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Research Article

Synthesis of Specifically Modified Oligonucleotides for Application in Structural and Functional Analysis of RNA

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Nowadays, RNA synthesis has become an essential tool not only in the field of molecular biology and medicine, but also in areas like molecular diagnostics and material sciences. Beyond synthetic RNAs for antisense, aptamer, ribozyme, and siRNA technologies, oligoribonucleotides carrying site-specific modifications for structure and function studies are needed. This often requires labeling of the RNA with a suitable spectroscopic reporter group. Herein, we describe the synthesis of functionalized monomer building blocks that upon incorporation in RNA allow for selective reaction with a specific reporter or functional entity. In particular, we report on the synthesis of 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilyl protected 3′-*O*-phosphoramidites of nucleosides that carry amino linkers of different lengths and flexibility at the heterocyclic base, their incorporation in a variety of RNAs, and postsynthetic conjugation with fluorescent dyes and nitroxide spin labels. Further, we show the synthesis of a flavine mononucleotide-N-hydroxy-succinimidyl ester and its conjugation to amino functionalized RNA.

1. Introduction

Over the past two decades, RNA synthesis has become a very active field. Synthetic RNAs are required for a large number of applications. Antisense oligonucleotides, ribozymes, aptamers, and siRNAs are required for medicinal diagnostic and therapy as well as for a wide variety of biochemistry and molecular biology studies [1, 2]. Furthermore, aptamers and reporter ribozymes are designed and applied in environmental diagnostics [3]. A number of other disciplines make use of synthetic oligoribonucleotides, for example, the field of material science, where novel materials from nanoparticle oligonucleotide conjugates are generated [4]. In parallel, RNA synthesis has developed to a degree that allows the synthesis of RNA oligonucleotides of any desired sequence from microgram to multigram scale. In addition to 2′-*O*-TBDMS chemistry, which may be considered as the standard procedure for RNA preparation, novel strategies mainly focussing on different 2′-*O*-protecting groups such as 2′-*O*-TOM, 2′-*O*-ACE or 2′-*O*-TC are available for laboratory use applying the specific monomer building blocks, or have been commercialized for custom RNA synthesis [5, 6].

Research in our laboratory is devoted to the chemistry and biochemistry of RNA with a strong focus on RNA aptamers and ribozymes. Therefore, we synthesize natural and modified RNA strands required for the design of functional RNAs that we want to investigate. Furthermore, we also prepare RNA molecules carrying site-specific modifications such as fluorescent dyes or other reporter groups to be used for studies into RNA structure. A number of monomer building blocks for the synthesis of modified RNA are commercially available. For example, amino linkers for 5′- and/or 3′-terminal labeling can be obtained ready for use in RNA assembly (Figure 1). Furthermore, 5′-*O*-DMT-2′-*O*-TBDMS protected 3′-*O*-phosphoramidites of 2′-aminouridine (**3**) and 4-thiouridine (**4**) (Figure 1) are available and after incorporation in RNA can be used for the specific attachment of desired molecular entities.

However, monomer building blocks of the purine nucleosides with functionalities suitable for postsynthetic conjugation are completely missing, and also in the pyrimidine series, the few existing derivatives of uridine do not offer much variety. Therefore, a currently very active part of our

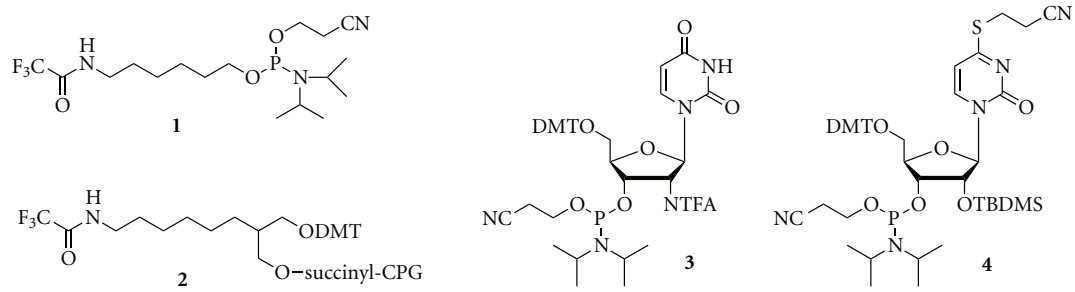


Figure 1: Structures of the commercially available building blocks used in our RNA functionalization studies.

research is focussed on the synthesis of 5′-*O*-DMT-2′-*O*-TBDMS protected 3′-*O*-phosphoramidites of nucleosides that carry functionalized linkers of different lengths and flexibility at the heterocyclic base, in particular at C5 of the pyrimidines as well as at C8 and C2 of the purines (Figure 2). Moreover, we currently work at the synthesis of modified nucleosides as they appear in tRNA as well as of isotope enriched nucleosides and their incorporation in specific RNA sequences (which will be reported elsewhere).

