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Solid-Phase Synthesis and On-Column Deprotection of RNA from 2′- (and 3′-) *O*-Levulinated (Lv) Ribonucleoside Monomers

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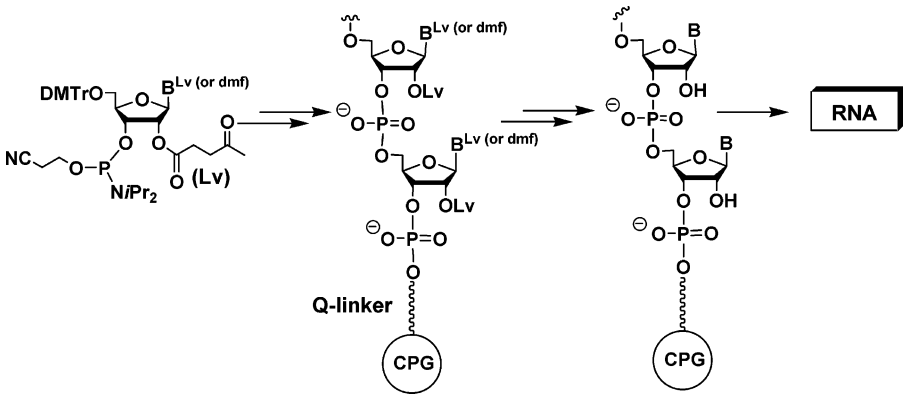
ABSTRACT



The solid-phase synthesis of oligoribonucleotides derived from ribonucleosides esterified at the 2′- (or 3′-) position with the levulinyl (Lv) group is described. The oligomers can be released from the solid support as 2′-*O*-Lv ester derivatives or fully deprotected while still attached to the solid support.

The advent of the RNA interference (RNAi)[1] methodology and its application to therapeutics has created an urgent and growing need for the synthesis of large quantities of native and chemically modified short interfering RNA (siRNA) for animal and human studies. For many years, RNA synthesis has been regarded as far more difficult than DNA synthesis because of the difficulty in finding a compatible 2′-protecting group that is stable throughout chain assembly and can be removed selectively at the end of synthesis without phosphodiester bond isomerization or degradation.[2] In fact, finding a satisfactory 2′-protecting group is a research problem that has spanned several decades.[3] The application of the 2′-*O*-*t*-butyldimethylsilyl group (TBDMS) from Ogilvie and co-workers has proved sufficiently robust to allow assembly of RNA chains at lengths suitable for RNA structure−function studies.[4]

Two other widely used RNA synthesis strategies (2′-*O*-ACE[5] and 2′-*O*-TOM[6]) allow oligoribonucleotides to be produced somewhat routinely and, together with the 2′-*O*-

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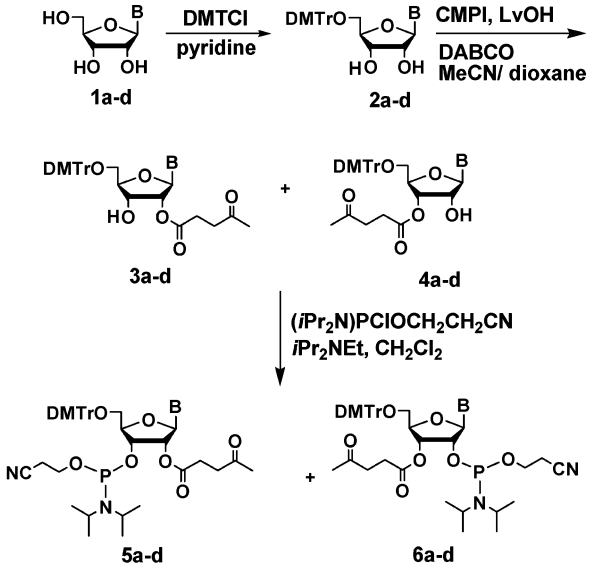
TBDMS strategy, satisfy the need for high-throughput RNA synthesis. A variety of other 2′-hydroxyl protecting groups have appeared in the literature.[7−11] The TBDMS, ACE, TOM, and most of the previously described 2′-protecting groups all share the same requirements for a manual solution-phase 2′-deprotection. The need for manual 2′-deprotection is time and labor intensive, particularly for large-scale synthesis, and a potential source for material losses and ribonuclease contamination.

