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Water-Soluble Leaving Group Enables Hydrophobic Functionalization of RNA

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Abstract

Attachment of hydrophobic groups to RNA is challenging because of their poor aqueous solubility. One-step acylation of RNA 2'-OH groups in water using a water-soluble imidazole leaving group is described. The effect of the hydrophobic groups on hybridization is reported. Furthermore, propargyl-functionalized RNA is shown to be readily labeled with a fluorophore. Lastly, heptyl-functionalized RNA is found to exhibit the unusual property of solubility in organic solvents.

Graphical Abstract

RNA is a versatile biomolecule with many biological functions, ranging from informational to catalytic.^{1–3} To study and modulate the properties and biological activity of RNAs, researchers have developed strategies for their chemical functionalization.^{4–11} For example, fluorescent labeling of RNAs can aid in imaging RNAs and analyzing their interactions.^{12–15} In a second example, the attachment of hydrophobic groups to biologically active short interfering RNAs has proven to be an important strategy for aiding cellular uptake.^{16–18}

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

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NMR data, NMR and MS spectra of compounds **1–12**, detailed RNA acylation and purification procedure, MS spectra of functionalized RNA, molecular beacon experimental procedure, procedure for labeling propargyl RNA, and description of solubility experiments with heptyl RNA (PDF)

While such applications of functionalized RNAs are useful, they are also limited by methods for synthesis.¹⁹ Varied groups can be incorporated into RNA during solid-phase synthesis, but this is only applicable to relatively short RNAs.^{19–21} Postsynthesis labeling of amine- or thiol-functionalized RNAs can be achieved readily in polar solvents such as dimethylformamide,^{22,23} but this solvent does not support or preserve RNA-folded structure.²⁴ As a whole, methods for reaction with nonfunctionalized RNAs in water are quite limited. Therefore, the development of new strategies for covalent attachment of groups to RNA in water remains a subject of interest.

Recently, we found that RNA can be functionalized in water at stoichiometric and superstoichiometric levels by acylation of the 2'-OH group on ribose moieties (Scheme 1).^{25,26} An acylimidazole reagent was used to form ester linkages at 2'-OH positions,²⁵ while imidazole carbamates were employed to form carbonate linkages.²⁶ This acylation strategy was used to establish external chemical or photochemical control over RNA function. One major advantage of this acylation strategy is that it can be applied in a single step to unmodified RNA of any length and does not require solid-phase nucleic acid synthesis. Subsequent studies revealed that this approach works efficiently when introducing hydrophilic groups into RNA. However, we found that with certain hydrophobic groups the water solubility of the activated acyl compounds was not sufficient to yield significant levels of acylation. This is a potential drawback of the method since many interesting and useful labels and tags are hydrophobic in nature.

To overcome this limitation, here we set out to develop a highly water-soluble imidazole leaving group that would enable functionalization of RNA with hydrophobic moieties. In previous work it was found that, while imidazole is not sufficiently activating, 2-chloroimidazole has the right electron-withdrawing properties to form an activated carbamate that can react with 2'-OH groups in RNA.²⁶ In the current work, it was anticipated that a carbonyl at the 2-position of imidazole would provide similar electron-withdrawing properties compared to chloride and would allow for further modification with a water-solubilizing group (Scheme 1). To this end, a tertiary amine was chosen to be appended to the carbonyl via an ester-, amide-, or *N*-methyl amide bond. These three imidazole derivatives were conveniently synthesized in one step from commercially available imidazole-2-carboxylic acid (see Supporting Information (SI)). The ester-linked compound **1** was found to be unstable during purification and was not considered further. Imidazole derivatives **2** and **3** were reacted with varied chloroformates to afford the corresponding activated carbamates. During this process it was found that the secondary amide in compound **2** could act as a nucleophile in the reaction with a chloroformate, leading to undesired side products. For this reason, compound **3**, with an *N*-methyl amide, was chosen as the most suitable leaving group.

