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Oligonucleotides |*Hot Paper*|

Chemical synthesis of long RNAs with terminal 5′-phosphate groups

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Abstract: Long structured RNAs are useful biochemical and biological tools. They are usually prepared enzymatically, but this precludes their site-specific modification with functional groups for chemical biology studies. One solution is to perform solid-phase synthesis of multiple RNAs loaded with 5′-terminal phosphate groups, so that RNAs can be concatenated using template ligation reactions. However, there are currently no readily available reagents suitable for the incorporation of the phosphate group into long RNAs by solid-phase synthesis. Here we describe an easy-to-prepare phosphoramidite reagent suitable for the chemical introduction of 5′-terminal phosphate groups into long RNAs. The phosphate is protected by a dinitrobenzhydryl group that serves as an essential lipophilic group for the separation of oligonucleotide by-products. The phosphate is unmasked quantitatively by brief UV irradiation. We demonstrate the value of this reagent in the preparation of a library of long structured RNAs that are site-specifically modified with functional groups.

The addition of a 5′-monophosphate group to oligodeoxyribonucleotides (DNA) and oligoribonucleotides (RNA) (oligonucleotides) is a key transformation that opens access to various biological and biochemical techniques. For instance, the enzymatic ligation of two oligonucleotides, of which one is 5′-monophosphorylated and the other bears a 3′-hydroxyl group[1,2], is an extensively used procedure in cloning and gene construction for assembly of long DNAs. The 5′-monophosphorylation of oligonucleotides can often be conducted enzymatically, whereby a kinase transfers a monophosphate from adenosine triphosphate (ATP) to the free 5′-hydroxyl of the oligonucleotide.[3] This process is easily performed on a small scale, however it is tedious to scale-up. Furthermore, efficiency of the conversion may be sequence- and enzyme-batch dependent, and oligonucleotides with structural modifications may be poor substrates. Thus, in these instances, a solid-phase chemical synthesis of the 5′-phosphorylated oligonucleotide would be preferred. Unfortunately, this approach is limited by the paucity of suitable phosphoramidites. For example, reagent **1** (Figure 1) is a commercially-available dimethoxytrityl (DMT)-substituted phosphoramidite of sulfonyldiethanol.[4] It is coupled to the terminal 5′-hydroxyl of a support-bound oligonucleotide in the final solid-phase coupling step under routine conditions. The terminal phosphate group is unmasked in a ß-elimination mechanism during oligonucleotide deprotection and cleavage of the solid support by treatment with aqueous ammonia.[5] Consequently, the crude oligonucleotide product does not carry the DMT group. This is inconvenient, because during conventional oligonucleotide purification by reverse-phase high-performance liquid chromatography (RP-HPLC) the lipophilic DMT protecting group provides an important means to separate efficiently the desired full-length "*n*-mer" oligonucleotide product from small amounts of contaminating "failure sequences" (i.e., *n*-1-mer, *n*-2-mer, etc.). This is a particular issue for long or modified oligonucleotides, for which failure sequences can be a major component of the crude product. Use of masked phosphate phosphoramidites to introduce the terminal phosphate is therefore limited to short oligonucleotides and produces products of moderate purity. This problem can be circumvented through the use of commercially available phosphoramidites **2** and **3**, because the DMT group is stable to conditions of ammonia deprotection and is able to separate full-length desired products from by-products.[6] The DMT group is then cleaved by mild acid treatment, however the release of the final 5′-phosphorylated

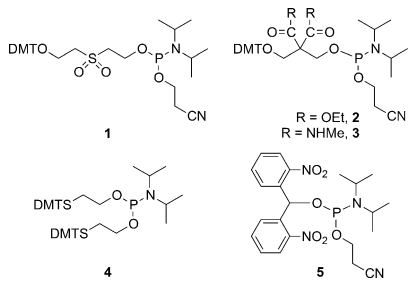

Figure 1. Structure of commercially available phosphoramidite reagents **1**, **2**, and **3**, phosphoramidite **4**, and the new dinitrobenzhydryl phosphoramidite **5**.