In this report, we have re-evaluated 2′- (and 3′-) *O*-acyl ribonucleosides, as possible synthons for RNA synthesis. At the onset, we recognized that 2′-*O*-acyl protecting groups (e.g., benzoyl) in RNA synthesis had been largely unsuccessful due to ease of 2′−3′ migration[9] and lack of specificity and compatibility with other groups. In fact, for these reasons, 2′-*O*-acyl protecting groups have only very rarely been used in oligoribonucleotide synthesis.[9,10] The levulinyl (Lv) group has been previously described by van Boom and others as a "transient" 5′-protecting group for ribonucleosides.[12] Ogilvie was the first to utilize the Lv group for transient 3′-protection in conjunction with the procedures their group developed for block coupling of 3′,5′-oligoribonucleotides.[13] The present study builds upon Ogilvie's work as we examine, for the first time, the application of 2′- (and 3′-) *O*-Lv ribonucleosides as direct synthons for RNA synthesis. As documented in an earlier study with 2′/3′-*O*-(2-chlorobenzoyl) ribonucleosides,[10b] we reasoned that *O*-phosphitylation of a mixture of interconverting 2′-*O*- and 3′-*O*-Lv ribonucleoside isomers would provide separable, regioisomerically stable, phosphoramidite monomers that would be suitable for RNA (and 2′,5′-RNA) synthesis.

The required adenosine and cytosine monomers **3b/c** and **4b/c** were synthesized by sequential *N*-levulination of the exocyclic amines (87−99%),[13b] 5′-dimethoxytritylation (DMTr; 88−90%), and 2′/3′-*O*-levulination (70−78%) using 2-chloromethyl-pyridinium iodide (CMPI) and 1,4-diazabicyclo-

Scheme 1. Synthesis of 2′/3′-Lv Monomers



1-6: B = (a) Ura; (b) Ade^{Lv}; (c) Cyt^{Lv}; (d) Gua^{dmf}

[2.2.2]octane (DABCO) (Scheme 1). Because *N*-levulination of guanine proceeded sluggishly (20%),[13b] the dimethyl formamidine (dmf) group[14] was used instead as it has been found that it can be efficiently introduced (>95%) and removed under the same conditions as the *N*-Lv and *O*-Lv groups.[15] Uridine was subjected to the same steps, except that it did not require base protection (Scheme 1). At this stage, we obtained, as expected, *inseparable* mixtures of 2′/3′-*O*-Lv regioisomers in favor of the 2′-*O*-Lv isomers (ca. 2:1 ratio for U, C, and A; 3:1 for G, after column chromatography as determined by ^{1}H NMR). The mixture of **3** + **4** was then carried on to the final phosphitylation step to afford mixtures of **5a**−**d** and **6a**−**d** in 77−85% yields (% **6a**−**d** > % **5a**−**d**). Separation of the 2′-*O*- and 3′-*O*-phosphoramidite regioisomers was possible by flash silica gel column chromatography yielding **5a** (19%), **6a** (42%), **5b** (10%), **6b** (7%), **5c** (29%), **6c** (35%), **5d** (22%), and **6d** (35%) in isomerically pure forms. These isolated yields are generally lower than those obtained with TBDMS nucleosides, particularly for the adenosine derivatives, and reflect the very similar (tlc) chromatographic properties of each **5**/**6** pair ($\Delta R_f \sim 0.1$). Nevertheless, we consider these results and those described below to be most encouraging. The identities of **5a**−**d** and **6a**−**d** were confirmed with ESI-MS in conjunction with 2D NMR (^{31}P−^{1}H) experiments (Supporting Information).

With phosphoramidite monomers in hand, a 3′,5′-linked RNA sequence (**8**) was synthesized on a 1 μmol scale using monomers **5a**−**d** (0.15 M in MeCN), 0.25 M 5-ethylthiotetrazole (ETT) as an activator, and 14 min coupling cycles (Table 1).

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Table 1. Data for Synthesized Oligoribonucleotides

no.	5'-sequence-3'	PG	CT min[a]	%[b]	av coup yield
8	GCUUGAAGUCUUUAAUUAAtt	Lv	14	72.5	98.5
9	GCUUGAAGUCUUUAAUUAAtt	Si	14	72.8	98.5
10	ttttttt*CUCUC*ttt[c]	Lv	14	72.5	97.8
11	U_{10}tt	Lv	1	69.6	97.0
12	U_{10}tt	Lv	10	71.2	97.2
13	U_{10}tt	Si	10	90.1	99.1
14	U_{10}tt	Si	1	60.7	95.9
15	$U^{(2'}$***O***Lv)$_{19}$tt	Lv	10	66.8	98.1
16	U_{19}tt	Lv	10	71.5	98.4
17	$U^{(3'}$***O***Lv)$U_3U^{(3'}$***O***Lv)$U_{12}U^{(3'}$***O***Lv)Utt	Si	10	nd[d]	nd[d]
18	*U*U_3*U*U_{13}*U*Utt[c]	Si	10	nd[d]	nd[d]

[a] Coupling time. [b]% yield of oligomer in crude material (HPLC). [c] *C* and *U* = 2′,5′-rC and rU, respectively. *t* = dT. [d]nd = not determined.

