8
Modified RNAs as Tools in RNA Biochemistry

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8.1
Introduction

RNA displays a vast variety of functions in that it carries genetic information, regulates gene expression, catalyzes reactions and participates in all facets of protein expression [1]. In addition to the four basic nucleosides (adenosine, guanosine, cytidine and uridine), many RNA molecules contain modified nucleosides essential for function. The fact that these modifications are essential for function in some RNAs, but entirely absent in others, indicates a significant layer of complexity in the hierarchy of RNA structure. With progress in the chemical synthesis of RNA over the last 15 years, modified nucleosides can now be readily incorporated at specific positions in RNA. These advances in solid-phase synthesis have promoted a cornucopia of experiments examining the influence of single-functional-group modification on the biological function of RNA.

Modified nucleosides have also been site-specifically incorporated into RNA as reporter groups for biochemical and biophysical structure–function analysis. There is a large diversity in these approaches. For example, fluorescent probes have been used to report internal changes during RNA folding [2] as well as to measure interhelical distances for determining the global structure of RNA [3–5]. Disulfide crosslinks have been used to restrict RNA helical elements to validate structural models based on other techniques [6]. These are but a few examples of site-specific incorporation of RNA structure–function probes.

The major goals of this chapter are to review the various types of modifications that can be incorporated site-specifically into RNA by chemical synthesis, and to provide a general method for the incorporation of reporter groups into RNA for biochemical and biophysical analysis. This includes comparison of the two central strategies for the incorporation of modified nucleosides into RNA, i.e. the phosphoramidite strategy and post-synthetic labeling. The phosphoramidite strategy utilizes chemical synthesis of a modified nucleoside phosphoramidite in conjunction with solid-phase synthesis, whereas post-synthetic labeling utilizes incorporation by the phosphoramidite method of a convertible nucleoside containing a reac-

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tive group, which is selectively modified after oligonucleotide synthesis with a labeling reagent. The advantages and disadvantages of each modification strategy will be described. Finally, a general and efficient modification strategy will be presented that utilizes post-synthetic labeling of 2′-amino groups with a wide range of reporter groups through a number of different coupling chemistries.

8.1.1 Modification Strategy: The Phosphoramidite Method

While enzymatic synthesis can be used to prepare uniformly labeled RNA, modified nucleosides can be incorporated site-specifically into RNA by solid-phase chemical synthesis using modified nucleoside phosphoramidites [7]. The main advantage of this method is that it allows for the incorporation of a desired modification or reporter group at a specific position in the RNA. While this is a highly effective and powerful method, it has several disadvantages. In most cases the synthesis of a modified phosphoramidite requires a lengthy and costly synthetic route. Furthermore, the reporter group must be stable to the conditions used in solid-phase oligonucleotide synthesis (e.g. incubation with acid, base and oxidizing solutions) as well as the deprotection conditions. However, phosphoramidites of many desirable modified nucleosides are commercially available, providing rapid, cost-effective access to a variety of modified RNAs (Table 8.1).

There are four basic categories of RNA modifications that can be incorporated into RNA via the phosphoramidite method: end (5′ and 3′), base, phosphate and sugar modifications. Figure 8.1 shows selected examples of such modifications, and Table 8.1 lists several of the RNA modifications that are commercially available as phosphoramidites and/or modified RNAs. Of those not commercially available (many of which are reviewed in [8–11]), other notable examples of RNA modification by the phosphoramidite method include the base modifications 2-deoxyribonolactone [12] and 5-ketone pyrimidine derivatives [13]. Internucleotide linkages include boranophosphates [14] and phosphoroselenoates [15]. Sugar modifications include 1′-deutero [16], 2′-modifications (*O*-(2-thioethyl) [17] and *O*-(2-aminoethyl) [18]), 5′-modifications (tallo or C-methyl [19, 20], chloro [21], amino [21]) and per-deuterated ribose [22]. Fluorescent labels have also been synthetically attached to the 2′-position via an ether linkage [23], a carbamoyl linker [24], an arabino carbamoyl linker [25] and an amido linkage [26].

The phosphoramidite method has been particularly useful in the investigation of ribozyme cleavage mechanisms. For example, incorporation of a 5′-C-methyl-modified nucleoside near the cleavage site of the hammerhead ribozyme resulted in a kinetically trapped intermediate in a crystal and provided information about a conformational change along the reaction pathway prior to transition state formation [20]. In another example, a crystal structure of the hairpin ribozyme containing a 5′-chloro group at the cleavage site provided structural information for comparison with the non-cleaved state (all RNA) and a vanadyl transition state mimic, providing valuable information about the entire mechanistic pathway [27].

Tab. 8.1. Commercially available modifications that can be incorporated into RNA by the phosphoramidite method