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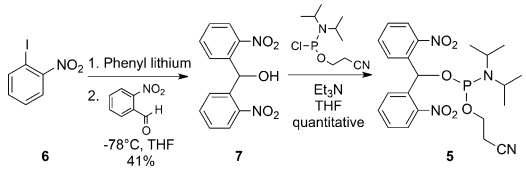
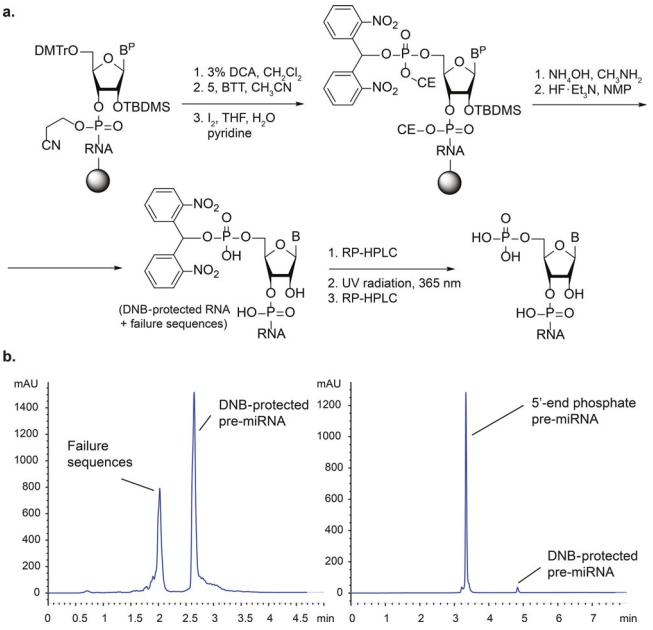
oligonucleotide requires an additional treatment under basic conditions. This is compatible with preparation of DNAs but it is unsuitable for synthesis of RNAs, which are unstable to base once the protecting group for the 2′-OH has been removed.[7] For the same reason phosphoramidite reagent **4**[8] is similarly unsuitable for the synthesis of long RNAs. Terminally-phosphorylated RNAs could alternatively be prepared using pre-phosphorylated (5′-phosphate) nucleoside phosphoramidites analogously to the approach of Lima et al. for phosphate analogs.[9] In addition to a tedious synthesis in which all four bases (or more in the case of modifications) are included, the lack of a lipophilic DMT-like group would hinder the isolation of full-length from failure sequences, essentially restricting its use to short oligonucleotides.

In this context, we aimed at developing a new phosphate moiety that i) can be introduced by conventional phosphoramidite coupling to the 5′-hydroxyl of a RNA sequence under standard conditions, ii) is stable to standard conditions of RNA synthesis and deprotection, iii) is sufficiently lipophilic to allow separation of long RNA from failure sequences, and iv) can be de-masked quantitatively after RP-HPLC purification under mild conditions. *o*-Nitrobenzyl protecting groups are labile upon UV irradiation and several studies have described their use as photolabile protecting groups in caged RNAs[10] and in linker groups of controlled-pore-glass (CPG) solid supports.[11] The dinitrobenzhydryl group (DNB), with two *o*-nitrobenzyl protecting groups, is highly lipophilic and is quickly deprotected under mild UV irradiation. The aforementioned criteria and the properties of the DNB group led us to a novel DNB-containing phosphoramidite **5** (Figure 1). Phosphoramidite **5** was easily prepared in two steps in 41% yield by addition of *o*-nitrophenyl lithium to *o*-nitrobenzaldehyde, followed by phosphitylation under standard conditions (Scheme 1).



Scheme 1. Preparation of dinitrobenzhydryl phosphoramidite **5** (see the Supporting Information for details).

We selected a microRNA precursor of the tumor suppressor let-7 family to assess the value of **5** as a means to introduce phosphate groups to the 5′-end of long RNAs. Pre-let-7a-2 is 67 nucleotides (nt) in length and has a well characterized stem-loop structure. Solid-phase synthesis of pre-let-7a-2 was initiated in conventional fashion. Following the coupling cycle of the final nucleotide to pre-let-7a-2, the CPG-bound RNA was detritylated, coupled with phosphoramidite **5**, capped, and oxidized under standard conditions (Scheme 2a, see the Supporting Information for details). After cleavage from the CPG solid support and removal of base and phosphodiester protecting groups, the DNB-protected 5′-phosphate pre-miRNA was purified by RP-HPLC. As with the DMT group, the lipophilic DNB