Next, the ability of compound **3** to act as a leaving group and enable functionalization of RNA with nonpolar groups was assessed. A range of activated hydrophobic groups was prepared by reacting the corresponding chloroformates with compound **3** in THF, yielding compounds **4–11** (Figure 1). The compounds were chosen to represent alkyl groups of varied length and branching, as well as an aryl example (a benzyl group). To functionalize RNA, the compounds **4–11** were dissolved in water at a concentration of 0.1 M and

incubated with a 15 nt RNA oligonucleotide (CAUAGGU-CUUAACUU, MW = 4696; 10 μ M) for 16 h. Functionalized RNA was purified by precipitation or filtration with a molecular weight cutoff filter with yields varying widely depending on substitution.

The relative degree of RNA functionalization employing leaving group 3 was analyzed by MALDI-TOF mass spectrometry (Figure 1 and Figures S1–S8), which is proven to be a suitable technique for determining relative abundance of similar species.²⁷ Reaction efficiency was found to be strongly influenced by the substitution pattern around the α -carbon. Reaction of the 15 nt RNA with methyl derivative **4** resulted in a median of 2–3 added groups per strand (Figure 1). Increasing the substitution decreased the number of groups to 1–2 for ethyl derivative **5**, 0–1 for isopropyl compound **6**, and virtually no functionalization with *tert*-butyl derivative **7**. Neopentyl compound **8** contains an additional methylene between the *tert*-butyl group and carbamate and resulted in increased levels of reaction compared to compound **7**. This suggests the importance of steric hindrance at the α -carbon in influencing reactivity with the relatively congested RNA 2'-OH. Interestingly, increasing the alkyl chain length seems to dramatically increase functionalization as is seen for *n*-heptyl derivative **9**, which exhibited a median of 4 added groups per RNA. Reacting RNA with the unsaturated propargyl derivative **10** resulted in a median of 1 added functional group, whereas incubation with the benzyl compound **11** resulted in 2–3 groups added. From this it can be concluded that the steric bulk around the α -carbon determines functionalization efficiency of small alkyl groups, but larger hydrophobic groups that are extended further (or branched beyond this point) apparently do not suffer from this steric issue.

Next, the effect of these different hydrophobic groups on hybridization was studied. Since few 2'-O-carbonates have been reported in RNA,²⁶ the effects of this type of substitution on its properties are largely unknown. Groups that do not inhibit hybridization may be useful in RNA-based probes, allowing binding while protecting them from nuclease activity.²⁸ On the other hand, groups that block hybridization are potentially useful in caging applications.^{25,26} To investigate these possibilities, we studied the RNAs functionalized above for their ability to hybridize to a DNA complement using a molecular beacon probe.²⁹ To this end, 120 nM-functionalized RNA was incubated with 100 nM of a complementary molecular beacon (see SI) in 100 mM Tris buffer, pH = 8.0, 25 °C, with 10 mM MgCl₂. Upon hybridization, the beacon linearizes, which separates the fluorophore and quencher, resulting in an increase in fluorescence. The experiments show that 2'-O-acylation with methyl, ethyl, isopropyl, neopentyl, and propargyl carbonates results in a fluorescent signal comparable to that with unmodified control RNA, indicating that these acylating groups (at the substitution levels achieved here) do not strongly affect hybridization (Figure 2A). This could potentially be exploited to protect RNA from nuclease degradation, while maintaining hybridization ability.²⁸ In contrast, acylation with heptyl derivative **9** resulted in almost complete loss of fluorescence, consistent with steric blocking of the complementary DNA by larger groups. Similarly, RNA functionalized with benzyl derivative **10** yielded ~50% of the fluorescent signal of control RNA. It must be noted that the different functionalized RNA strands do not have the exact same number of modifications, making direct comparison difficult. However, these early experiments do indicate that larger hydrophobic groups substantially perturb

hybridization, and smaller groups have less effect if any. Furthermore, the data suggest that increased levels of functionalization, not surprisingly, result in increased perturbation to duplex formation.

To validate the utility of the water-soluble leaving group to functionalize RNA with hydrophobic groups, a control compound using the previous 2-chloroimidazole as a leaving group was prepared. Benzyl derivative **11** and the analogous 2-chloroimidazole compound **12** were incubated at 0.1 M concentration with 10 μ M RNA in water for 16 h. After purification, the amount of added benzyl carbonate groups was determined by mass spectrometry (Figure 2B and 2C). Control compound **12** showed only minor functionalization, while compound **11** yielded up to 5 added benzyl carbonate groups, underlining the importance of a water-soluble leaving group.