Site	*Modification*	*Commercial Source*[1]	*Reference*[2]
End-labeling			reviewed in 74–76
5′ end	fluorescent dyes	CG, Dh, GR	3–5
	amino groups	CG, Dh, GR	77, 78
	biotin	CG, Dh, GR	
	photo-cleavable biotin	Dh, GR	
	5′-thiol	Dh, GR	
	acridine	GR	
3′ end	fluorescent dyes	CG, Dh, GR	
	amino groups	CG, Dh, GR	
	inverted abasic	Dh	
	Puromycin	CG, Dh	79
	dideoxy G,C	Dh	
	biotin	CG, GR	
	acridine	GR	
	psoralen	CG, GR	
	cholesterol	CG, GR	
	DNP	CG, GR	
Internucleotide			
	S (non-bridging)	CG, Dh, GR	29–31
	3, 9, 18 atom spacers	CG, Dh, GR	
Sugar			reviewed in 9, 10
1′	abasic	Dh, GR	
2′	NH_2 U,C	CG	37
	F U,C	CG, Dh	
	OCH_3	CG, Dh, GR	
	SCH_3 U	GR	80
	$OCH_2CH_2CH_2NH_2$	CG	65
	$NHCOCH_2CH_2CH_2$py rU	Dh	36
	LNA	GR	81
Purine			reviewed in 9, 10
	N^6,N^6-dimethyl A	Dh	
	inosine	CG, Dh, GR	
	purine ribonucleoside	CG, Dh	
	ribavirin	Dh	
	7-deaza A,G	CG	
	2-aminopurine	CG, Dh, GR	2
	2,6-diaminopuridine	Dh	
	8-bromo A	CG	
Pyrimidine			reviewed in 9, 10
	N^3-methyl U,rT	CG	
	N^3-thiobenzoyl ethyl U	CG	
	4-triazoylyl U	CG	
	N^4-ethyl C	CG	
	pyridine-2-one	CG	
	pyrrolo-C	GR	

Tab. 8.1. *(continued)*

Site	*Modification*	*Commercial Source*[1]	*Reference*[2]
	2,2′-anhydro U	CG	
	5-methyl U,C	Dh	
	4-thio uridine	CG, Dh, GR	71
	5-fluoro U	CG, Dh, GR	
	5-bromo U,C	CG, Dh, GR	
	5-iodo U	CG, Dh, GR	
	pseudouridine	CG, Dh, GR	
	5-$CHCHCH_2NH_2$ U	Dh	

[1] For commercial sources: CG, ChemGenes; Dh, Dharmacon; GR, Glen Research. Please note that chemical suppliers are subject to change and this list is a representative example at time of publication. Several other companies exist which sell modified RNA and modified RNA phosphoramidites.
[2] References are select examples and may be reviews, applications or synthetic procedures.

8.1.2
Modification Strategy: Post-synthetic Labeling

Post-synthetic modification of convertible nucleosides enables the site-specific incorporation of a wide variety of reporter groups into RNA. The main advantage of this approach is that once the RNA has been prepared, it enables the rapid and efficient production of a wide variety of modified RNAs. Another advantage is that sensitive reporter groups, which would otherwise be unstable to the conditions of solid-phase oligonucleotide synthesis, can be incorporated into RNA. Possible disadvantages of this strategy are that in some cases additional purification steps are necessary and that it may be necessary to synthesize the convertible nucleoside phosphoramidite if the desired one is not commercially available.

Post-synthetic modification strategies have been developed for attachment of re-

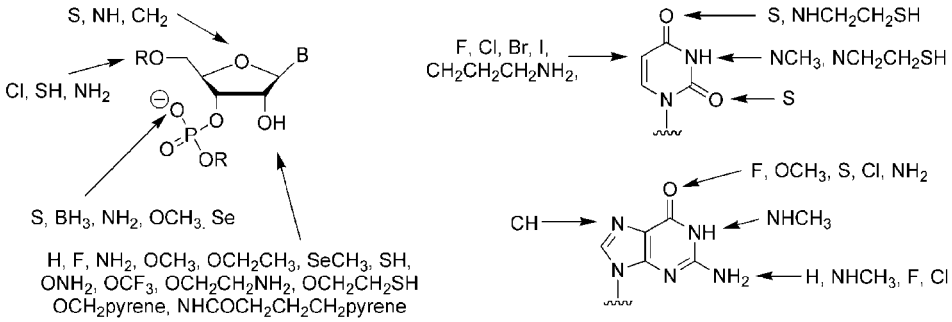

Fig. 8.1. Selected examples of modifications that can be incorporated at the sugar (left), phosphodiester backbone (left) and base (right) using the phosphoramidite method.

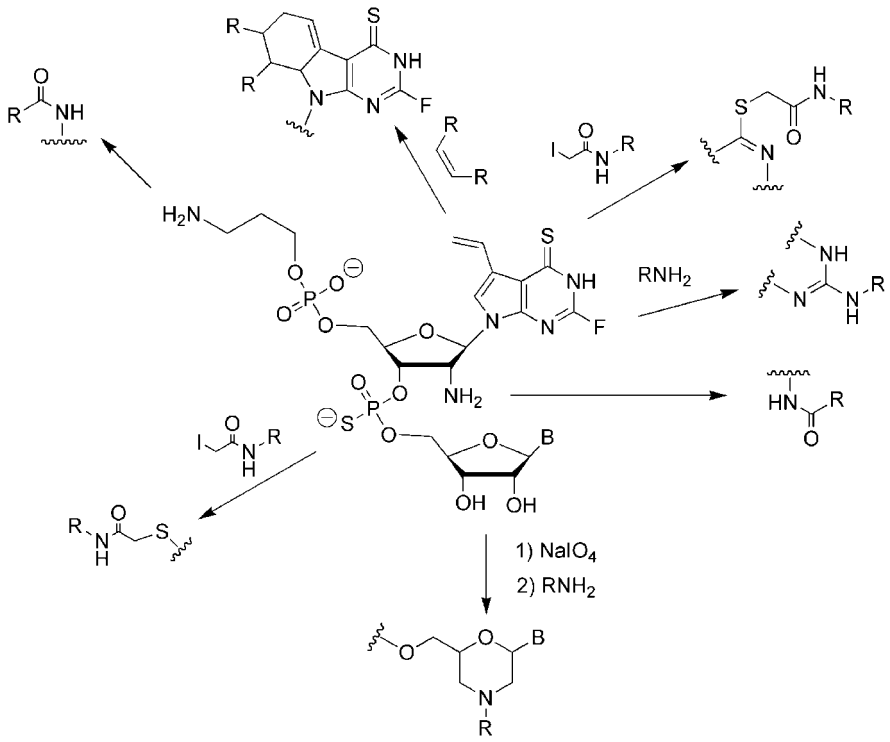

Fig. 8.2. Selected examples of RNA post-synthetic labeling.

