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RNA synthesis via dimer and trimer phosphoramidite block coupling

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ABSTRACT

The solid-phase synthesis of oligoribonucleotides using dimer and trimer phosphoramidite blocks is described. This method significantly reduces the total number of steps required in the synthesis of a target RNA sequence, provides more material, and simplifies separation of the product from shorter failure sequences. The procedure is illustrated by the synthesis of UpU, ApA, and UpUpU phosphoramidite blocks and their use in the rapid synthesis of oligoribonucleotides on a solid support. Dimer and trimer amidite blocks will likely find use in the large scale solution (or solid)-phase synthesis of siRNA drugs.

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1. Introduction

Current methods for RNA synthesis rely on stepwise addition of monomeric phosphoramidite units on solid supports. Careful chromatographic purification of the final product is required after the oligomer is detached from the solid support and deprotected because of the inevitable presence of $n-1$ mers arising from incomplete coupling. An alternative approach is to assemble oligonucleotide chains more rapidly through 'block' condensation reactions, as exemplified by the early work of Khorana and coworkers in the synthesis of a DNA gene fragment from phosphodiester intermediates.[1,2] This has not been applied to solid phase RNA synthesis because of the difficulty of obtaining the blockmer phosphoramidites and the perceived inefficiency of block condensation on solid-phase supports.

The generation of oligoribonucleotide blocks is made difficult by the presence of the 2′-hydroxyl group and the protection it requires. Ogilvie and co-workers described the synthesis of 5′-*O*-MMTr-2′-*O*-TBDMS-3′-*O*-levulinyl ribonucleoside monomers and their use in the assembly of a hexadecauridylic acid via the phosphodichloridite procedure in 51% yield.[3] This and other[4,5] reports utilized earlier coupling methods, which generally provided far too low yields to be considered effective synthetic strategy especially for large scale. Since then, the phosphoramidite method has proven to be the coupling method of choice,[6] offering higher yields and faster reactions. Although block-wise solid-phase synthesis of DNA using phosphoramidite block-mers has been demonstrated,[7,8] there has been only one conference proceeding describing the synthesis RNA phosphoramidite block-mers for use in solid-phase synthesis.[9] Herein we describe a facile method to construct dimer/trimer RNA phosphoramidites that can be readily applied to current synthesis approaches.

Our method builds upon the contributions of Ogilvie and coworkers,[3] who introduced the levulinyl (Lev) ester group as a transient and orthogonal protection for the 3′-position. Initially, the synthesis of UpU amidite was attempted by coupling commercially available 5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-*O*-cyanoethyl phosphoramidite with 2′-*O*-TBDMS 3′-*O*-levulinyl-uridine **1**. While the desired product could be obtained, we observed up to 15% decyanoethylation during column chromatography caused by the basic eluent system necessary to avoid activation of the amidite moiety. This led us to switch to the less base labile methyl protecting group (Scheme 1).[10–13]

In addition, it was found that the removal of the 3′ levulinyl protecting group caused migration of the 2′-TBDMS group to the 3′-position resulting in a mixture of regioisomers **4** and **5** (Scheme 1). The isomerization was confirmed by preparing the 3′-silyl regioisomer **5** independently and comparing its ^{1}H and ^{31}P NMR chemical shifts to compounds in the isolated mixture. We also established that these regioisomers could not be resolved by TLC which may explain why previous reports[3] did not detect TBDMS isomerization under these conditions.

The synthetic standard **5** allowed the evaluation of various conditions for the removal of the levulinyl group for the degree of isomerization (^{31}P NMR detection limit: 0.4 mol %). Variation of pH, concentration, mole equivalence of hydrazine, workup conditions, solvent and temperature all led to similar results: 5–10% formation of the inseparable 3′-isomer **5** as assessed by ^{31}P NMR. This result came as a surprise as the monomeric nucleoside **1** did not isomerize under identical conditions (and even longer reaction

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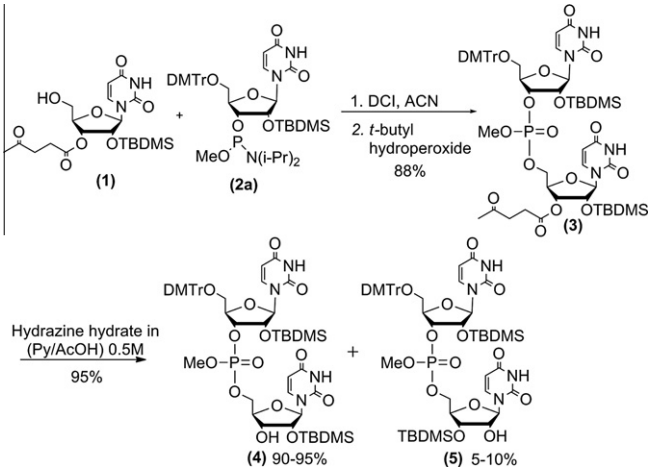