The solid support used was controlled pore glass (500 Å CPG) with a 5′-*O*-DMTr-thymidine unit (45 μmol/g) appended through a hydroquinone-*O*,*O*′-diacetic acid linker (Q linker).[16] This linker, unlike the standard succinyl linker, makes it possible to release an oligonucleotide chain with fluoride ions under conditions that do not lead to internucleotide cleavage (1 M TBAF in THF; rt, 15 min; 92% recovery; see below). Oxidation of the phosphite triester intermediates was achieved using the standard 0.1 M iodine/pyridine/water treatment. These conditions resulted in an average stepwise coupling yield (98.5%) that was comparable to that obtained by 2′-*O*-TBDMS chemistry (Figure 1).

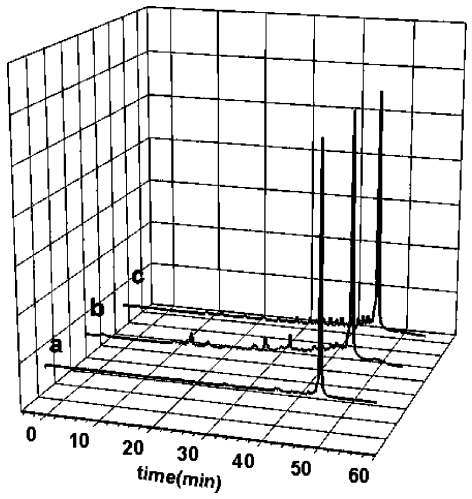

Figure 1. Ion-exchange HPLC analysis of 5′,3′-linked siRNA (a) **8** prepared via 2′-*O*-Lv monomers (after purification), (b) **8** prepared via 2′-*O*-Lv monomers (crude), and (c) **9** prepared via 2′-*O*-TBDMS monomers (crude).

Following chain assembly, the fully deprotected oligomers were obtained following a deprotection scheme composed of: (1) treatment with anhydrous 2:3 v/v NEt_3/CH_3CN (rt, 60 min) to deblock the phosphates' β-cyanoethyl groups; (2) washing the solid support (acetonitrile, 5 min); (3) hydrazinolysis (0.5 M hydrazine hydrate in 3:2 v/v pyridine/acetic acid, 25 min, rt) to simultaneously deprotect bases and hydroxyl positions; and (4) washing the solid support to fully remove hydrazine. At this stage, the deprotected RNA strand is attached to the CPG support through the Q linker. A final fluoride treatment (1 M TBAF, rt, 15 min) releases the RNA chain now ready for purification (HPLC). For comparison, an RNA oligomer with the same sequence was prepared under the same conditions, except that 2′-TBDMS monomers and the conventional succinyl LCAA-CPG were used (entry 9, Table 1). The two oligomers were identical, as shown by PAGE, HPLC, and MALDI-TOF (calcd 6616, found 6617 [M + H^+]). T_m values of duplexes formed by the hybridization of **8** and **9** with a complementary RNA strand synthesized via TBDMS chemistry were 51.7 and 49 °C, respectively.

To further confirm the integrity of the phosphodiester linkages during RNA synthesis/deprotection, dTTTT-[3′,5′-rU]-dTTTT and dTTTT-[2′,5′-rU]-dTTTT were prepared using monomers **5a** and **6a** and commercial dT amidite. These oligomers were separable by HPLC (rt, 42.8 and 44.2 min, respectively) and were free from their their isomeric oligomer (detection limit <1%; Supporting Information).

We also evaluated the activity of siRNAs synthesized with monomers **5a**−**d** in an RNAi assay that targets luciferase mRNA.[17] The siRNA duplex [**8**/complement] had the same gene silencing activity [IC_{50} ~ 0.08 nM] as the reference siRNA duplex prepared via TBDMS chemistry [**9**/complement], further confirming the integrity of the synthesized RNA strand (Figure 2).

