An Efficient Method for the Incorporation of Molecular Probes at Multiple/Specific sites in RNA: Levulinyl Protection for 2'-ACE®, 5'-Silyl Oligoribonucleotide Synthesis

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Abstract

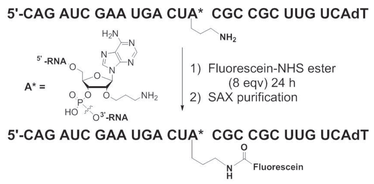
Molecular probes have found wide application in the study of biomolecules within living systems. Oligonucleotides that are labeled with molecular probes are an invaluable tool for monitoring DNA and RNA processing for both *in vitro* and *in vivo* applications. Solid-phase oligonucleotide synthesis facilitates relatively straightforward and efficient incorporation of molecular probes at the 5'-end of DNA or RNA. However, modifying the 3'-end of an oligonucleotide generally requires either post-synthetic strategies or immobilization of the molecular probe to the solid support. The former process is subject to low yields due to potentially inefficient coupling while the latter strategy is restricted by the stability of the modification to repeated exposure to synthesis reagents. Similarly, internal labeling of oligonucleotides with molecular probes is largely limited to post-synthetic processing and subject to coupling efficiencies associated with this process for these labeling steps. Finally, the need to differentially label oligonucleotides with distinct moieties in specific terminal and internal positions adds yet another layer of complexity in the generation of these important molecular tools.

In order to improve the labeling efficiency and ease of preparation of internal or 3'-terminal sites of oligoribonucleotides, we have developed a method for labeling these positions while the oligonucleotide remains immobilized on the solid support. We have applied a method to selectively de-block a levulinyl-protected hydroxyl group at a variety of different sites within an oligonucleotide and to selectively label these positions by the use of phosphoramidite-activated molecular probes. Conditions used to remove the levulinyl protecting group are mild and compatible with the 2'-ACE®, 5'-Silyl oligoribonucleotide synthesis platform, resulting in excellent yields of high quality, full length modified oligoribonucleotides.

Introduction

Traditional incorporation of molecular probes onto oligonucleotides can be accomplished through the coupling of activated esters to amines. This approach requires that the oligonucleotide be labeled post-synthetically and suffers from several common problems.

- A large excess of expensive active ester is generally required to drive the reaction to > 90% completion; in some cases, only a modest (50% or less) conjugation efficiency is routinely achieved.
- Active esters are not stable in aqueous solution for extended reaction periods and are consumed in competing side reactions that breakdown the reactive species.
- One or more steps of purification are required to isolate the labeled oligonucleotide from the excess conjugating reagent. Also, it can often be difficult to rigorously purify labeled product from unlabeled material.



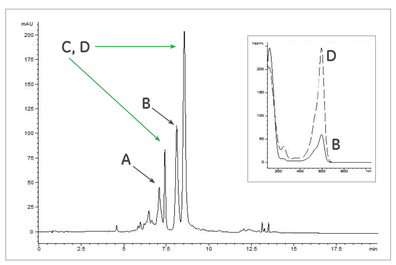


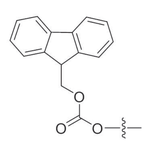
Figure 1. Representative example of labeling oligonucleotides internally using activated ester approaches. Key: A = Unlabeled oligonucleotide; B = Fluorescein-labeled oligonucleotide; C,D = Residual unconjugated fluorescein species

Labeling oligonucleotides during chain assembly while they are immobilized on the solid support has several advantages:

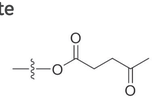
- Phosphoramidite derivatives react quickly using only minor modifications of standard coupling cycles.
- Coupling efficiencies are typically > 90%.
- Excess conjugating reagent is easily removed by washing.

Traditionally this strategy has been used to label the 5'-terminus of the oligonucleotide where the phosphoramidite derivative of the desired conjugating reagent is available. Applying the phosphoramidite conjugation approach at sites other than the 5'-OH is possible but requires a new synthetic strategy. Phosphoramidites or supports that contain hydroxyl groups protected with moieties that are stable to the synthesis conditions but that can be specifically removed under conditions that do not affect the other protecting groups on the oligonucleotide are needed. Two examples of such orthogonal protecting groups in oligonucleotide synthesis are the Levulinyl (Lev) protecting group and the 9-Fluorenylmethyloxycarbonyl (Fmoc) protecting group.

