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Efficient Automated Solid-Phase Synthesis of DNA and RNA 5′-Triphosphates

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Dedicated to Prof. Dr. Dr. h.c. mult. Wittko Francke, University of Hamburg on the occasion of his 75th birthday

Abstract: A fast, high-yielding and reliable method for the synthesis of DNA- and RNA 5′-triphosphates is reported. After synthesizing DNA or RNA oligonucleotides by automated oligonucleotide synthesis, 5-chloro-saligenyl-*N*,*N*-diisopropylphosphoramidite was coupled to the 5′-end. Oxidation of the formed 5′-phosphite using the same oxidizing reagent used in standard oligonucleotide synthesis led to 5′-*cyclo*Sal-oligonucleotides. Reaction of the support-bonded 5′-*cyclo*-Sal-oligonucleotide with pyrophosphate yielded the corresponding 5′-triphosphates. The 5′-triphosphorylated DNA and RNA oligonucleotides were obtained after cleavage from the support in high purity and excellent yields. The whole reaction sequence was adapted to be used on a standard oligonucleotide synthesizer.

Introduction

5′-Triphosphorylated (5′-ppp-)oligonucleotides have a wide range of applications in biochemistry. While DNA 5′-ppp-oligonucleotides may be used in ligation reactions,[1] 5′-triphosphorylated RNA and their conjugates have a broader spectrum of applications. For example, RNA 5′-triphosphates were used for ligation reactions,[2,3] as antiviral compounds,[4] in the enzymatic synthesis of m^{7}G-capped-5′-triphosphorylated RNA,[5–7] in the induction of antiviral immunity,[8] as well as in the detection of viral responses by activation of the RIG-I protein.[9] Moreover, triggering of immune responses by RIG-I can result in siRNA-mediated gene silencing and potential target knockdown (synergistic effect).[10] There are only a few methods described so far for the efficient synthesis of 5′-triphosphorylated oligonucleotides and they are usually derived from the methods used for nucleoside triphosphate ((d)NTP) synthesis involving *H*-phosphonates[11] and the Ludwig/Eckstein phosphitylation reagent.[12] Zlatev et al. reported on a *H*-phosphonate-based method producing a 5′-phosphorimidazolidate that is converted with pyrophosphate to the corresponding 5′-triphosphate using ultramild base protecting groups.[13] Later this method was adjusted to be used on an ABI-394 DNA/RNA synthesizer.[14] The major product is the 5′-triphosphated oligonucleotide but the side products were some lower phosphorylated compounds and the non-phosphorylated oligonucleotide (5′-OH, 5′-phosphonate, 5′-phosphate and 5′-diphosphate).

Following the method developed by Ludwig and Eckstein,[12] Nagata et al. showed that a solid-phase-bonded 25 mer RNA-oligonucleotide could be phosphitylated using 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl phosphorochloridite method) which was subsequently reacted with pyrophosphate to yield a 5′-cyclotriphosphitylated oligonucleotide followed by oxidation to give the 5′-triphosphate.[15] When tris(tetra-*n*-butylammonium) hydrogen pyrophosphate was used, the major product was the desired 5′-triphosphate with still a significant amount of 5′-OH and 5′-phosphonate oligonucleotide. However, when bis(tri-*n*-butylammonium) dihydrogen pyrophosphate was used only a low triphosphorylation efficiency was observed. In this case high amounts of the 5′-phosphonate were obtained.

Ludwig et al. later improved this approach by first oxidizing the 5′-cyclotriphosphite to yield the 5′-cyclotriphosphate form using *tert*-butylhydroperoxide.[16] A subsequent ring-opening with aliphatic amines such as *n*-decylamine led to lipophilically tagged γ-phosphoramidate RNAs. After cleavage and deprotection with AMA (concentrated ammonia in aqueous methylamine) for 10 min at 65 °C, the tagged RNAs were purified by reversed-phase ion-pairing chromatography as these oligonucleotides elute with markedly higher retention times. After purification, the lipophilic tag was cleaved by hydrolysis of the P–N bond using a TEMED buffer (pH 3.8) at 60 °C for 70 min. Surprisingly, from the paper it seemed that the oligonucleotide triphosphates proved to be stable under these conditions, although we have observed marked degradation of the triphosphate at acidic conditions.

Although these methods work in principle, all of these methods have one significant disadvantage: they are not transferable to the conditions of the automated oligonucleotide synthesis because the needed reagents are too reactive[14] and not designed to be used on standard synthesizers respectively.

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Furthermore, lower phosphorylated byproducts as mono- and diphosphorylated oligonucleotides may be formed during the synthesis, which are often difficult to separate from the target triphosphorylated product by chromatographic means.