porter groups to the 5′ and 3′ ends and at internal sites on the base, phosphate and sugar (Fig. 8.2 and Table 8.2). The main focus of this chapter is the general strategy of post-synthetic labeling of 2′-amino containing RNA and this approach will be described in detail in the next section. In addition to the modifications shown in Fig. 8.2 and Table 8.2, various groups can be attached to the 5-position of pyrimidines via on column Pd-catalyzed coupling reactions [28]. A variety of molecules have also been attached to the phosphodiester backbone through phosphorothioate [29–31] or phosphoramidate linkages [32]. However, these labeling strategies are problematic for RNA due to the inherent instability of these linkages in the presence of 2′-hydroxyl groups; consequently, this problem is overcome by incorporation of a 2′-deoxy or 2′-methoxy group at the nucleotide 5′ of the modification.

8.2
Description of Methods

8.2.1
Post-synthetic Modification: The 2′-Amino Approach

Post-synthetic labeling of the 2′-amino group (Fig. 8.3a and b) has emerged as an effective approach for the site-specific incorporation of reporter groups into RNA.

Tab. 8.2. Select examples of modifications for post-synthetic RNA derivatization

Modification	*Molecular handle*	*Commercially available?*[1]	*Labeling reactants*	*Reference*[2]
1	3-amino modifiers	CG, Dh, GR	activated esters	77, 78
2[3]	diene	No	dienophile	82
3	sulfur-containing bases	CG, Dh, GR	iodoacetamides, disulfides	69–71
4	convertible F or ClΦ nucleosides	No	amines	83
5	2′-amino	CG, Dh	isothiocyanates, NHS esters, isocyanates	37
6	2′,3′-diols	All (RNA)	$NaIO_4$, amines	84
7	non-bridging phosphorothioates	CG, Dh, GR	iodoacetamides	29–31

[1] For commercial sources: CG, ChemGenes; Dh, Dharmacon; GR, Glen Research. Please note that chemical suppliers are subject to change and this list is a representative example at time of publication. Several other companies exist which sell modified RNA and modified RNA phosphoramidites.
[2] References are select examples and may be reviews, applications or synthetic procedures.
[3] This modification strategy has only been applied to DNA thus far, but is of select interest.

Several notable examples include the incorporation of disulfide crosslinking reagents for the evaluation of RNA helical orientation [6, 33, 34], the incorporation of fluorescent probes for the study of RNA folding and ligand binding [35, 36] and the incorporation of EPR active probes [37] for the study of RNA internal dynamics [38–40] and for distance measurements [41]. RNAs containing 2′-amino groups at specific pyrimidine nucleotides (Dharmacon) and 2′-amino-modified pyrimidine phosphoramidites (ChemGenes) are now commercially available. Because the 2′-amino group is an aliphatic amine, it is more reactive (i.e. nucleophilic) than the aromatic amines or hydroxyl groups native to RNA, making this method of post-synthetic labeling highly selective. The major advantage of the 2′-amino group over other amino-based modifiers (e.g. 5′- and 3′-amino modifiers, 5-alkylamino modified pyrimidines) is that it offers a minimal linker length. Several chemical conjugation approaches exist, including reaction with succinimidyl esters (often referred to as to NHS esters) to produce amide modified RNA [33], reaction with aromatic isothiocyanates to form thiourea linked RNA [6, 42] and reaction with aliphatic isocyanates to prepare urea tethered RNA [42, 43]. These three methods will be described in detail below, and examples employing these methods to address biochemical and biophysical questions will be provided. Of notable importance for this modification strategy is an alternative 2′ protection approach that has been developed based on a photo-cleavable protecting group in place of the standard 2′-trifluoroacetyl group; after removal of the protecting group, the 2′-amino group may be derivatized on-column, providing many advantages over solution-based post-synthetic modification of deprotected oligonucleotides [44]. Other