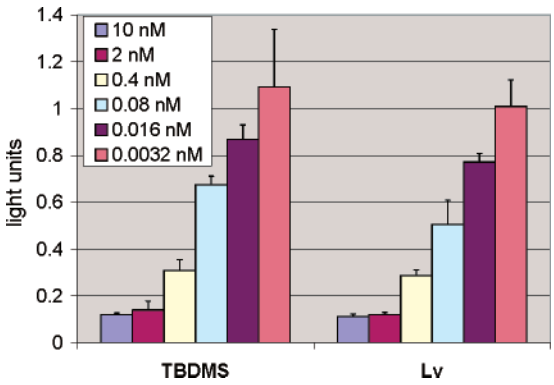

Figure 2. Silencing of luciferase mRNA expression by siRNA duplexes (light units are relative to a scrambled siRNA control). The antisense strands were synthesized via TBDMS and Lv chemistries (sequences **8** and **9**, respectively, Table 1), whereas the sense strand was obtained via TBDMS chemistry.

A number of 2′,5′-linked RNA sequences of moderate length were also successfully prepared by this approach (e.g.,

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entry 10, Table 1; and data not shown). The fully levulinated sequence, 5′-$(U^{2'OLv})_{19}dT_2$-3′ (entry 15, Table 1), was also synthesized. In this case, the solid support bound oligomer was treated with (a) anhydrous NEt_3/CH_3CN (2:3 v/v; rt, 60 min) to deblock the phosphate's β-cyanoethyl groups and (b) 1 M TBAF (15 min) to release the *protected* 2′-*O*-Lv RNA oligomer from the Q-CPG solid support. The average stepwise coupling efficiency was 98.1% based on the purity of the crude product isolated (Supporting Information). A small amount of this material was deprotected in solution by hydrazinolysis (25 min; rt). After quenching the excess hydrazine with acetone, the fully deprotected product was analyzed by HPLC. The purity of U_{19}tt matched that of the 2′-*O*-Lv precursor (**15**), indicating that delevulination proceeds very cleanly (see Supporting Information, Figure S4). Finally, the Lv and TBDMS chemistries were combined to produce *partially* esterified RNA strands (oligomer **17**, Table 1; calcd 6657, found 6659 [M + H^+]). Deprotection of **17** involved on-column decyanoethylation with NEt_3/CH_3CN (2:3 v/v; rt, 60 min) followed by fluoride treatment to desilylate and release the oligomer from the Q-CPG solid support.

Partially or fully esterified RNAs may find applications in RNAi gene silencing by virtue of their enhanced stability against serum nucleases and, possibly, uptake by cells or tissues. For instance, monomers **5** and **6** may be used in combination with a *N*-Lv ribonucleoside containing a different 2′-ester group (e.g., one derived from linoleic acid) to produce, after removal of all *N*/*O*-Lv groups, a partially esterified siRNA.[18] To study the impact of esters on the nuclease and thermal stability of duplexes, we compared the rate of degradation of oligomer **15** ($t_{1/2} \sim 2.5$ h) in fetal bovine serum to that of the native deprotected sequence **16** ($t_{1/2} \sim 15$ min; Supporting Information). To assess the effect of the *O*-Lv groups on the thermal stability of RNA duplexes, the T_m values of modified hybrids **15**/rA_{21} and **17**/rA_{21} were compared to those of the controls **18**/rA_{21} (34.7 °C) and **16**/rA_{21} (35.7 °C). The duplex **17**/rA_{21} showed a single cooperative transition at 34.5 °C, whereas a 1:1 mixture of **15** and rA_{21} showed a much weaker and broader transition that is derived from the rA_{21} alone. Thus, provided that the number of ester groups remains small, the impact of interdispersed 3′-ester groups on an RNA duplex appears to be small.

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After completion of the above studies, we found that a 1 min coupling time is sufficient to provide good yields of sequences 11 and 12 , which resulted in 70% of desired products (HPLC analysis). The use of the bulkier 2′-TBDMS required a 10 min coupling time to achieve excellent results (entries 13 and 14; Table 1).

In conclusion, we have shown that the Lv group is a suitable protecting group for the 2′-hydroxy functions of ribonucleoside building blocks. Its major advantage over other 2′-protecting groups is in the on-column unblocking step at the end of the synthesis which greatly simplifies and speeds up postsynthesis processing. With regard to the introduction of the Lv group, the reagent required, namely, 4-oxopentanoic acid, is inexpensive and readily available (prepared in >70% yield by treating starch or cellulose with acid).[19] Although this provides a clear cost advantage over some current protection schemes, the arduous separation of derivatives such as **5**/**6** must be taken into account. The orthogonal deprotection conditions of 2′-TBDMS and a transient 2′-Lv group lend themselves to the synthesis of branched RNA.[20] Furthermore, the Lv strategy should make it possible to synthesize (and deprotect) RNA directly on microarrays.

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Supporting Information Available: Experimental procedures, compound characterizations, and selected ^1H and ^{31}P NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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