In previous reports we synthesized various nucleotide bioconjugates applying the *cyclo*Sal-method[17,18] using activated *cyclo*Saligenylchlorophosphites of the general structure **1** (Figure 1).

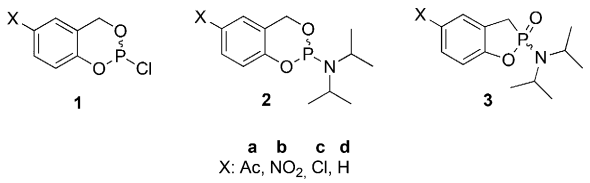


Figure 1. General structure of *cyclo*Sal-chlorophosphites **1**, *cyclo*Sal-phosphoramidites **2** and their Arbuzov isomerized form **3**.

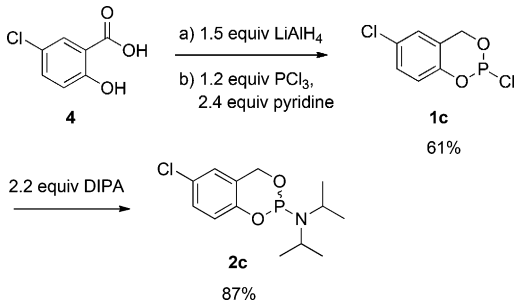
With regard to an automated 5′-pppDNA/RNA synthesis these compounds cannot be used in commercially available synthesizers because of their high reactivity. Nevertheless, although the difference to the salicyl phosphorochloridite method reported by Ludwig/Eckstein to the *cyclo*Saligenylchlorophosphite seems to be small, the latter compounds showed in the past already clear advantages.[17,18]

Therefore, our aim was to develop a method that is compatible with reagents used in the standard phosphoramidite chemistry protocol and a protocol that allows that the whole process can be used in an automated oligonucleotide synthesizer based on the *cyclo*Sal-method. We focused our attention on the preparation of *cyclo*Sal-phosphoramidites that have a lot of advantages compared to the currently published methods (compounds **2**, Figure 1): 1) *cyclo*Sal-phosphoramidites may be coupled and oxidized with the same reagents used in oligonucleotide synthesis, 2) in principle the same excellent coupling efficiencies can be expected as with standard DNA phosphoramidites, and 3) the subsequent phosphorylation reaction proceeds in a short reaction time at room temperature due to the increased reactivity caused by an electron-withdrawing substituent in the *cyclo*Sal-moiety.[17]

Results and Discussion

After the preparation and evaluation of *cyclo*Sal-phosphoramidites **2** with different electron-withdrawing groups at the 5-position, compound **2c** bearing a 5-chloro substituent was selected to be used in all further coupling reactions. In principle, for a fast phosphorylation reaction with pyrophosphate it is desirable to have a strong electron-withdrawing group attached to the 5-position of the *cyclo*Sal-ring because of the high electrophilicity at the phosphorus atom.[17,21] However, we observed that strong electron-withdrawing substituents, for example, the 5-acetyl- and 5-nitro-group in the *cyclo*Sal derivatives led to a rapid isomerization to give the thermodynamically more stable Arbuzov products **3** (Figure 1).[22] In contrast, the 5-unsubstituted and 5-chloro-*cyclo*Sal-phosphoramidites were found to be stable (Cl/H $\ll$ Ac $<$ NO_2). In addition, the chemical stability of the 5-acetyl- and 5-nitro-*cyclo*Sal compounds with regard to hydrolysis was markedly low which made the purification difficult. Therefore, we decided to focus on 5-chloro-saligenyl-*N*,*N*-diisopropylphosphoramidite (**2c**) for further studies because it is the most stable compound of the *cyclo*Sal-phosphoramidites **2a**–**c** and the starting materials used for the synthesis are readily available. The 5-unsubstituted phosphoramidite **2d** was found to be only poorly reactive towards the phosphorylation with pyrophosphate and thus has not been further considered.

The synthesis of 5-chloro-saligenyl-*N*,*N*-diisopropylphosphoramidite (**2c**) was achieved in 3 steps in an overall yield of 53% (Scheme 1).



Scheme 1. Synthesis of the *cyclo*Sal-phosphoramidite **2c** used for the synthesis of DNA- and RNA-5′-triphosphates. DIPA = diisopropylamine.

First, 5-chlorosalicylic acid **4** was reduced with lithium aluminumhydride to give 5-chlorosaligenol, which was then reacted with phosphorus trichloride in the presence of pyridine to give 5-chloro-*cyclo*Sal-chlorophosphite **2**. After purification by Kugelrohr distillation, compound **2** was reacted with 2.2 equivalents of diisopropylamine. The resulting 5-chloro-saligenyl-*N*,*N*-diisopropylphosphoramidite (**2c**) was purified by silica gel filtration and was obtained as a colorless solid after evaporation of the solvent. This material was found to be suitable to be used in a DNA/RNA synthesizer.

