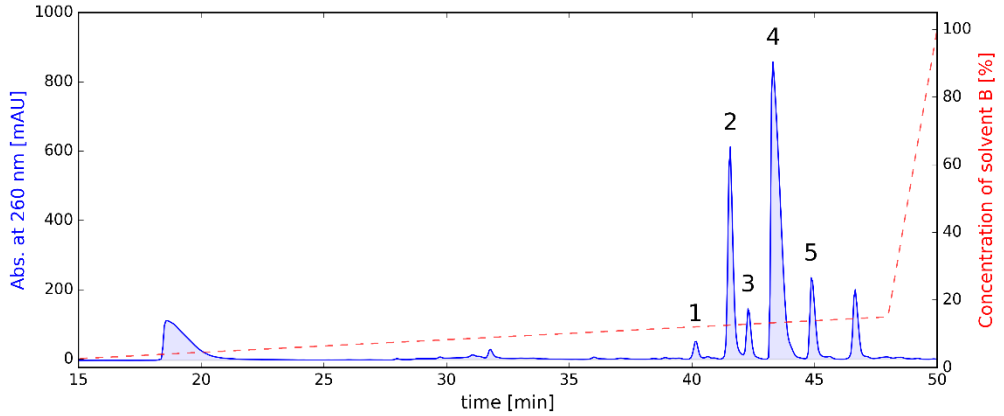

Fig. 1: Representative chromatogram of a 7',5'-bc-TTP purification by RP-HPLC; The peaks correspond to: **1**) 7',5'-bc-T-monophosphate; **2**) 7',5'-bc-T-diphosphate; **3**) 7',5'-bc-T-phosphonate; **4**) 7',5'-bc-T-triphosphate; **5**) 7',5'-bc-tetraphosphate.

3.3 Purification by AE-HPLC

1. Repeat the same steps as described in 3.2 but using a linear gradient reaching 30% of eluent B in 30 min (see Note 6).

3.4 10% PAGE analysis

1. Mix equal volumes of the 20% Acrylamide solution and the diluent in a beaker.

2. Add 1% of the volume 10% APS solution to initiate the radical reaction and continue stirring the mixture.

3. Add 0.1% of the volume TMED to begin the polymerization.

4. Use the solution to cast a gel and let the gel polymerize for 4h at rt.

5. Fix the gel plates on the gel chamber and load 1x TBE buffer by diluting the 10x running buffer.

6. Preheat the gel for 30 min at 20W to remove unpolymerized acrylamide (see Note 9).

7. Using a syringe, wash the wells with the running buffer to remove any bubbles or polyacrylamide particles trapped in them.

8. Load the samples.

9. Run the gel at 40W for 2.5h.

10. Take down the gel, remove the gel plates carefully and transfer the gel on a Whattman® blotting paper. Dry the gel for 1h under vacuum using a gel dryer.

11. Visualize the gel on a Phosphorimager (Typhoon FLA 7000 by GE).

3.5 Labeling of DNA primers

1. In a 1.5 mL Eppendorf tube add 30 pmol of primer, 3 μL buffer A, 3 μL ^{32}P-γ-ATP, 1 μL PNK and fill up with H_2O up to 30 μL total volume. Mix at rt.

2. Incubate the Eppendorf tube at 37°C for 40 min using a heating block.

3. Heat the sample to 95°C for 5 min to quench the enzyme.

4. Purify the labeled primer using a spin column and following the instructions of the manufacturer.

5. Store the label in the freezer.

3.6 Primer Extensions

1. In a 1.5 mL Eppendorf tube containing the annealed oligonucleotides (final concentration of 500 nM) add 1x buffer, 1 mM $MnCl_2$, 100 μM dNTPs, 2U of enzyme and H_2O for a total reaction volume of 20 μL and mix at rt (see Note 11).