Applying this strategy to 5'-Silyl-2'-ACE® RNA synthesis requires that the removal of the Levulinyl group does not interfere with other protecting groups that are being used. The 5'-Silyl-2'-ACE® synthesis platform utilizes TEA-HF to deblock the 5' position and mild acid to remove the 2'-ACE® protection group. The Levulinyl protecting group is stable to these conditions and is removed using a solution of N_2H_4. The N_2H_4 solution does not interfere with phosphate protection (POMe), the 5'-Silyl group (BZH), or the 2'-ACE® group.



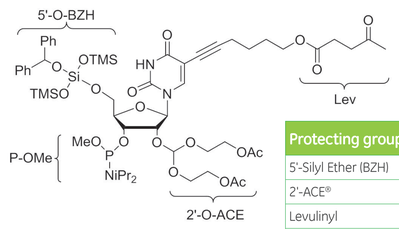
Fmoc-Carbonate



Levulinate Ester

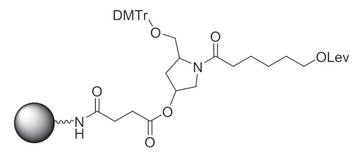
Methods

Phosphoramidites containing the Lev Protecting Group were designed in a manner such that minimal disruption in hybridization would occur. Amidtes were synthesized using standard 5'-Silyl-2'-ACE® RNA phosphoramidite synthesis conditions.

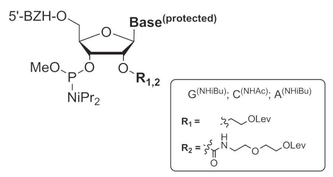


5-Lev-U Amidite

Protecting group	Deprotection Conditions
5'-Silyl Ether (BZH)	TEA-HF
2'-ACE®	300 mM AcOH-TEMED
Levulinyl	0.3 M N_2H_4 in 3:2 Pyridine:AcOH
P-OMe	0.2 M S_2Na_2 in DMF

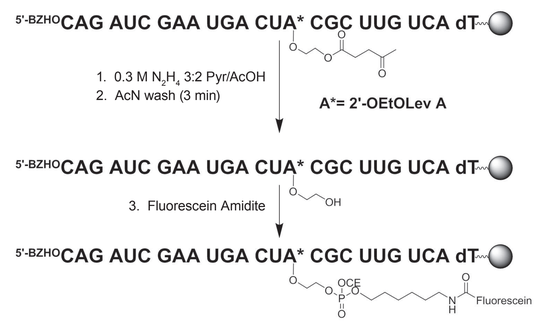


3'-Lev Linker Support

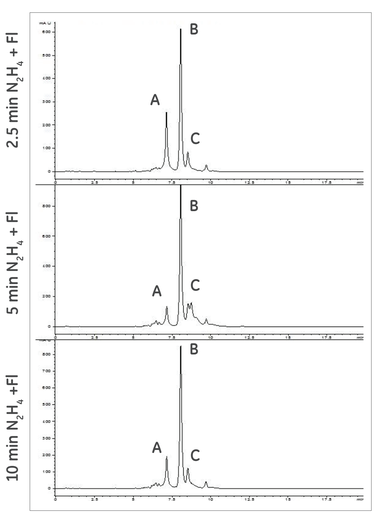


2'-Modified-Lev Amidites

RNA Synthesis Conditions



After synthesis of 25 mer is complete (5'-BZH intact), the Levulinyl group is deprotected using 0.3 M N_2H_4 in 3:2 pyridine:AcOH at room temperature for 2.5, 5, and 10 minutes. The column is washed with AcN and treated with the Fl amidite (single isomer).



- Peak A is unlabeled—due to incomplete Lev deprotection or Fluorescein coupling.
- Peak B is the labeled product.
- Peak C is doubly labeled with Fluorescein resulting from a small amount of the 5'-BZH group coming off during the N_2H_4 treatment.
- Decreasing the concentration of the hydrazine solution slows the rate down. Using 0.1 M N_2H_4 shows little difference with the backside impurities.