Scheme 2. a) Synthetic procedure for the preparation of 5′-phosphate-capped oligoribonucleotides (B^P = uracil, N4-acetyl-cytosine, N6-benzoyl-adenine, N2-isobutyryl-guanosine; CE = cyanoethyl; DCA = dichloroacetic acid; BTT = 5-Benzylthio-1-H-tetrazole). b) Left: HPLC spectra of the crude DNB-protected 5′-phosphate pre-let-7a-2; Right: HPLC spectra of the free 5′-phosphate pre-let-7a-2 upon 5 min irradiation at 365 nm (see the Supporting Information for details).

group extended the retention time on the column to allow efficient separation of the DNB-protected 5′-phosphate pre-miRNA from failure sequences (Scheme 2b). The 5′-phosphorylated pre-miRNA was obtained by irradiation of the DNB-protected RNA at 365 nm for 10 min (>95% conversion in 5 min) and was then purified by RP-HPLC. This procedure worked equally efficient with the pre-miR-20b sequence (60 nt).

Very long RNAs can be assembled from shorter chemically synthesized sequences by enzyme-mediated ligation of the 5′-phosphorylated terminus of one sequence to the 3′-OH group of another.[12,13,14] We demonstrated the practical value of phosphoramidite **5** for the preparation of various sequences of lengths larger than 100 nt. They included a pri-miRNA (miR-122), an *anti*-EGFR aptamer,[15] a small nucleolar RNA (ACA45),[16] and four spliceosomal RNAs (U1, U2, U4, and U6). These RNA are too long to be synthesized under automated solid-phase synthesis using conventional 2′-*O*-TBDMS phosphoramidites. The sequences were divided into two parts of similar length corresponding to the 5′-end phosphorylated RNA donor and the unmodified 3′-end hydroxyl RNA acceptor (Table 1).

Phosphoramidite **5** coupled with high efficiency to A, C, G, and U yielding phosphorylated RNAs of lengths up to 96 nt. In our hands, this represents the technical limits of solid-phase synthesis using conventional TBDMS phosphoramidites. Ligation of RNA pairs was carried out using T4 RNA ligase 2 and a DNA template. In accordance with previous reports,[17] 20 nt-long templates produced only low yields of full-length products, whereas 40 nt-long templates covering 20 nt on the

Table 1. RNAs prepared for this study (Table S1 in the Supporting Information).

ORN[a]	RNA	Acceptor [nt]	Donor [nt]	Label (position, nt)	Length ORN[a] [nt]
1	Pre-let-7a-2	–	–	–	67
2	Pre-miR-20b	–	–	–	60
3	Pri-miR-122	55	54	–	109
4	Anti-EGFR	58	59	–	117
5	ACA45	65	67	–	132
6	ACA45	65	67	Cy5/Cy3 (42/88)	132
7	U6	68	65	–	134
8	U6	68	66	Bio/Pso (17/105)	134
9	U4	73	68	–	141
10	U1	84	80	–	164
11	U2	94	96	–	190

[a] ORN = oligoribonucleotide

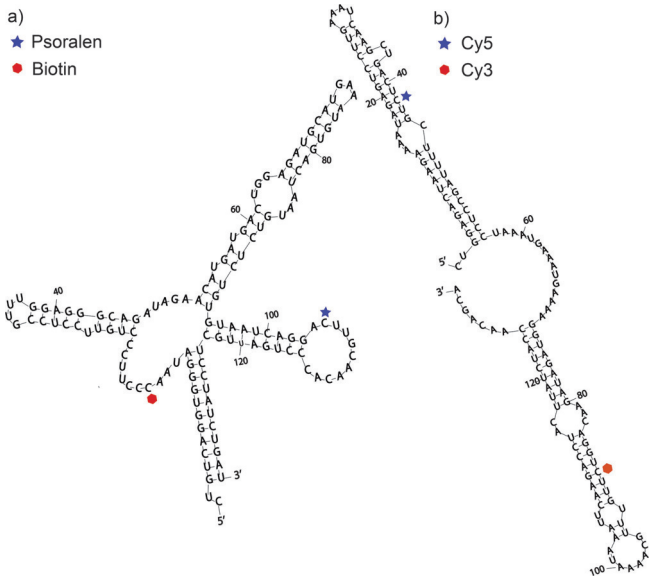


Figure 2. Secondary structures of a) U6 (psoralen/biotin) and b) Cy3/Cy5-ACA45 as provided by Mfold Web Server.