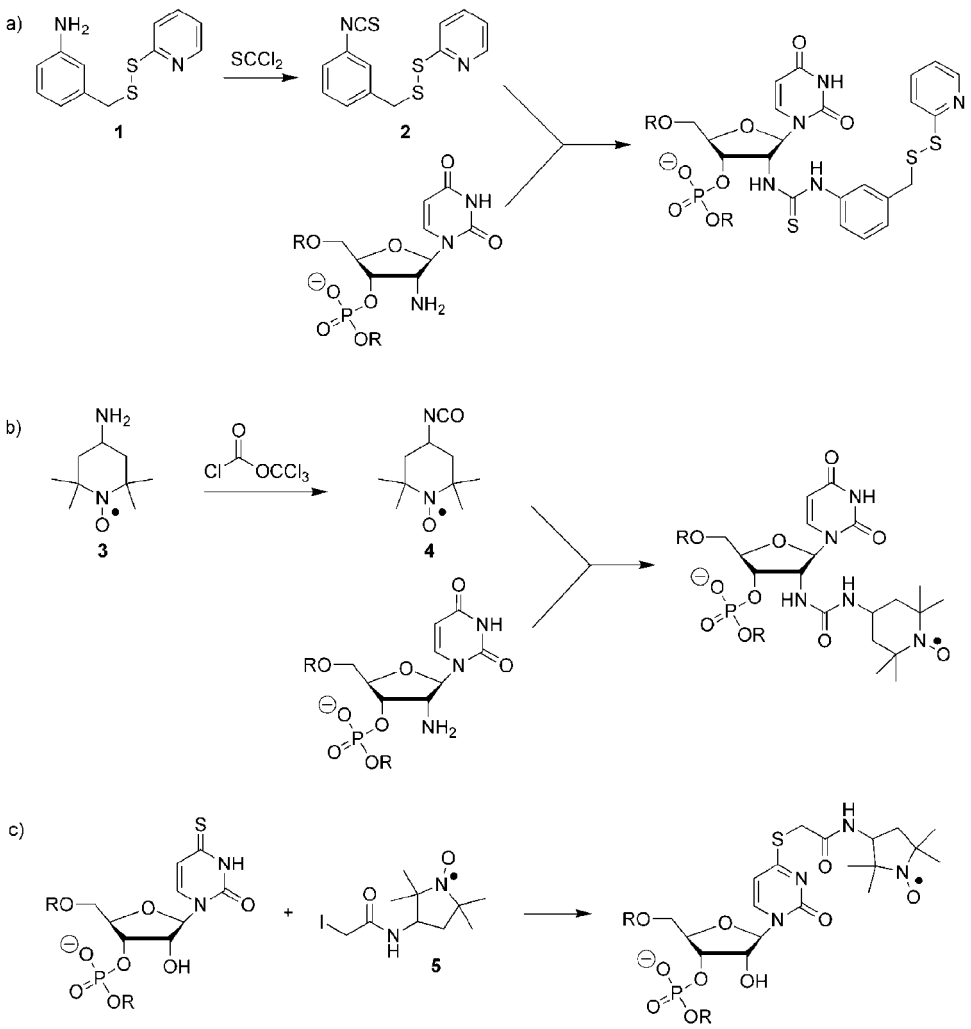

Fig. 8.3. Preparation of an isothiocyanate crosslinking reagent **2** (a) and an EPR spin-labeling reagent 4-isocyanato TEMPO **4** (b) from the corresponding amines using thiophosgene and diphosgene, respectively, and their subsequent incorporation into 2′-amino-modified RNA. (c) Post-synthetic modification of 4-thiouridine by alkylation with spin-label **5**.

approaches for the attachment of reporter groups to the 2′-position of oligonucleotides that will not be addressed in depth include chelation of metal ions such as ruthenium to 2′-amino modified oligonucleotides [45, 46], incorporation of fluorescamine at a 2′-amino group using a Michael addition and rearrangement reaction [47], reaction of amines with a 2′-*O*-(acetaldehyde) group [48] and reduction of thiol containing compounds with 2′-*O*-(2-thioethyl) to form disulfide linked modi-

fied RNAs [17]. The attachment of sterically hindered compounds to the 2′-amino group may be difficult and can be overcome by use of the 2′-*O*-(2-aminoethyl) modification [18].

8.2.1.1 Reaction of 2′-Amino Groups with Succinimidyl Esters

The reaction of 2′-amino groups with succinimidyl esters to produce amido-linked modified RNAs has been used to incorporate disulfide crosslinks to evaluate RNA conformational dynamics [33, 49], to convert the hammerhead ribozyme from a ribonuclease to a ligase ribozyme [50], to incorporate photocrosslinking reagents to evaluate RNA tertiary structure [51], to identify base-pair mismatches [52, 53], and to incorporate fluorescent pyrene labels to study RNA folding and ligand binding [36, 54–56]. In conjunction with the isocyanate method described below, this method has been used to probe steric interference in the hammerhead ribozyme [57]. Catalysis of this chemistry by the phosphodiester on the 3′-position adjacent to the 2′-amino-containing nucleoside and/or the 3′-oxygen has been reported [58]. One advantage of this method is that many succinimidyl esters are commercially available (e.g. Molecular Probes and ChemGenes have a wide variety of amine-reactive succinimidyl ester dyes available). The major drawback of this method is that this chemistry often suffers from low labeling efficiency, e.g. the pyrene succinimidyl esters typically coupled with only 20–26% yield after purification [36]. However, in some cases it is possible to overcome this low coupling efficiency by the use of the corresponding carboxylic acid with an activating agent, such as a carbodiimide, which may result in nearly quantitative coupling [18, 44, 49]. Another disadvantage of this modification approach is that 2′-amido modifications destabilize RNA when incorporated at internal positions ($\Delta T_m \sim -5$ to 12 °C per modification) [26, 59, 60]. However, some 2′-amido linked modifications located at end positions increase RNA stability [26, 61], which is likely a result of these particular modifications that contain large aromatic groups (e.g. fluorescent labels), which may stack onto the end of the helix. Nevertheless, this destabilizing effect of 2′-amido groups at internal sites should be kept in mind when incorporating reporter groups into RNA.

8.2.1.2 Reaction of 2′-Amino Groups with Aromatic Isothiocyanates

The reaction of 2′-amino groups with aromatic isothiocyanates (Fig. 3a) has been used to incorporate fluorescent probes [26, 35], disulfide cross-links [6, 34] and photocrosslinking agents [62] into RNA. The main advantage of this method is the highly efficient chemistry, which has resulted in reported conversion yields in excess of 90% in all cases. In addition, fewer equivalents of the isothiocyanate labeling reagent are required than for the succinimidyl ester chemistry. The main drawback is that although some isothiocyanates are commercially available, most must be prepared from the corresponding amine and thiophosgene; however, this synthetic conversion is relatively straightforward [42, 63]. There is limited available UV thermal stability data for 2′-thioureido modifications and all of the data involves incorporation of large fluorescent probes (fluorescein and rhodamine). Following a similar pattern to that observed for the 2′-amido modifications, these

modifications are rather destabilizing at internal positions, but have a stabilizing effect at end positions [26, 35]. Isothiocyanates have also been used to selectively incorporate metal ion chelators at 5-amino-derived pyrimidines [64] and at 2′-(*O*-propylamino)-derived nucleotides [65].