Scheme 1. Dimer phosphoramidite synthesis (TBDMS protection).

times), as also reported by Ogilvie and co-workers.[3] To directly compare the extent of isomerization between monomer and dimer units, the delevulination reaction was performed in an NMR tube and the isomerization was monitored over time (see Supplementary data). Under dilute conditions, isomerisation of 5′-*O*-DMTr 2′-*O*-TBDMS uridine was detectable after 2.3 h whereas isomerisation of dimer **4** was observed after 80 min (Fig. 1A). The dimer is more susceptible to isomerization than the monomer nucleoside under identical conditions [see Table 2, Supplementary data].

An alternative approach to the synthesis of dimer nucleotides was to circumvent the use of 3′-protection completely and rely on the greater steric bulk around the secondary hydroxyl to reduce its reactivity, allowing selective coupling with a phosphoramidite **2a** at the less unhindered 5′-hydroxyl (Scheme 2). This was achieved by pre-activating the phosphoramidite with 4,5-dicyanoimidazole (DCI) and adding that solution dropwise to a stirred solution of either nucleoside **6** or **7** in ACN at 0 °C, and then allowing the mixture to warm up to room temperature. The solution was then oxidized with *tert*-butyl hydroperoxide in situ affording the desired dinucleoside phosphotriesters. The use of iodine/water for the oxidation should be avoided as premature demethylation via an Arbuzov-like reaction can occur under these conditions.[14]

The method outlined in Scheme 2 proved to be successful only when 2′-*O*-triisopropylsilyl (TIPS) protection was used, as the presence of DCI in solution caused the terminal 2′-*O*-TBDMS group to isomerize during coupling and/or work up. Furthermore, the 2′-TIPS protecting group increases the steric bulk around the 3′-hydroxyl group significantly so that the regiospecific coupling proceeded with greater efficiency. Also, unlike TBDMS, the TIPS group is uniquely resistant to silyl isomerization during the delevulination conditions. The increased stability of the TIPS protected dimer over the TBDMS dimer was confirmed by NMR. The TBDMS dimer **3** (Fig. 1A) and TIPS dimer **11a** (Fig. 1B) were subjected to 5 equiv of hydrazine (0.045 M) and their conversion into **4** and **8a**, respectively, monitored by ^{31}P NMR over time.

Under these conditions, addition of hydrazine resulted in complete cleavage of the levulinyl group from **3** and **11a** within 2.5 and 10 min, respectively. Detectable isomerization of the TBDMS group was observed within 40–80 min. This is in contrast to the TIPS dimer **11a**, which was resistant to silyl isomerization for at least 68 h under the same conditions (^{31}P NMR detection limit: 0.4 mol %). Neither TIPS nor TBDMS protected monomers underwent isomerization (up to 10 h) under these conditions. As shown in Figure 1, the emergence of demethylated products became apparent after 40 min in the case of the 2′-TBDMS protected dimer (−0.84 and −1.1 ppm) and 2.3 h in the case of the 2′-TIPS protected dimer (single resonance at −1.00 ppm). The presence of a single peak at around −1.00 ppm for the TIPS demethylated dimer also confirmed that no isomerization occurred during the delevulination step. These experiments confirmed that the use of TIPS protection is significantly more likely to produce isomerically pure dimers within the time required for delevulination. Since removal of the levulinyl group of TIPS dimer is complete within 10 min, there is little chance of either isomerization or demethylation during the conversion of **11a**–**8a** and neither was observed.