donor and acceptor yielded superior couplings, which suggests that in some cases invasion of the RNA secondary structure by the template may be critical for reaction efficiency. Extending the template to 60 nt did not improve significantly the yields, but did make product purification by RP-HPLC more difficult. Templates of RNA or 2′-*O*-methylated RNA did not have a great influence on ligation efficiencies, however DNA templates were easier to remove by RP-HPLC purification. Optimally, ligation reactions were conducted in a (1:1:2) mixture of 5′-phosphorylated donor, acceptor RNA, and DNA template. Ligation conversions were generally greater than 70% after 2 h. Interestingly, we found that pri-miR-122 could be ligated without a template sequence, presumably because the stem-loop acted as its own template.

One application of this approach of particular value is the synthesis of long structured RNAs that are site-specifically labelled with functional groups for chemical biology investigations such as FRET studies[18] or intracellular cross-linking experiments.[19] We have previously described two multi-labelling approaches for structured RNAs. Here, we demonstrate how access to phosphoramidite **5** facilitates synthesis of multi-labelled RNAs by prior incorporation of different functional groups into different RNA subsequences prior to their ligation. Hence, we prepared ACA45 and U6, bis-labelled with Cy5/Cy3 and biotin/trioxsalen (psoralen), respectively (Figure 2). We used our previously optimized methods for site-specific labelling, whereby groups are conjugated to 2′-*O*-propargyl nucleosides either on solid support[20] or in solution.[18]

The target RNA was constructed from two smaller RNAs of similar lengths, one of which was 5′-phosphorylated (the donor). A 2′-O-alkyne modification was site-specifically incorporated into both RNAs. Preliminary experiments suggested that post-synthetic labelling of the donor RNA can be carried out before or after DNB deprotection as long as the label tolerates UV light. Thus, a Cy3 group was conjugated to the ACA45 donor prior to DNB removal. This sequence of reactions was not possible with attachment of a psoralen, which crosslinks RNA under UV irradiation.[21] The psoralen-labelled U6 donor was therefore prepared by reaction of psoralen azide with the fully deprotected phosphorylated donor in solution. Cy3-labeled ACA45 and biotin-U6 acceptors were prepared in a straightforward manner by conjugation of the labels on solid support, as previously described.[20] The two pairs of labelled RNA were then ligated following the protocol reported herein to yield Cy5/Cy3 ACA45 (ORN-6) and biotin/psoralen U6 (ORN-8) with good purity (>80%).

In conclusion, we report herein the synthesis and the use of a novel, easily-accessible phosphoramidite for the incorporation of 5′-phosphate groups into RNAs by chemical solid-phase synthesis. Combining enzymatic ligation with labelling techniques that we have described previously has opened access to site-specifically modified structured RNAs of lengths larger than 130 nt.

Experimental Section

Oligoribonucleotides were synthesized on an MM12 synthesizer (BioAutomation Corp., Irving, TX) on a 50 nmol scale with 2′-*O*-TBDMS-phosphoramidites (Thermo Fisher), using a 1000 Å UnyLinker support (ChemGenes, Wilmington, MA). Coupling time was 2× 90 s for regular 2′-*O*-TBDMS phosphoramidites, 2×180 s for the DNB phosphoramidite **5**, and 3×240 s for 2′-*O*-propargyl rC phosphoramidite. Biotin and Cy3 labels were introduced on support by click chemistry. Oligonucleotides were deprotected from support with AMA (40% aq. methylamine, 25% aq. ammonia; 1:1) and dried prior to RP-HPLC purification. Isolated 5′-DNB-protected oligonucleotides were dried in a SpeedVac and redissolved in 200 µl RNAse-free water prior to irradiation for 10 min at 365 nm in a Vilber Lourmat Bio-link BLX Crosslinker (5×8 W). Complete removal of the DNB group was monitored by LC-MS. Final RP-HPLC purification yielded 5′-phosphate oligoribonucleotides ready for the introduction of functional groups by click chemistry (e.g., psoralen, Cy5), and for enzymatic ligation experiments with T4 RNA Ligase 2 (M0239, New England Biolabs) (see the Supporting Information for details).

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Conflict of interest

The authors declare no conflict of interest.

Keywords: dinitrobenzhydryl · phosphate · phosphoramidite · photolabile protecting group · RNA

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