Figure 2. Schematic Representation of RNA processing and optimization of Lev Deprotection while on solid-support.

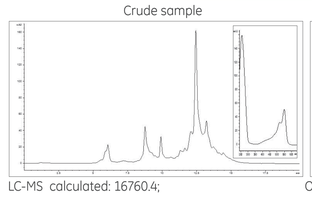
Results

Table 1. Sequences and labeling positions using the Levulinate protection strategy for the incorporation of molecular probes at defined locations.

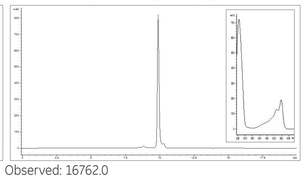
Description	RNA sequence (5' → 3')	Length
3'-End-labeled (ˆ = phosphoro-thioate)	Dabcylˆ GˆCˆG GAG ACA GCG c3mGˆmGˆmU AAC UAG AGA UCC CUC AGA CˆmGc3G GCA GGA AˆGˆAˆ ACy3	50
Dual-labeled (two internal positions)	CCA UUU GAU ACA CUA 5-Lev-U(5,6-Fl)UU AUC AA5-Lev-U(Cy3) GG	26
Dual-labeled (5'-and internal positions)	Cy3CC GGU AUA ACC UCA AUA AUA 5-Lev-U (5,6-Fl)GG UUU GAG GGU GUC UAC CAG GAA CCG UAA AAU CCU GAU UAC CGG	66

3'-End Labeled

Crude sample



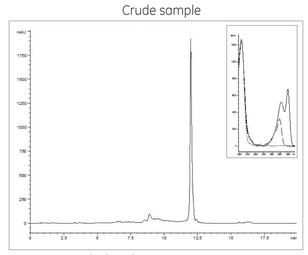
Gel Purified (15% PAGE)



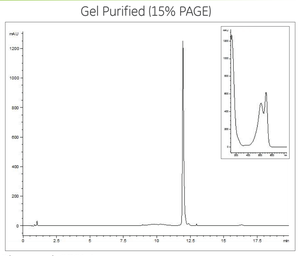
LC-MS calculated: 16760.4; Observed: 16762.0

Dual-Labeled (Two internal positions)

Crude sample



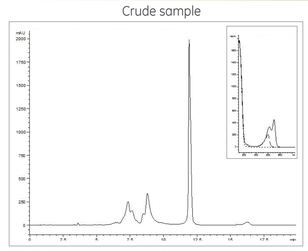
Gel Purified (15% PAGE)



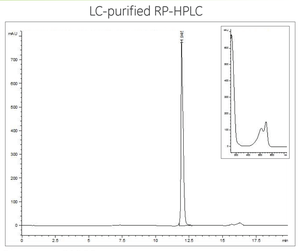
MALDI-TOF calculated: 9761.4; Observed: 9761.1

Dual-Labeled (5'-and internal positions)

Crude sample



LC-purified RP-HPLC



MALDI-TOF calculated: 21989; Observed: 21988

Conclusion

Incorporation of molecular probes at defined sites within synthetic RNA was accomplished using the 2'-ACE®-5'-Silyl RNA synthesis platform in combination with the Levulinyl protecting group. Selective removal of the Levulinyl group with hydrazine was accomplished while the RNA molecule is retained on solid support allowing for efficient coupling of phosphoramidite activated molecular probes at specific sites within the RNA molecule. This process results in excellent yields of modified full length RNA and superior qualities.

References

1. S.A. Scaringe, F.E. Wincott and M.H. Caruthers, Novel RNA synthesis method using 5'-silyl-2'-orthoester protecting groups. *J. Am. Chem. Soc.*, **120**, 11820-11821 (1998).
2. S.A. Scaringe, D. Kitchen, et al., Preparation of 5'-silyl-2'-orthoester ribonucleotides for oligonucleotide synthesis. *Current Protocols of Nucleic Acid Chemistry.* 2.10.1-2.10.16 (2004).
3. S.A. Hartsel, R.J. Kaiser, and M.O. Delaney, Polynucleotide synthesis labeling strategy. US 8,026,349 B2.

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