The dimer and trimer amidites **9a** and **14** were synthesized according to Schemes 3 and 4, respectively. Coupling of **2a** with 2′-*O*-TIPS 3′-*O*-Lev nucleoside **10a** in the presence of DCI followed by in situ oxidation with 6 M *tert*-butyl hydroperoxide afforded dimer **11a** in excellent yields (Scheme 3). After purification, dimer **11a** (1 mmol/ml of ACN) was treated with a 0.5 M solution of hydrazine hydrate in pyridine/acetic acid (3:2 v/v) cleaving the levulinyl group quantitatively within 10 min. The reaction was cooled to 0 °C and the excess hydrazine was quenched by addition of 2,4-

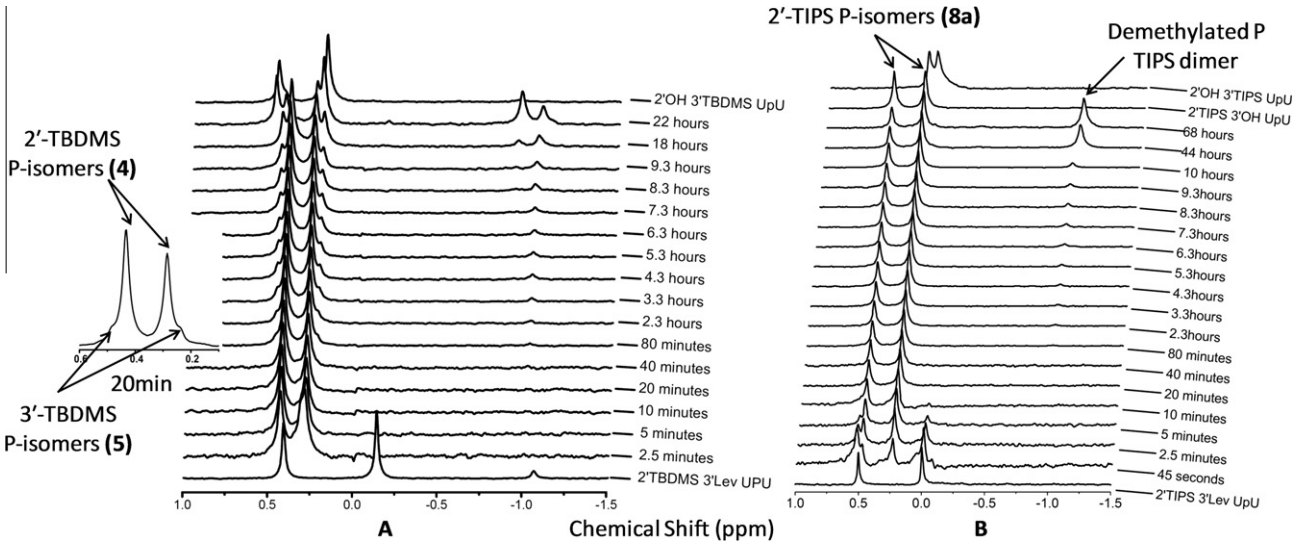
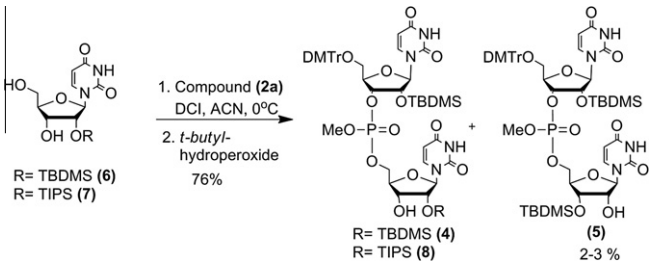
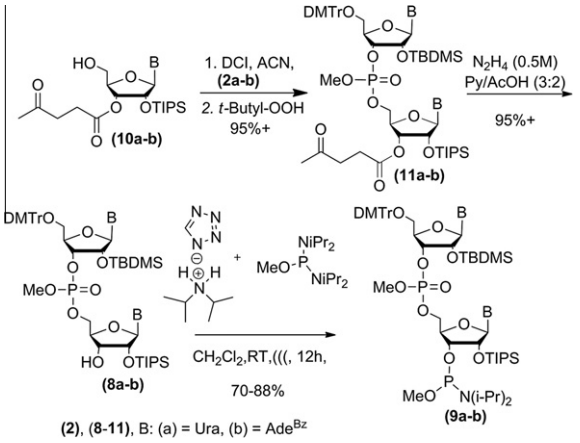


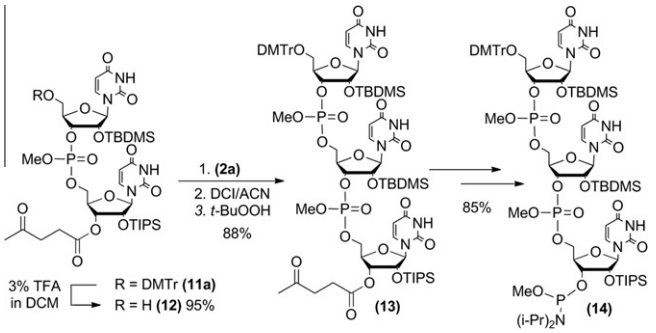
Figure 1. ^{31}P NMR analyses of (A) UpU TBDMS protected dimer (**3**), (B) UpU TIPS protected dimer (**11a**) under delevulination conditions: 5 equiv of hydrazine hydrate (0.045 M) in pyridine/acetic acid (3:2, v/v) in $CDCl_3$.