8.2.1.3 Reaction of 2′-Amino Groups with Aliphatic Isocyanates

The reaction of 2′-amino groups with aliphatic isocyanates (Fig. 8.3b) is a versatile platform for the incorporation of biochemical and biophysical reporter groups into RNA. This method has been used to incorporate disulfide crosslinks [34, 43, 66]; an activated disulfide that can be used to conjugate a wide variety of groups such as cholesterol [67], glutathione and bimane [43], nitrophenol [44], pyrene [18] and nitroxide spin-labels [37]. Like the isothiocyanate coupling, this chemistry is highly efficient, typically displaying quantitative yields for unhindered isocyanates [43, 44]. Unlike the succinimidyl ester coupling, there is no leaving group for the isocyanate (and isothiocyanate) coupling chemistry, and therefore good yields have been reported for structurally hindered isocyanates (e.g. 90% yields are routinely observed for the secondary isocyanate, 4-isocyanato-TEMPO [37, 38]). Due to the high selectivity and efficiency of this reaction, the crude, deprotected RNA can be labeled directly, allowing for only one purification and therefore high yields. Another advantage is the relatively fast coupling time (15–60 min). In addition, 2′-ureido modifications are not as destabilizing as 2′-amido modifications [43, 66], e.g. incorporation of the EPR spin probe TEMPO through a 2′-urea linkage at internal base-pairing nucleotides resulted in a minor decrease in stability as measured by a small decrease in melting temperature of 1–3 °C [37]. The main drawback is that usually the isocyanate labeling reagent must be prepared from the corresponding amine; however, this chemistry is straightforward and pure isocyanates are obtained in high yields after purification by extraction [42, 43, 68].

8.3 Experimental Protocols

The general experimental protocols for two representative examples of RNA labeling by the 2′-amino approach will be detailed: incorporation of a crosslinking reagent (Fig. 8.3a) for validation of existing structural models [6, 42] and incorporation of an EPR spin-probe (Fig. 8.3b) for biophysical analysis of structure [41] and dynamics of RNA molecular recognition [37–40]. We have also included an example of base-labeling using 4-thiouridine for RNAs that cannot be modified in the 2′-position due to loss of function (Fig. 8.3c).

8.3.1 Synthesis of Aromatic Isothiocyanates and Aliphatic Isocyanates

The 2′-amino post-synthetic labeling approach often requires the synthesis of the desired labeling reagent from the corresponding amine, which may be commer-

cially available. The isothiocyanate crosslinking reagent **2** was prepared from the corresponding amine **1** and thiophosgene (Fig. 8.3a) [6, 42].

(1) Add a solution of amine **1** (for synthesis, see [6]; 8.20 g, 33 mmol) in chloroform (250 ml) drop-wise to a solution of thiophosgene (4.17 g, 36.3 mmol) in chloroform (50 ml) over 10 min at room temperature.
(2) Stir for 1 h at room temperature.
(3) Dilute the mixture with methylene chloride (330 ml).
(4) Wash with NaOH (1 M aq, 165 ml).
(5) Extract the aqueous phase with additional methylene chloride (40 ml).
(6) Combine the organic phases.
(7) Dry the combined organic phases (Na_2SO_4) and filter off the salt.
(8) Remove the solvent *in vacuo*.
(9) Purify the crude product by flash column chromatography (CH_2Cl_2).
(10) This procedure produces an oil (in our hands 8.80 g, 92% yield).

The isocyanate spin-labeling reagent, 4-isocyanato TEMPO **4**, was prepared from 4-amino TEMPO **3** (Acros and Sigma-Aldrich) and diphosgene (Fig. 8.3b) [37].

(1) Pre-cool a solution of **3** (198 mg, 1.15 mmol) in anhydrous CH_2Cl_2 (1.5 ml) in a rock-salt ice-water bath at -8 °C.
(2) Separately, pre-cool in the same bath a solution of trichloromethyl chloroformate (diphosgene, 25 µl, 0.21 mmol) in CH_2Cl_2 (1.5 ml).
(3) Rapidly (around 8 s), add the solution of amine under a positive pressure of nitrogen to the stirred solution of trichloromethyl chloroformate at -8 °C.
(4) Remove the cooling bath and allow the reaction to stir for 2 min.
(5) Dilute the crude reaction mixture to 20 ml with CH_2Cl_2.
(6) Wash the organic layer successively with NH_4Cl (1 M aq, 4×20 ml) and NaOH (1 M aq, 20 ml).
(7) Dry the organic layer with Na_2SO_4 and filter off the salt.
(8) Remove the solvent *in vacuo*.
(9) This protocol typically yields a peach colored solid (66 mg, 29% based on starting amine or 87% maximum theoretical yield).
(10) Store the isocyanate desiccated at -20 °C in CH_2Cl_2 (0.5 mg/50 µl). Small quantities of isocyanates hydrolyze slowly when stored concentrated at -20 °C (around 30% after 4 weeks) and rapidly when stored in DMF at -20 °C [43]. However, isocyanates can be stored in CH_2Cl_2 solutions as described above for several months after preparation.