*Cyclo*Sal-phosphoramidite **2c** showed comparable stability to normal nucleoside 3′-*O*-phosphoramidites and it can be stored in the refrigerator for months without degradation. When dissolved in acetonitrile *cyclo*Sal-phosphoramidite **2c** was stable for at least a few days when kept under dry conditions and no formation of the Arbuzov product was observed. In a separate control experiment, 5-chloro-*cyclo*Sal-phosphonamidate **3c** was used together with the *cyclo*Sal-phosphoramidite **2c** in the coupling reaction but **3c** did not couple to the 5′-end of the oligonucleotides. Thus, even in the case that some degree of rearrangement of **2c** to give **3c** occured, no formation of side products during the coupling reaction has to be considered. In contrast to the *cyclo*Sal-phosphoramidites one might consider to convert the Ludwig/Eckstein phosphitylation reagent into its phosphoramidite form in order to obtain a more selective phosphitylation reaction. To the best of our knowledge, this compound hasn't been described before. Consequently, we synthesized and purified it on a route compara-

ble as used for the *cyclo*Sal-phosphoramidites **2**. However, the resulting phosphoramidite could not be coupled to a 5′-OH nucleoside. The Ludwig/Eckstein-phosphoramidite obtained showed high stability in acetonitrile (no Arbuzov isomerization) but in contrast to the *cyclo*Sal-phosphoramidite **2c** no coupling reaction at all which might be due to an electron-deficient phosphorus atom caused by the adjacent carbonyl group. Therefore, the Ludwig/Eckstein procedure cannot be applied to standard automated oligonucleotide synthesizers.

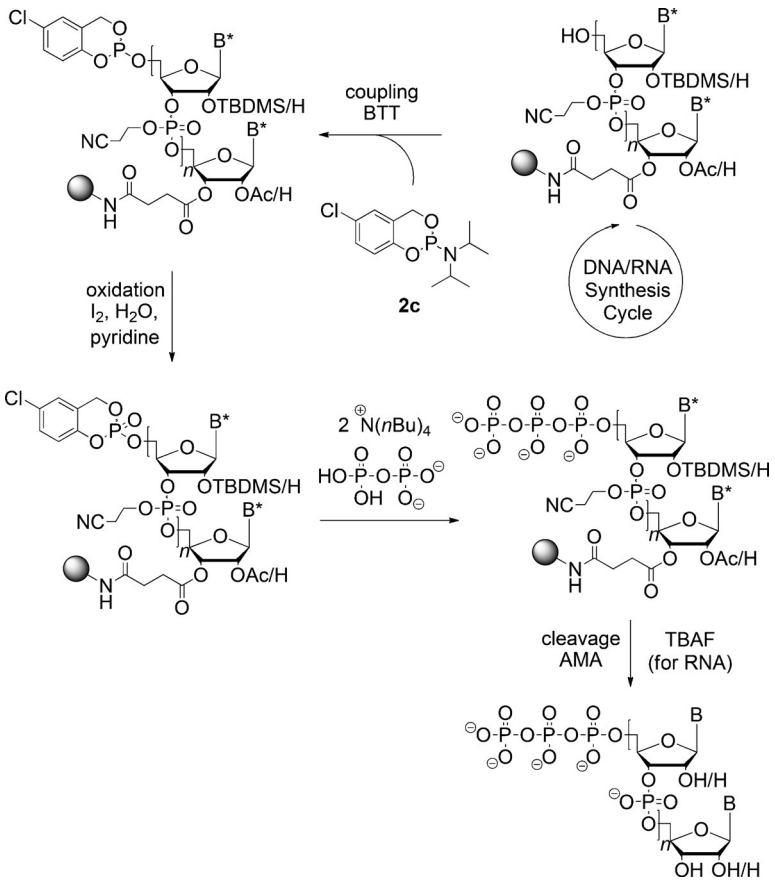
The general synthesis route for both DNA and RNA 5′-triphosphorylated oligonucleotides using *cyclo*Sal-phosphoramidite **2c** is depicted in Scheme 2.

Briefly, an oligonucleotide sequence was synthesized on support and the 5′-*O*-dimethoxytrityl protecting group was removed by standard conditions. Next, the coupling and oxidation of the *cyclo*Sal-phosphoramidite **2c** was carried out with the reagents used on the synthesizer. After the pyrophosphorylation, which was still conducted on the synthesizer, the resulting 5′-triphosphorylated oligonucleotide was deprotected and cleaved from the support. Overall this process makes the method convenient to be applied to any automated oligonucleotide synthesizer.