2. Incubate the reactions at 55°C with continuous stirring for 1h.

3. Stop the reactions by adding equal volume of loading buffer.

4. Denature the DNA strands by heating the reaction tubes at 95°C for 5 min and rapidly cooling them down to 0°C. Centrifuge the reactions for a two seconds before loading them on the gel.

5. Load 8 μl of each reaction on a 10% polyacrylamide gel well.

6. Visualize the radiolabeled extended primers with phosphorimaging.

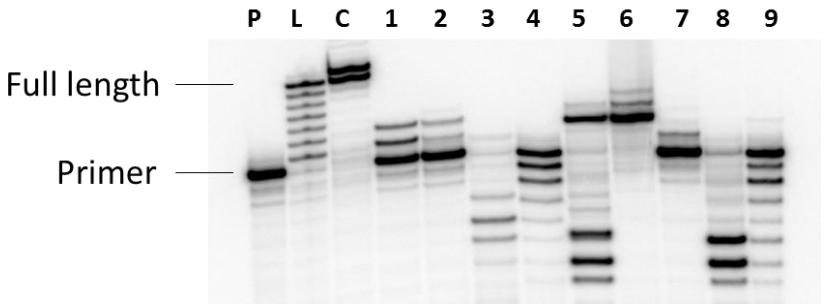

Fig. 2: Representative 20% PAGE image as visualized by phosphorimaging showing several primer extension reactions performed with different polymerases (lanes 1-9). P contains only the primer, L is a ladder formed to assist the determination of the number of incorporations and C is a control reaction performed with the wild type dTTP to exclude possible error factors attributed to any of the additives.

3.7 Solid phase synthesis of an oligonucleotide containing bicyclo-DNA residues

Capping, oxidation and detritylation should be performed with standard conditions and following the protocols recommended by the manufacturer of the Gene Assembler. Natural DNA phosphoramidites are coupled using a 1.5 min step. The modified bc-DNA requires an extended coupling time of 12 min. To ensure optimal yields, the acetonitrile, phosphoramidite and coupling agent solutions should be maintained under strict anhydrous conditions and the system should be kept under an inert atmosphere of argon.

1. Flush the DNA synthesizer for 10 min with argon and then fix the reagent bottles. Add a fine layer of grease to the top of the vials and fix them. Make sure the DNA synthesizer is under a slight overpressure of argon.

2. Wash the system by running acetonitrile. Prime all the reagent lines and then wash the system again with acetonitrile.

3. Run a test sequence: place a column filled with 1.3 µmol of solid support into the reactor chamber and run a sequence having each natural nucleobase in a consecutive manner (e.g.: 5'-AAT TCC GGG-3'). Monitor the coupling efficiency by recording the trityl absorbance at 436 nm during the detritylation step. The coupling efficiency should be over 98% (see Note 12).

4. Run the solid phase synthesis: enter the desired sequence (e.g.: 5'-GGX ACA XGT CAT AGC TGT TTC CTG-3'). Make sure that the correct protocols are selected for each nucleobase and that the final detritylation will be performed. Place a column filled with 1.3 µmol of solid support into the reactor chamber. Run the synthesis and monitor the coupling efficiency as before.

5. Dry the solid support: at the end of the synthesis, take out the column from the reactor chamber and place it into a 1.5 mL Eppendorf tube. Centrifuge for 2 min at 4000 rpm.

6. Deprotect and cleave the oligonucleotide from solid support: open the column and transfer the solid support into a 1.5 mL Eppendorf tube equipped with a screw cap. Add 1.5 mL of a 33% ammonia solution (see Note 13). Incubate the Eppendorf tube at 55°C shaking at 300 rpm for 16h.

7. Cool down the Eppendorf tube to 0°C, centrifuge it for 10 min at 4000 rpm and collect the supernatant. To further wash the solid support, add 0.5 mL H_2O to the beads, shake the Eppendorf tube, centrifuge it for 10 min at 4000 rpm and collect the supernatant. Repeat this step a second time. Freeze-dry the combined supernatant, resuspend the crude oligonucleotide

in 1 mL H_2O and filter the resulting solution with a 13 mm syringe filter (0.45 µm pore size). The crude oligonucleotide can be purified by ion-exchange HPLC or by 20% PAGE.