The syntheses of the isothiocyanate and isocyanate can be readily performed on a scale ranging from 25 mg to several grams. Preparation of isothiocyanates and isocyanates produces acid (HCl), which combines with the starting amine to produce an unreactive ammonium salt. This is particularly problematic for isocyanates where it is only possible to convert one-third of the amine to the corresponding isocyanate using this protocol. Alternatively, the non-nucleophilic base Proton

Sponge® (1,8-bis((dimethyl)amino)naphthylene, 2.5 equivalents; Sigma-Aldrich) can be used, which is especially advantageous if the starting amine is expensive or only available in small quantities. If the light-sensitive Proton Sponge® is used, the reaction should be performed in the dark. After the reaction, Proton Sponge® can be removed by extraction using the protocol described above.

Like most chemicals commonly used in any chemistry laboratory, thiophosgene and diphosgene are harmful, but since they are liquids and used in small quantities, they are relatively simple to handle. However, these reactions should be carried out in a well-ventilated area. Likewise, the isothiocyanates and isocyanates are toxic chemicals, but they are simple to use and require only standard laboratory safety equipment (e.g. nitrile gloves).

8.3.2
Post-synthetic Labeling of 2′-Amino-mdified RNA

RNA modified with 2′-amino groups can be purchased from several companies. The standard 2′-trifluoroacetyl protecting group is readily cleaved under standard RNA deprotection conditions and thus no additional deprotection step is necessary. Reaction of 2′-amino modified RNA with isothiocyanates or isocyanates is typically done under conditions where the RNA is denatured, in aqueous DMF and/or formamide. The organic solvents also act as co-solvents for dissolving the isothiocyanates or isocyanates. It is pertinent that highly pure amine-free anhydrous DMF be used in these reactions, due to the high reactivity of succinimidyl esters, isothiocyanates and isocyanates toward amines. Furthermore, we recommend ethanol precipitation of 2′-amino-modified RNAs, effectively converting ammonium salts of RNA from chemical synthesis into sodium salts, prior to reaction with these amine-reactive compounds as a precaution against unwanted side reactions.

Labeling of 2′-amino-modified RNA with aromatic isothiocyanates

(1) Dissolve the 2′-amino-containing RNA in 5 μl of 50 mM borate buffer, pH 8.6 (RNA concentration around 2 mM).
(2) Add **2** (100 mM in DMF, 5 μl).
(3) Incubate at 37 °C for 28 h (final concentrations: 1 mM 2′-amino RNA, 50 mM isothiocyanate **2**; 50% aqueous DMF, v/v). This reaction proceeds more slowly at room temperature.

Labeling reactions with the aliphatic isocyanates were carried out in a salt, ice-water bath (−8 °C) in a cold room (5 °C). If performed at higher temperatures, increased rates of isocyanate hydrolysis result in lower yields. Furthermore, non-specific labeling has been observed at 37 °C [43]. Analytical-scale reactions can be performed using the following procedure, provided reaction amounts are scaled down in such a way that all concentrations of reactants and buffer remain constant.

Preparative scale reactions of 2′-amino-modified RNA with aliphatic isocyanates

(1) Dissolve the crude (i.e. not yet gel or HPLC purified), deprotected 2′-amino-containing RNA (one-quarter of a 1-μmol synthesis) in 100 μl 70 mM boric acid buffer, pH 8.6.
(2) Cool the solution in a salt, ice-water bath (−8 °C) in a cold room (5 °C).
(3) Treat the solution sequentially with pre-cooled solutions of formamide (60 μl, 0 °C) and freshly prepared isocyanate in anhydrous DMF (75 mM, 40 μl, −8 °C). Final concentrations: 1 mM 2′-amino RNA, 15 mM isocyanate **4**; 50% aqueous borate buffer, 30% formamide, 20% DMF, v/v/v.
(4) Incubate for 1 h at −8 °C.
(5) Treat the oligoribonucleotide solution with a second aliquot of freshly prepared isocyanate (40 μl, 75 mM in DMF).
(6) Incubate for 1 h at −8 °C.
(7) Wash the solution with $CHCl_3$ (2 × 300 μl) at room temperature.
(8) Add sodium acetate (3.0 M, 20 μl, pH 5.3).
(9) Add absolute ethanol (−20 °C, 1.3 ml).
(10) Precipitate the RNA by storage at −20 °C for 4 h.
(11) Centrifuge the sample (11 500 r.p.m., 15 min, 5 °C).
(12) Remove the supernatant.
(13) Wash the pellet with cold absolute ethanol (2 × 50 μl).
(14) Dry the pellet *in vacuo*.
(15) Dissolve the pellet in water (50 μl).
(16) Dilute with aqueous urea (8 M, 150 μl).
(17) Purify the RNA by 20% denaturing PAGE (20-cm gel for short oligos up to 20 nt in length, 20 h at 400–600 V or three-quarters the length of the gel; 40-cm gel for longer oligos up to 50 nt in length, up to 72 h at 600 V or less time if higher voltage, e.g. 1200–1800 V, can be used).
(18) Yields typically range from 100–170 nmol for one-quarter of a 1.0-μmol synthesis, depending on the length and quality of the RNA synthesis.