As a first example, a 5′-triphosphorylated 7 mer-thymidine oligonucleotide (5′-pppT_7) was synthesized. After the synthesis of the 7 mer-thymidine oligomer by routine phosphoramidite chemistry and detritylation, **2c** was coupled with 5-benzylthio-1*H*-tetrazole (BTT) as an activator to form the corresponding 5′-*O*-phosphite. The subsequent oxidation with the iodine oxidizer solution led to the supported 5′-*O*-*cyclo*Sal-oligonucleotide, which was then phosphorylated using a bis(tetra-*n*-butylammonium) dihydrogen pyrophosphate solution in DMF for 2 h at room temperature. The whole synthesis as well as the phosphorylation reaction was carried out in a fully automated way. The cleavage from the support as well as the base deprotection was achieved in this case with aqueous conc. ammonia. To analyze the quality of the 5′-triphosphorylated T_7-oligonucleotide it was necessary to use ion exchange (IEX) HPLC because in reversed-phase (RP) HPLC 5′-triphosphorylated oligonucleotides could not be separated from their 5′-di- or 5′-monophosphorylated forms. After IEX-HPLC purification, an additional desalting step (Sephadex G-25 DNA grade) was needed to remove the salts that were present in IEX-HPLC buffers. The IEX-HPLC chromatogram of crude 5′-pppT_7 is shown in Figure 2.

No non-phosphorylated 5′-OH-T_7-oligonucleotide was detected, showing that a quantitative coupling of the *cyclo*Sal-phosphoramidite **2c** was achieved. It was proven that a coupling time of 45 seconds was enough for a quantitative reaction. The only byproducts formed were very minor amounts of the mono- and diphosphorylated oligonucleotides (in total $<4\%$), which proved the very high conversion rate (crude HPLC purity of the triphosphorylated product was 90%). The very minor formation of pT_7 was attributed to an incomplete phosphorylation with pyrophosphate and ppT_7 was formed because commercially available pyrophosphate salts may also contain monophosphate, which led to the formation of 5′-*O*-diphosphorylated oligonucleotides. In first experiments, the quality grade of the used pyrophosphate was 97% and the concentration was 0.4 M. For all further reactions only ACS grade pyrophosphate with a purity of $\geq 99\%$ and a concentration of 0.5 M was used to avoid 5′-*O*-diphosphorylated products and to obtain a complete phosphorylation reaction.

Using this slightly changed protocol, a triphosphorylated 20 mer cytosine-DNA oligonucleotide was synthesized and the crude material was analyzed by ESI-MS. The 5′-ppp$(dC)_{20}$ oligonucleotide showed no mono- or diphosphorylated side products at all (Figure 3). These results proved that a highly efficient reaction to the 5′-*O*-tri-



Scheme 2. Automated solid-phase synthesis of DNA- and RNA-5′-triphosphates.

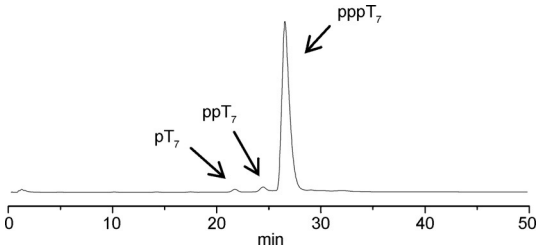


Figure 2. IEX-HPLC Chromatogram of crude 5′-pppT$_7$.

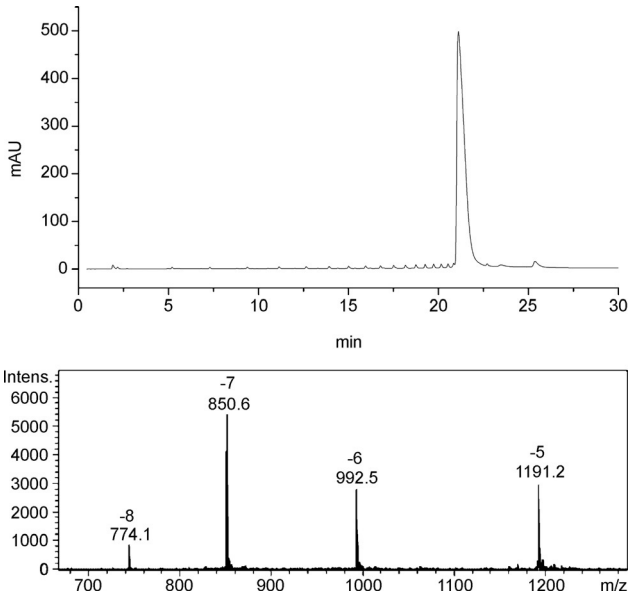


Figure 3. IEX-HPLC chromatogram (top) and ESI mass spectrum (bottom) of crude 5′-ppp(dC)$_{20}$. Deconvoluted mass: 5959.09 (calcd: 5961.62).

phosphates has been achieved. The lower phosphorylated oligonucleotides (5′-OH, 5′-phosphonate, 5′-phosphate and 5′-diphosphate) which are formed as side products in the method reported by Zlatev[13,14] are usually difficult to remove by chromatographic means.