Next, the utility of functionalizing RNA with hydrophobic groups was studied by testing a fluorescence labeling strategy. Alkyne groups are proven to be exquisite handles for bioconjugation, and therefore it was envisioned that RNA treated with propargyl compound **10** would render it useful for bioorthogonal labeling.^{10,11,30} Furthermore, the hybridization experiments showed that the propargyl carbonate groups are minimally perturbing for duplex formation, making it a potentially interesting group for use in biological studies. The azide-substituted coumarin **13** was synthesized following literature procedures.³¹ This fluorogenic compound exhibits a dramatic increase in fluorescence upon cycloaddition with an alkyne, making it an interesting substrate for labeling propargyl RNA (Figure 3A).

Coumarin **13** was incubated at 20 μ M with 100 μ M propargylated RNA in the presence of 5 mM CuSO₄ and 50 mM sodium ascorbate in phosphate-buffered saline (PBS) for 15 min at room temperature. This mixture was diluted 10-fold in PBS, and an emission spectrum was acquired, showing a strongly emissive band at $\lambda_{\text{max}} = 458$ nm (Figure 3B). The experiment was repeated with control RNA that was not treated with propargyl compound **10**, and ~10-fold lower emission was observed (Figure 3B), indicating that the propargyl carbonate groups on RNA can indeed be used as reactive handles for fluorescence labeling. The formation of coumarin-labeled RNA was confirmed by mass spectrometry (Figure S9).

Lastly, it was hypothesized that hydrophobic groups such as *n*-heptyl might give the RNA extraordinary properties, such as improved solubility in organic solvents. Control unmodified RNA was mixed with water, ethanol, or acetonitrile at a theoretical concentration of 4 μ M, and solubility was measured by RNA absorbance at 260 nm. Only when mixed with water was RNA absorbance observed (Figure 3C). However, when the heptyl-functionalized RNA was mixed with the different solvents, strong absorbance was observed in all three (Figure 3D), confirming solubility. The heptyl RNA remained fully soluble in water. The absorbance in ethanol was 86% of that in water, and the absorbance in acetonitrile was 22% compared to water. This significantly improved solubility might enable new reactivities and applications of RNA in organic solvents. For example, it could prove useful when using RNA as a chiral source in asymmetric organic transformations^{32,33} or in anchoring RNAs at membrane surfaces.¹⁷

In conclusion, we have developed a new water-soluble leaving group that can be used to activate hydrophobic groups to acylate RNA at the 2'-OH position in water. It was found that the efficiency of hydrophobic functionalization is determined by the substitution pattern around the α -carbon. Small hydrophobic groups did not seem to affect the hybridization ability of RNA, whereas larger groups were more perturbing. RNA functionalized with propargyl groups could be labeled with an azide-coumarin dye using a copper-catalyzed cycloaddition, underlining the potential of hydrophobic labeling. Finally, heptyl-functionalized RNA exhibited surprising solubility in polar organic solvents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of hydrophobic acylating groups **4–11** and RNA acylation efficiency, as measured by the number of added labels on a 15 nt RNA. Acylation was achieved by incubating compounds **4–11** at 0.1 M in water with 10 μ M RNA for 16 h at room temperature. See SI for original MS data.

Figure 2.

(A) Measuring the ability of hydrophobic-functionalized RNAs (Figure 1) to hybridize to a DNA molecular beacon probe. Note that RNAs have varied levels of substitution (see Figure 1). Fluorescence is normalized to 100% (the level of fully hybridized unmodified RNA). Error bars reflect SD from 3 experiments. (B) Mass spectrum of RNA reacted with benzyl carbamate activated with the previous leaving group (compound **12**) and (C) the new water-soluble leaving group (compound **11**).

Figure 3.

Applications and properties of hydrophobic-substituted RNAs. (A) Scheme for labeling 2'-O-propargyl RNA with fluorogenic azide-coumarin **13**. (B) Fluorescence emission spectra of control and propargylated RNA incubated with azido-coumarin **13**. (C) UV-vis absorption spectra in water, ethanol and acetonitrile of control RNA and (D) heptyl RNA, showing organic solubility of the latter.

Scheme 1.

Acylation of RNA at 2'-OH Groups with Activated Imidazole Carbamates