To monitor the extent of labeling:

(1) Remove an aliquot (1.0 μl) of the reaction mixture from step 15 of the above protocol.
(2) Dilute with water (19 μl).
(3) Wash with chloroform (2 × 75 μl) to remove excess labeling reagent.
(4) Analyze the reaction by one of the following three methods:
    (a) Reversed-phase HPLC on an analytical column (C_{18}, 4.6 × 250 mm, 5-μm column) at 1.5 ml/min using the following protocol: solvent A, 50 mM Et_3NHOAc (pH 7.0); solvent B, 70% CH_3CN/30% of 50 mM Et_3NHOAc (pH 7.0); 15-min linear gradient from 0 to 23% B, 5-min linear gradient to 100% B, isocratic for 10 min, 3-min linear gradient to initial conditions, 15 min equilibrium time between runs. A representative example is given

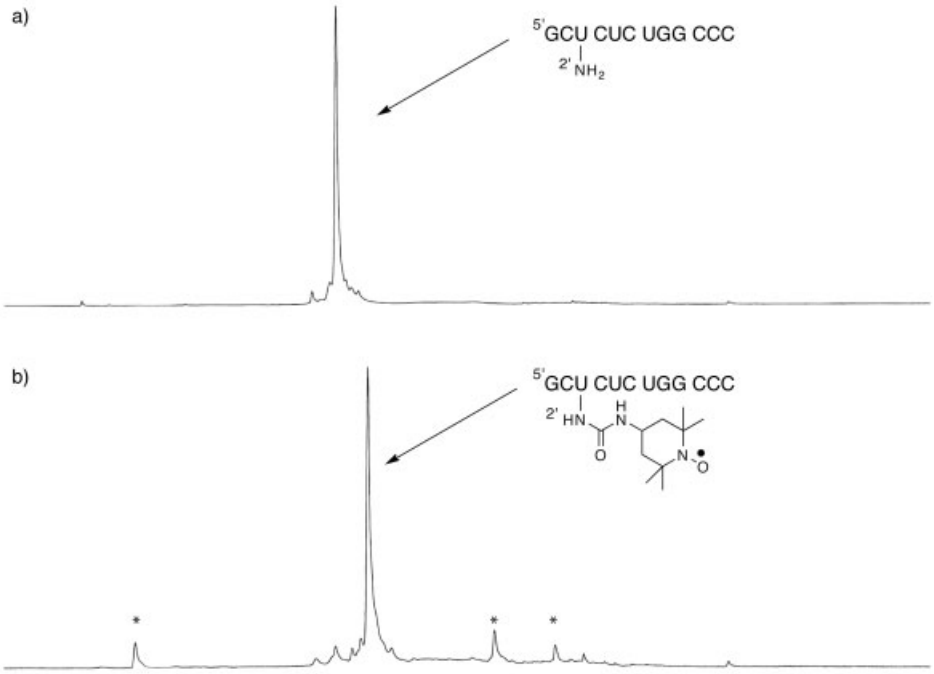

Fig. 8.4. HPLC analysis of 5′-GC(2′-NH_2 U) CUC UGG CCC before (a) and after (b) reaction with **4** which shows 95% conversion to the labeled RNA of later retention time. The asterisks correspond to **4** and its hydrolysis products. HPLC chromatograms were obtained at 260 nm using an analytical column (C18, 4.6 × 250 mm, 5-μm column) run at 1.5 ml/min according to the following protocol: solvent A, 50 mM Et_3NHOAc (pH 7.0); solvent B, 70% CH_3CN/30% of 50 mM Et_3NHOAc (pH 7.0); 15-min linear gradient from 0 to 23% B, 5-min linear gradient to 100% B, isocratic for 10 min, 3-min linear gradient to initial conditions, 15 min equilibrium time between runs.

in Fig. 8.4, which shows the reaction of **4** with 5′-GC(2′-NH_2 U) CUC UGG CCC.

(b) 20% denaturing PAGE (20-cm gel, 400 V for 3.5 h) by UV shadow visualization.

(c) Analytical ion exchange HPLC on a Dionex DNA Pac PA-100 4 × 250 mm analytical column heated at 50 °C by a column warmer. Separation will not be achieved without heating the column. Solvent gradients for analytical IE-HPLC were run at 1.0 ml/min as follows: solvent A, 25 mM Tris–HCl, pH 8.0; Solvent B, 1.0 M NaCl, 25 mM Tris–HCl, pH 8.0; 35-min linear gradient from 10% B to 80% B, 5-min linear gradient to 10% B.

Short labeled RNAs (up to 20 nt in length) can also be purified utilizing these HPLC protocols, although we recommend 20% denaturing PAGE purification, since the hydrolysis products of some isocyanates may co-elute with the labeled RNA using RP-HPLC.

8.3.3
Post-synthetic Labeling of 4-Thiouridine-modified RNA

If one knows *a priori* that modification of the 2′-hydroxyl group will likely interfere with biological function (e.g. 2′-OH is involved in an essential hydrogen bond), it may be necessary to label using an alternative post-synthetic labeling strategy. In this case, another simple, straightforward method is the labeling of 4-thiouridine residues with iodoacetamides [69, 70] or sulfur-based compounds [71]. One of the advantages of this method is that the labeling reaction can be followed by monitoring the consumption of UV signal at 320 nm, which corresponds to the thiocarbonyl. This labeling strategy changes the base-pairing properties of this residue. However, UV thermal denaturation melting temperature and hypochromicity data as well as NMR structural data indicate that 4-thiouridine residues can be labeled in this manner without disruption of helical stacking [71]. Labeling of the 4-amine group of cytidine with a crudely analogous modification, however, resulted in severe thermal instability of DNA [72]. Therefore, caution should be exercised when choosing such a labeling strategy for base-pairing residues.