3.8 Purification of oligonucleotides by ion-exchange HPLC

1. Bring buffers A and B to rt and degas them for 20 min by stirring them under vacuum prior to their usage.

2. Equilibrate the semi-preparative DNAPac 200 column with a solvent mixture of 20% B.

3. Inject 300 µL of the crude oligonucleotide solution per round and use the following gradient conditions for the purification: 0 to 7 min: 20% B; 7 to 35 min: 20-55% B; 35 to 40 min: 100% B.

4. Collect the top of the peak corresponding to the full-length oligonucleotide in a 15 mL Falcon™ tube. Equilibrate the column with the starting condition before performing another round.

4. Desalt the combined fractions with a Sep-Pak® C18 Cartridge following the manufacturer's instructions. Freeze-dry the collected fractions and resuspend the purified oligonucleotide in 1 mL H_2O.

5. Determine the concentration by measuring the absorbance at 260 nm with a NanoDrop™ spectrophotometer, using the extinction coefficient of the corresponding natural DNA oligonucleotide.

6. Confirm the mass by ESI^- mass spectrometry or LC-MS.

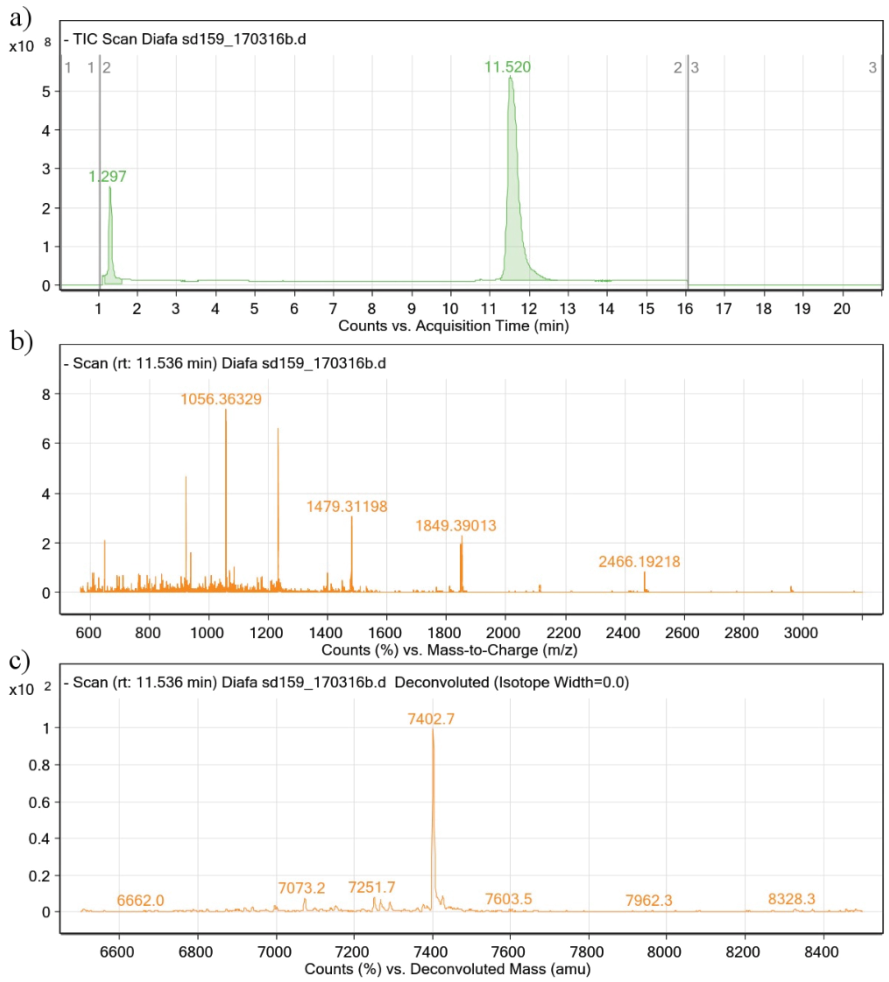

Fig. 3 Representative result of an oligonucleotide characterization by LC-MS: a) Chromatogram of the sample: the peak with a R_t of 11.52 min corresponds to the oligonucleotide; b) Mass spectrum: the higher peaks should correspond to m/z of the most abundant oligonucleotide isotope; c) Deconvoluted mass spectrum: the higher peak should correspond to the molecular weight of the oligonucleotide.

4 Notes

1. The stock solution can be stored in the fridge for a few months. Eventually the CO_2 will escape the flask and lead to an increase of the pH, which will cause difficulties to the purification of the triphosphate. We recommend measuring the pH value of the solution every time prior to the preparation of the elution buffers.

2. We find that the reaction works best in the range of 20-60 mg of nucleoside (i.e. 60-190 µmol).

3. The nucleophilic bis-(tri-*n*-butylammonium) pyrophosphate is a key reagent in the synthesis. Since it is a rather hygroscopic solid, it is advised to thoroughly dry the reagent for at least 16 hours under vacuum prior to use. Alternatively, a 0.5 M solution in DMF can be prepared which can be dried overnight with activated 4 Å molecular sieves. Lastly, if low triphosphate yields are regularly obtained, the reagent should be prepared freshly prior to use as described in the literature.(Sarac and Meier 2015; Sarac and Meier 2016)