After we showed that the method exclusively gave the 5′-*O*-triphosphorylated oligonucleotides, the purification was achieved by RP-HPLC using triethylammonium acetate (TEAA) as a volatile buffer instead of ion-exchange chromatography, which simplified the purification process.

When we applied this method to the synthesis of mixed DNA sequences ESI and MALDI-MS experiments revealed that in the phosphorylation reaction the formed *o*-quinone methide was able to react with adenosine and guanosine residues leading to covalently modified side products (Figure 4).

This formation of byproducts was not observed in the case of 5′-ppp(dC)$_{20}$ and 5′-pppT$_7$. Such a possible Michael-type reaction was previously reported for acrylonitrile and *p*-tolylvinyl sulfone during the deprotection of the 2′-*O*-cyanoethyl (CE),[23] 2′-*O*-(2-cyanoethoxymethyl) (CEM)[24] and 2′-*O*-2-(4-tolylsulfonyl)-ethoxymethyl (TEM)[25] group in RNA synthesis when using tetra-*n*-butylammonium fluoride (TBAF).

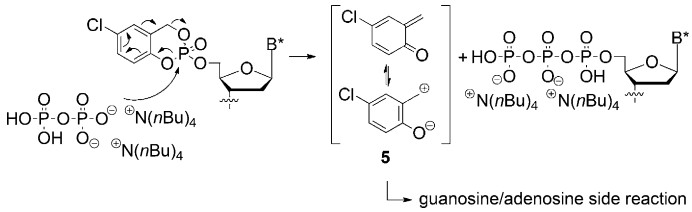


Figure 4. Formation of 4-chloro-6-methylene-2,4-cyclohexadien-1-one (**5**) in the pyrophosphorylation step.

Therefore, we tried different counterions than tetra-*n*-butylammonium for the pyrophosphate reagent that might act as a scavenger for the cleaved intermediate **5**. Bis(tri-*n*-butylammonium) dihydrogen pyrophosphate might be an alternative because in comparison to tetra-*n*-butylammonium, the tri-*n*-butylammonium counterion acts as a weak acid. In the case of bis(tri-*n*-butylammonium) dihydrogen pyrophosphate, which is often used in the literature for triphosphorylation reactions,[16–19] we proved by pH measurements that only 1.2 counterions of tri-*n*-butylammonium remained on the final product instead of the assumed two equivalents.

Although tri-*n*-butylamine has a boiling point of 214 °C, it is volatile enough and can be evaporated while drying to a fine powder due to its relatively weak ionic bond with pyrophosphate. The prepared tri-*n*-butylammonium pyrophosphate was soluble in DMF, but not nucleophilic enough for the phosphorylation reaction. By adding an additional 0.8 equivalents of anhydrous tri-*n*-butylamine to that solution, a two-phase system appeared. 1,4-Dioxane had to be added to the mixture to obtain a homogeneous phase; however, it didn't affect the reactivity. In conclusion, the preparation of a fine powder of bis(tri-*n*-butylammonium) dihydrogen pyrophosphate was not possible because the expected number of counterions were not obtained resulting in a less reactive pyrophosphate ion which was not suitable with the *cyclo*Sal-method because of its insufficient nucleophilicity.

Next, different scavengers such as nitromethane[26] and amines were tested and added to the solution of bis(tetra-*n*-butylammonium) dihydrogen pyrophosphate in DMF. Because tertiary amines as triethylamine and tri-*n*-butylamine are not soluble in DMF, secondary amines were screened. Sterically hindered amines such as diisopropylamine led to a higher *o*-quinone methide induced byproduct formation than more basic and nucleophilic amines. Finally, morpholine and piperidine were selected, which were also used to trap *p*-tolylvinyl sulfone that was formed during the cleaving of the 2′-*O*-TEM group in RNA synthesis.[27] Thus, in principle either morpholine or piperidine as 5% volume additive can be used in the phosphorylation reaction to prevent byproduct formation. Nevertheless, although no difference in the scavenger efficiency between morpholine and piperidine was observed, we decided to use the less basic morpholine to prevent potential chain cleavage in RNA synthesis.[27]

Morpholine as a 5% volume additive was successfully used in the phosphorylation reaction to avoid any byproduct

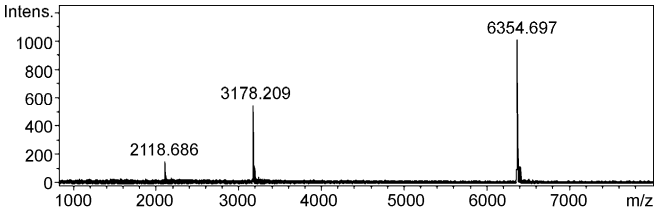


Figure 5. MALDI mass spectrum of RP-HPLC purified 5′-ppp[d(GTC AGA GCT TAG CTA GAC CT)]. Mass found: 6354.697 (calcd: 6355.913).

formation for a mixed-sequence 20 mer-DNA oligonucleotide (Figure 5).