Labeling of 4-thiouridine with the iodoacetamide spin-labeling reagent 3-(2-iodoacetamido)-proxyl 5 (modified procedure from that reported in [70])

(1) Dissolve 4-thiouridine-modified RNA (one-quarter of a 1-μmol synthesis) in 166 μl of buffer (100 mM sodium phosphate, pH 8).
(2) Acquire UV spectrum of an aliquot of the above mixture, monitoring at 260 and 320 nm.
(3) Dissolve 6 mg of 3-(2-iodoacetamido)-proxyl, **5** (Sigma) into 14 μl of ethanol and 20 μl of anhydrous DMF (0.5 M labeling reagent).
(4) Mix **5** and 4-thiouridine-modified RNA; final concentrations: around 1 mM RNA, 85 mM **5**, 83% phosphate buffer/7% ethanol/10% DMF (v/v/v).
(5) Due to light sensitivity of 4-thiouridine residues, cover samples with aluminum foil.
(6) Vortex vigorously until absorbance at 320 nm disappears (typically 18–28 h).
(7) Once the reaction is complete, precipitate and purify RNA as described above.

8.3.4
Verification of Label Incorporation

Whenever a modification is introduced into RNA, either by solid-phase chemical synthesis using a phosphoramidite or by post-synthetic modification, several steps are necessary to verify that the incorporation was successful. Not all modifications are incorporated as intended. For example, the 5-trifluoromethyl-2′-deoxyuridine phosphoramidite was prepared for the purpose of ^{19}F-NMR spectroscopy of nucleic acids; however, standard oligonucleotide deprotection conditions converted the 5-trifluoromethyl group to a 5-cyano group, prompting the use of alternate mild deprotection conditions [73]. Incorporation of the modified nucleoside should

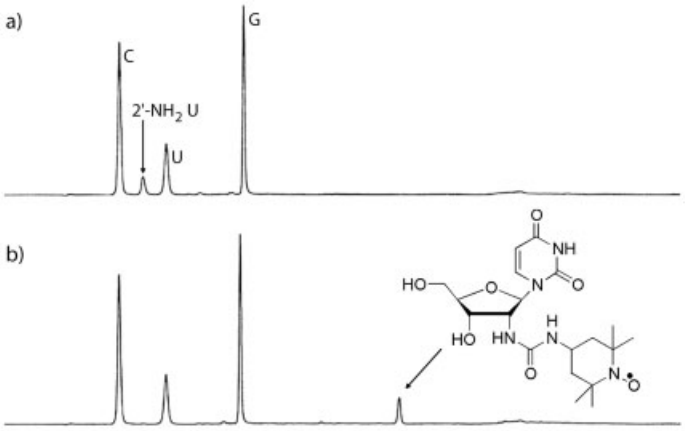

Fig. 8.5. HPLC analysis of enzymatically digested RNA (a) enzymatic digestion of 5′-GCU C(2′-NH_2 U)C UGG CCC; (b) enzymatic digestion of the product of the 2′-NH_2-modified oligonucleotide from a after reaction with isocyanate **4**. HPLC chromatograms were obtained as in Fig. 8.4.

be verified by mass spectrometry and enzymatic digestion in conjunction with RP-HPLC analysis. The RNAs (2.0 nmol or around 0.2 OD_{260}) should be digested with snake venom phosphodiesterase (0.5 U) and calf intestinal alkaline phosphatase (8 U) at 37 °C for 5 h in 5 mM Tris–HCl, pH 7.4 (20 µl) and then analyzed by analytical RP HPLC using the same protocol as that listed above for monitoring the extent of labeling. For example, HPLC analysis of the enzymatic digestion of 5′-GCU C(2′-NH_2 U)C UGG CCC resulted in peaks corresponding to C, 2′-NH_2 U, U and G (Fig. 8.5a), whereas after labeling with the spin-label isocyanate **4** HPLC analysis revealed the absence of the 2′-NH_2 U peak and the presence of a new peak (Fig. 8.5b) that co-eluted with the expected modified spin-labeled nucleoside prepared by chemical synthesis [37].

8.3.5
Potential Problems and Troubleshooting

It is always important to determine if the modification interferes (intentionally or unintentionally) with the structure and function of the molecule using a standard structural (e.g. UV thermal denaturation and/or other biophysical spectroscopy or crystallography) and functional (binding or enzymatic) assay. For example, the effect of incorporation of nitroxide spin-labels at the 2′-position on RNA has been investigated by UV thermal denaturation [37], whereas their effect on RNA–protein complex formation was investigated by electrophoretic mobility shift analysis [38].

If the labeling reaction does not work or the yields of the labeling reactions are low, this is generally a result of one of four problems.

(1) The isocyanate may be hydrolyzed or not prepared properly. The quality of the isocyanate can be determined by spectroscopic methods (e.g. NMR) and/or tested by reaction with a simple aliphatic amine such as benzylamine (30 min in CH_2Cl_2 at room temperature).
(2) The 2′-trifluoroacetyl protecting group may not have been fully removed, which may not be readily apparent because 2′-trifluoroacetamido and 2′-amino modified RNAs often have similar mobility on HPLC or in gels. However, this can be readily investigated by enzymatic digestion of the RNA, followed by HPLC analysis as described above. For example, if the 2′-trifluoroacetyl group is not fully removed a new peak will be observed by HPLC analysis with a retention time of around 5 min corresponding to the 2′-trifluoroacetamido uridine nucleoside (e.g. in the order of C, 2′-NH_2 U, U, 2′-$NHCOCF_3$ U, G, A).
(3) If the temperature of the reaction is not low enough, the yields are lower, presumably because of the competing hydrolysis of the isocyanate. Therefore, it is important to monitor the temperature of the ice-salt bath with a thermometer.
(4) Lower yields will be obtained if the RNA is not completely dissolved at the beginning of the reaction.

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