4. The reaction is significantly influenced by the nature of this reagent. We find that it works best when it is in the form of colorless crystals than in the form of a powder. Additionally, upon oxidation, a white coat can be formed around the crystals, preventing them from reacting. In such case, it is advised that the surface of the crystal be scratched prior to the reaction.

5. Ensure that the temperature of the water bath does not exceed the 30°C to avoid thermal degradation of the triphosphate moiety

6. The R_ts of the di- and triphosphate are close to each other. Depending on the resolution of the column, the corresponding peaks might be overlapping. In this case, the separation can be improved by reducing the volume of the crude solution injected and by adjusting the elution conditions. Alternatively, purification can be performed by AE-HPLC where a better separation of the diphosphate from the triphosphate is usually observed. The use of the volatile ammonium acetate buffer system precludes a tedious desalting protocol of the resulting nucleotide analogs.

7. Although an ESI-MS could confirm the presence of the triphosphate as well, a peak for the presence of the diphosphate would render it difficult to distinguish between fragmentation pattern and contamination of the sample. By minimizing the fragmentation of the molecule, it will be easier to ensure that successful separation has taken place.

8. We recommend preparing a fresh APS solution prior to use or to prepare small aliquots that can be stored in the freezer to avoid problems with the polymerization of the gels.

9. Depending on the temperature and the power source available, the power can be adjusted to higher or lower values.

10. For longer templates (>60 nucleobases) it is preferable to use lower concentrations of annealed oligonucleotide (150 nM-200 nM) to avoid overloading of the gel wells and get a better resolution of the gel.

11. The enzyme should be added last as it initiates the reaction.

12. If the DNA synthesizer has not been used over an extended period of time, the first couplings can be affected due to the remaining presence of moisture. If the initial test sequence does not lead to satisfactory yields, run a second test sequence. If one natural DNA phosphoramidite is not coupling efficiently, we advise to discard the solution and prepare a fresh phosphoramidite solution in a new vial.

13. To ensure proper deprotection of oligonucleotides, it is important to use a fresh solution of concentrated ammonia. For general use, we advise to prepare 1.5 mL aliquots from a new concentrated ammonia solution.

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