In contrast, morpholine was not used in further RNA triphosphorylation reactions because in some cases some byproduct formation was observed even in the presence of morpholine for unknown reasons. Fortunately, the RP-HPLC purification is easy because the *o*-quinone methide induced byproducts elute with markedly higher retention times as compared to the 5′-ppp-oligonucleotides (Figure 6). In summary, the addition of the scavenger should be used for 5′-ppp-DNA to prevent byproduct formation while in the case of the more labile triphosphorylated RNA-oligonucleotides some other side reactions were observed because of the added scavenger and therefore here the addition of morpholine should be avoided.

For RNA deprotection and cleavage from the support, it is critical not to use AMA at 65 °C for 10 min, which is the widely used method for the deprotection of regular RNA oligonucleotides and it has been used for RNA 5′-triphosphate synthesis as well.[16] When using high temperatures, serious degradation of the 5′-*O*-triphosphate moiety to the 5′-*O*-di- and 5′-*O*-monophosphorylated forms was observed. In contrast, when we compared the deprotection of a normal base-protected 5′-OH-RNA oligonucleotide with AMA for 2 h at room temperature or for 10 min at 65 °C, we did not detect any difference in the purity of the resulting deprotected RNA oligonucleotides. Consequently, in case of the 5′-ppp-RNA oligonucleotides, the deprotection should be carried out at low temperatures, otherwise mono- and diphosphorylated oligonucleotides will be formed. When standard base protection (G(*i*Bu), A(Bz) and C(Ac)) was used, AMA-driven deprotection at room temperature for 2 h was found to be the best condition to ensure the 5′-ppp moiety remained intact. No use of ultramild protecting-group chemistry was necessary. After evaporation of the solvent, 2′-*O*-desilylation of RNA strands was carried out at 65 °C for 2.5 h using triethylamine trihydrofluoride (TEA*3 HF) which is the most widely used reagent for RNA desilylation.

However, serious degradation of the 5′-triphosphate moiety to its 5′-*O*-di- and 5′-*O*-monophosphorylated form was observed. In accordance, Zlatev et al. reported on this degradation for a $pppU_7$ oligonucleotide as well.[13] To achieve a milder desilylation reaction, two additional equivalents of triethylamine may be added to the desilylation solution to fully neutralize hydrogen fluoride and the desilylation should be carried out at room temperature overnight as well. Similar to DMT-on RNA deprotection approximately one additional equivalent of triethylamine is used for this purpose to avoid premature cleavage of the acid labile DMT-group. As solvents, DMSO and *N*-methyl-2-pyrrolidone (NMP) are commonly used. However, not more than one equivalent of TEA is soluble in DMSO or NMP. As a possible alternative base to TEA, *N*-methylmorpholine was tried but unfortunately showed the same solubility problems. Nonetheless, when a mixture of NMP, TEA and TEA*3HF in the ratio 6:3:4 (v/v)[28] was used as the desilylation reagent for 16 h at 23 °C degradation to the 5′-*O*-di- and 5′-*O*-monophosphorylated oligonucleotide was minimized to a low amount (Figure 7).

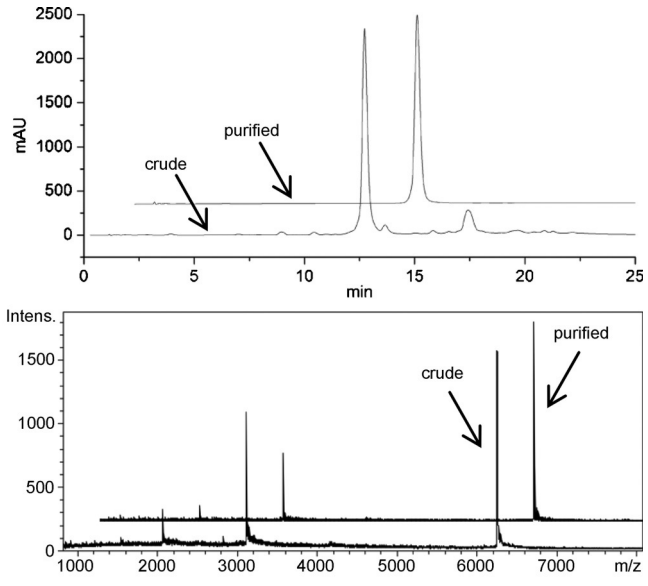


Figure 6. RP-HPLC chromatograms (top) and MALDI MS spectra (bottom) of crude and purified 5′-pppACU GUU UCA ACG UCA UGU UGU G.