Scheme 2. Regioselective dimer phosphoramidite synthesis.



Scheme 3. Dimer phosphoramidite synthesis (TIPS protection).



Scheme 4. Trimer phosphoramidite synthesis.

pentanedione. Chromatography was performed, avoiding the use of base, and the dimer was phosphitylated with bis(*N*,*N*-diisopropylamino)methoxyphosphine and *N*,*N*-diisopropylammonium tetrazolide, with sonication to speed up the reaction, yielding dimer 3′-phosphoramidite **9a** in isomerically pure form. The bis(*N*,*N*-diisopropylamino)methoxyphosphine reagent was required for this step as the use of the corresponding phosphonamidic chloride led to incomplete reaction and silyl migration.

The trimer amidite could be prepared by treatment of **11a** with 3% TFA in DCM to deprotect the 5′-hydroxyl (95%), followed by coupling (**2a**) and in situ oxidation to afford **13** in good yield (88%). Delevulination and phosphitylation afforded the desired trimer amidite in 80% yield (over two steps) without any detectable 2′/3′ isomerization (Scheme 4; ^{31}P NMR detection limit: 0.4 mol %).

Of the two routes used to prepare dimer amidite **9a** (Schemes 2 and 3), use of levulinyl protection was preferred as the coupling step is more straightforward to carry out and provides higher yield of the desired product (75% vs 70%). The ApA dimer phosphoramidite **9b** was synthesized in a similar manner in good yields (Scheme 3), demonstrating the general applicability of our procedures.

With dimer and trimer amidites in hand, their coupling efficiency were assessed on a solid support through the synthesis of 5′-(rU)$_{18}$dT-3′ using the following conditions: 1 μmol scale; dimer and trimer (0.15 M) blocks in MeCN, 0.25 M DCI as an activator, and 20 min coupling cycles (Table 1) (see Supplementary data). Oxidation of the phosphite triester intermediates was achieved using a 3 M solution of *tert*-butyl hydroperoxide in toluene.[13,15] Following chain assembly, the fully deprotected oligomers were obtained by: (1) treatment with 5-*tert*-butyl-2-methylthiophenol (rt, 120 min) to deblock the phosphate methyl groups; (2) washing the solid-support (ACN, 10 ml); (3) ammonolysis with NH_4OH/EtOH (3:1) (rt, 60 h) to release the oligomers from the support, and (4) fluoride treatment (1 ml of 1 M TBAF in THF, rt, 48 h) to deblock the TBDMS and TIPS protecting groups. The three oligomers were identical as shown by HPLC and MALDI-TOF (Calcd 5753.3, found: 5752.9 *m*/*z*).

Yields of oligomers were calculated from the HPLC traces (Supplementary data), from which coupling efficiencies of the amidites were estimated at 98.7%, 98.3%, and 97.2% for monomer, dimer, and trimer amidite, respectively (Table 1; entries II, IV, VI). The overall yield of 5′-(rU)$_{18}$dT-3′ prepared from trimer couplings (6× at 97.2% efficiency) was 84.7% (Fig. 2), which was superior to the 80% overall yield obtained by coupling rU monomers (18× at 98.7% efficiency) (Table 1; entry II vs VI). Reducing the trimer amidite concentration to 0.10 M and the coupling time by 50% reduced the coupling efficiency by 11% (Table 1; entry V). These conditions afforded, as expected, a mixture of the full length product and a series of well resolved *n*−3 'failure' sequences (Fig. 3). A second poly-py/pu sequence, 5′-(rAAUU)$_4$dTdT-3′, was assembled from dimers **9a** and **9b** in 88.8% overall yield (98.5% per coupling). This contrasts the 72.5% overall yield obtained when this sequence was assembled via monomer coupling (98.0% per coupling; Table 1, entry VII vs VIII). Thus in this case, the block coupling strategy yielded ca. 16% more product than the standard method.