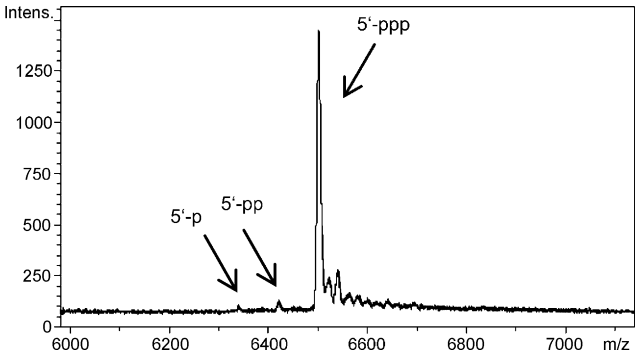


Figure 7. MALDI MS spectra of RP-HPLC purified 5′-ppp UCU CUA UAC GCU AGC ACU GU. Mass found: 6502.248 (calcd: 6500.713).

By using 1 M TBAF in THF within 16 h at room temperature no damage of the 5′-triphosphate moiety was detected. Therefore, TBAF remains the best desilylation reagent that ensures no degradation of the 5′-triphosphate, probably due to its neutral to slightly basic conditions (Figure 6). Overall isolated yields for different sequences of 5′-ppp DNA- and RNA-oligonucleotides are summarized in Table 1.

Table 1. Yields for isolated DNA- and RNA 5′-*O*-triphosphates of different sequences and lengths.

Sequence	Scale	OD_{260}	Isolated yield [%]
pppTTT TTT T	0.5	22.9	80[a]
ppp[d(TCT ATG T)]	0.5	23.2	71[a]
ppp[d(CCC CCC CCC CCC CCC CCC CC)]	0.5	26.5	37[a]
pppTTT TTT TTT TTT TTT TTT TT	0.5	41.0	50[a]
pppTTT TTT TTT TTT TTT TTT TTT TTT T	0.5	51.7	51[a]
ppp[d(GTC AGA GCT TAG CTA GAC CT)]	0.5	34.4	36[b]
pppACU GUU UCA ACG UCA UGU G	0.2	10.6	28[b]
pppUUG UCU CUG GUC CUU ACU UA	0.2	10.1	27[b]
pppGAC GCU GAC CCU GAA GUU CAU	0.2	9.3	23[b]
pppAGA AAU UAU UCA UGG CAG ACU U	0.2	10.7	23[b]

DNA oligonucleotides have been synthesized on polystyrene supports, RNA on CPG. [a] Purified by IEX-HPLC. [b] Purified by RP-HPLC.

Conclusions

In summary, we disclose here a method by which DNA- and RNA-oligonucleotide 5′-triphosphates are accessible using a *cyclo*Sal-phosphoramidite. The advantages of the method are the use of: 1) "standard" DNA- or RNA-phosphoramidites, 2) standard reagents in automated oligonucleotide synthesis, 3) AMA deprotection at room temperature and 4) standard RP-HPLC purification. It was very important that the deprotection with AMA was carried out at room temperature, otherwise 5′-p and 5′-pp formation will be observed.

An important advantage as compared to other methods is that the complete reaction sequence can be performed in an automated way using a standard oligonucleotide synthesizer. The method allows the reliable and inexpensive preparation of 5′-triphosphorylated oligonucleotides with efficient coupling reactions and extremely high phosphorylation efficiencies. In addition to the reaction of the 5′-*cyclo*Sal-oligonucleotides with pyrophosphate, the method should allow also the reaction with other nucleophiles, for example, nucleoside phosphates to synthesize cap-RNA-structures or (oligo)saccharide-phosphates to synthesize carbohydrate–oligonucleotide conjugates. Thus, this approach represents a new route to the efficient chemical synthesis of the important oligonucleotide 5′-triphosphates and derivatives thereof.

Acknowledgements

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Keywords: 5′-triphosphates · *cyclo*Sal · oligonucleotides · phosphoramidite chemistry · solid-phase synthesis

[1] K. Gao, L. E. Orgel, *Origins Life Evol. Biospheres* **2000**, *30*, 45 – 51.
[2] E. Ekland, J. Szostak, D. Bartel, *Science* **1995**, *269*, 364 – 370.
[3] J. C. Haugner III, B. Seelig, *Chem. Commun.* **2013**, *49*, 7322 – 7324.
[4] E. De Clercq, *Rev. Med. Virol.* **2009**, *19*, 287 – 299.
[5] Y. Thillier, E. Decroly, F. Morvan, B. Canard, J.-J. Vasseur, F. Debart, *RNA* **2012**, *18*, 856 – 868.
[6] F. Peyrane, B. Selisko, E. Decroly, J.-J. Vasseur, D. Benarroch, B. Canard, K. Alvarez, *Nucleic Acids Res.* **2007**, *35*, e26.
[7] J. Jemielity, J. Kowalska, A. M. Rydzik, E. Darzynkiewicz, *New J. Chem.* **2010**, *34*, 829 – 844.
[8] R. Allam, R. D. Pawar, O. P. Kulkarni, V. Hornung, G. Hartmann, S. Segerer, S. Akira, S. Endres, H.-J. Anders, *Eur. J. Immunol.* **2008**, *38*, 3487 – 3498.
[9] V. Hornung, J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K.-K. Conzelmann, M. Schlee, S. Endres, G. Hartmann, *Science* **2006**, *314*, 994 – 997.
[10] H. Poeck, R. Besch, C. Maihoefer, M. Renn, D. Tormo, S. S. Morskaya, S. Kirschnek, E. Gaffal, J. Landsberg, J. Hellmuth, A. Schmidt, D. Anz, M. Bscheider, T. Schwerd, C. Berking, C. Bourquin, U. Kalinke, E. Kremmer, H. Kato, S. Akira, R. Meyers, G. Hacker, M. Neuenhahn, D. Busch, J. Ruland, S. Rothenfusser, M. Prinz, V. Hornung, S. Endres, T. Tuting, G. Hartmann, *Nat. Med.* **2008**, *14*, 1256 – 1263.
[11] Q. Sun, J. P. Edathil, R. Wu, E. D. Smidansky, C. E. Cameron, B. R. Peterson, *Org. Lett.* **2008**, *10*, 1703 – 1706.
[12] J. Ludwig, F. Eckstein, *J. Org. Chem.* **1989**, *54*, 631 – 635.
[13] I. Zlatev, T. Lavergne, F. Debart, J.-J. Vasseur, M. Manoharan, F. Morvan, *Org. Lett.* **2010**, *12*, 2190 – 2193.
[14] I. Zlatev, J. G. Lackey, L. Zhang, A. Dell, K. McRae, S. Shaikh, R. G. Duncan, K. G. Rajeev, M. Manoharan, *Bioorg. Med. Chem.* **2013**, *21*, 722 – 732.
[15] S. Nagata, K. Takagaki, T. Wada, *Chem. Pharm. Bull.* **2012**, *60*, 1212 – 1215.
[16] M. Goldeck, T. Tuschl, G. Hartmann, J. Ludwig, *Angew. Chem. Int. Ed.* **2014**, *53*, 4694 – 4698.
[17] S. Warnecke, C. Meier, *J. Org. Chem.* **2009**, *74*, 3024 – 3030.
[18] V. C. Tonn, C. Meier, *Chem. Eur. J.* **2011**, *17*, 9832 – 9842.
[19] S. Shah, S. H. Friedman, *Nat. Protoc.* **2008**, *3*, 351 – 356.
[20] M. Greig, R. H. Griffey, *Rapid Commun. Mass Spectrom.* **1995**, *9*, 97 – 102.
[21] S. Wolf, T. Zismann, N. Lunau, C. Meier, *Chem. Eur. J.* **2009**, *15*, 7656 – 7664.
[22] E. E. Nifantiev, S. B. Khrebtova, Y. V. Kulikova, D. A. Predvoditelv, T. S. Kukhareva, P. V. Petrovskii, M. Rose, C. Meier, *Phosphorus Sulfur Silicon Relat. Elem.* **2002**, *177*, 251 – 259.
[23] H. Saneyoshi, K. Ando, K. Seio, M. Sekine, *Tetrahedron Lett.* **2007**, *63*, 11195 – 11203.
[24] T. Ohgi, Y. Masutomi, K. Ishiyama, H. Kitagawa, Y. Shiba, J. Yano, *Org. Lett.* **2005**, *7*, 3477 – 3480.
[25] C. Zhou, D. Honcharenko, J. Chattopadhyaya, *Org. Biomol. Chem.* **2007**, *5*, 333 – 343.
[26] T. Umemoto, T. Wada, *Tetrahedron Lett.* **2005**, *46*, 4251 – 4253.
[27] C. Zhou, W. Pathmasiri, D. Honcharenko, S. Chatterjee, J. Barman, J. Chattopadhyaya, *Can. J. Chem.* **2007**, *85*, 293 – 301.
[28] F. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, D. Tracz, C. Workman, D. Sweedler, C. Gonzalez, S. Scaringe, N. Usman, *Nucleic Acids Res.* **1995**, *23*, 2677 – 2684.

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