While we have not yet fully optimized deprotection conditions, we have noted, in some instances, the presence of very small

Table 1
Synthesis of oligonucleotides via monomer and block coupling

Entry	Oligomer 5′-to-3′	Amidite	Concd (M)	# of couplings	Time (min)	Coupling efficiency (%)	Yield[a] (%)
I	(rU)$_{18}$dT	rU (**2a**)	0.10	18	10	98.5	76.5
II	(rU)$_{18}$dT	rU (**2a**)	0.15	18	20	98.7	80.1
III	(rU)$_{18}$dT	rUU (**9a**)	0.10	9	10	97.2	77.8
IV	(rU)$_{18}$dT	rUU (**9a**)	0.15	9	20	98.3	85.9
V	(rU)$_{18}$dT	rUUU (**14a**)	0.10	6	10	86.5	41.8
VI	(rAAUU)$_4$dTdT	rUUU (**14a**)	0.15	6	20	97.2	84.7
VII	(rAAUU)$_4$dTdT	rU (**2a**), rA (**2b**)	0.15	16	20	98.0	72.5
VIII	(rAAUU)$_4$dTdT	rUU (**9a**), rAA (**9b**)	0.15	8	20	98.5	88.8

[a] Yield % of oligomer in crude material (HPLC). Solid supports: dT or dTT functionalized succinyl-LLAA-CPG. 5′-(rAAUU)$_4$dTdT-3′ was deprotected by a modification of fast deprotection;[18] 5′-(rU)$_{18}$dT-3′ was deprotected as described in this manuscript.

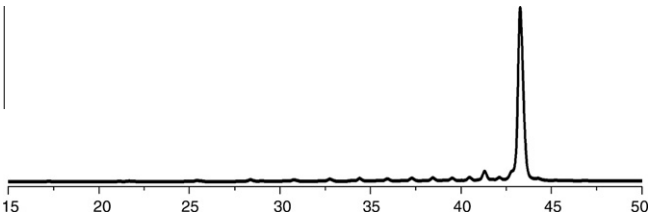


Figure 2. Polypyrimidine, 5′-(rU$_{18}$)-dT-3′ made from trimer amidite **14** under optimized conditions (0.15 M, 1200 s coupling time).

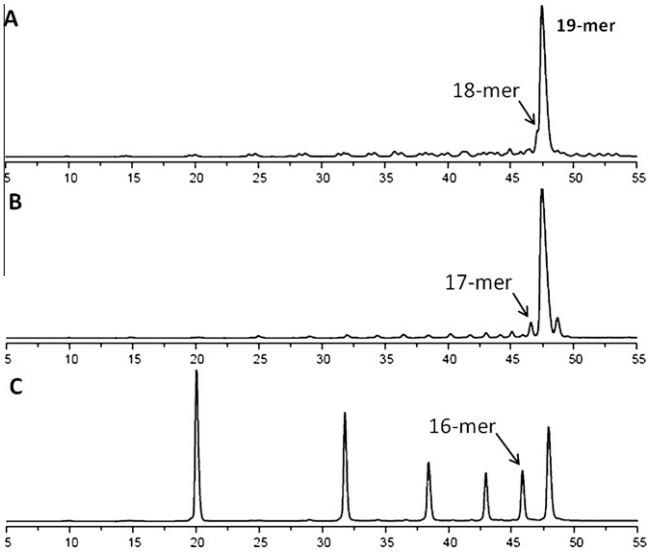


Figure 3. Polypyrimidine sequence 5′-(rU$_{18}$)-dT-3′ made from (**A**) monomer amidite **2a** (18× couplings), (**B**) dimer amidite **9a** (9× couplings) and (**C**) trimer amidite **14** (6× couplings), under, purposefully, un-optimized conditions (0.1 M, 600 s coupling).

amounts of $n-1$ and $n-2$ sequences using block amidites. This likely arises from premature silyl cleavage during ammonia treatment, as first reported by Ogilvie and co-workers for a poly-rU sequence.[16,17] Conditions that greatly minimize this problem have been described[16] and will be adopted when deprotecting sequences of mixed base composition (work in progress).

In conclusion, we have developed a viable route for the synthesis of regioisomerically pure dimer and trimer RNA phosphoramidites that couple with similar efficiency as monomeric phosphoramidite units. The method increases the overall yield of the target oligoribonucleotide sequence by decreasing the number of coupling steps required for chain assembly and has the potential of significantly simplifying the final purification of RNA sequences. For instance, dimer and trimer synthons can be utilized either in solution or solid-phase in conjunction with monomer synthons in the final stages of chain assembly, affording $n-2$ or $n-3$ failure sequences that are more readily resolved. Protected trinucleotides may also find applications in codon and anticodon construction of combinatorial libraries of mRNA and tRNA mimics.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.03.042.

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