Herein, we focus on the synthesis of amino-modified monomers, their incorporation into RNA, and the postsynthetic labeling with fluorescent dyes. Furthermore, we report on strategies for preparation of specifically spin labeled RNAs for EPR studies, and on the preparation of a flavinmononucleotide (FMN) derivative that is postsynthetically attached to an amino-modified hairpin ribozyme derivative. The modified RNAs described herein are used in collaboration with spectroscopists for structural analysis of functional RNAs, such as RNA thermometers [7] and RNA four-way junctions [8] or riboswitches [9], or in RNA engineering projects like the design of an FMN-dependent aptazyme [10].

2. Materials and Methods

2.1. General Information. All reactions were carried out in dry solvents under argon atmosphere. All solvents and reagents were purchased from commercial sources and used as supplied unless otherwise stated. The solvents used in Pd coupling reactions were freed from oxygen. All products were visualized on TLC plates (aluminium sheets coated with silica gel 60 F 254, 0.2 nm thickness) at 254 nm ultraviolet light. The linker molecules L1, L2, and L3 (Figure 3) were synthesized according to the literature: the alkinyl linker L1 was synthesized according to Cruickshank and Stockwell [11], and alkenyl linkers were synthesized as described by Dey and Sheppard [12] and McKeen et al. [13]. All modified phosphoramidites were freshly prepared prior to use in RNA synthesis. Progress of reaction was monitored by TLC (hexane/ethyl acetate 1 : 1 containing 1% NEt_3). Phosphoramidites were coevaporated several times with dry DCM and kept under vacuum over P_2O_5 in a desiccator overnight. A 0.1 M solution of the modified phosphoramidite in anhydous acetonitril was freshly prepared right before use in solid-phase RNA synthesis.

2.2. MALDI-TOF Analysis. For nucleoside derivatives: 1-2 mg sample was dissolved in 100 μL methanol or water. 1.5 μL of the sample solution and 1.5–3.0 μL of the matrix solution (30–40 mg 3-hydroxypicolinic acid (3-HPA) in 500 μL MeCN/H_2O 1 : 1, vigorously mixed for 30 s) were mixed, and 1-2 μL of that mixture was loaded on a MALDI-plate, dried, and allowed to crystallize. The sample was analyzed on a Bruker microflex mass spectrometer. The program was set as reflective positive mode with 40–50 shoots/measurement. The laser intensity was adjusted such that the intensity of the desired signals was about 1000.

For RNA: RNA samples were desalted by gel filtration on Sephadex G25 fine (GE Healthcare). After desalination, samples were dissolved in autoclaved micropore water to a concentration of about 100–500 pmol/μL. 1 μL of this RNA solution was mixed with 1-2 μL of matrix solution (30–40 mg 3-hydroxypicolinic acid (3-HPA) in 500 μL MeCN/H_2O 1 : 1, vigorously mixed for 30 s) and treated with cation-exchange beads (Dowex 50 WX8, NH_4^+-form, 100–200 mesh, ServaFeinbiochemica). Therefore, 4–7 μL suspension of the beads in autoclaved micropore water was pipetted in a 250 μL tube, and water was removed, followed by addition of the sample and matrix solutions. The mixture was left at rt for 10 min then loaded on the MALDI-plate and dried on air. The sample was measured using the linear negative (or positive) mode with 40–50 shoots/measurement. In addition to the main peak $[M-1]^-$ (negative mode) or $[M+1]^+$ (positive mode), occasionally the signals corresponding to $[M-2]/2$ were observed.

2.3. Synthesis of Amino-Modified Monomer Building Blocks. Amino linker-modified uridine derivatives were synthesized as described [14]. The detailed synthesis of linker-modified adenosine derivatives with focus on the problem of double bond isomerization will be reported elsewhere (H. Nguyen, B. Appel, and S. Müller, manuscript in preparation).

2.4. 8-(3-Trifluoroacetamidoprop-1-ynyl)guanosine 16. 8-Bromoguanosine-hydrate **15** (98%) (1448.4 mg. 4.0 mmol) was dissolved in dry DMF (32 mL). Traces of dissolved oxygen in the solution were removed by iterative cycles of reduced pressure and passing in argon (3 times). Then, $Pd(Ph_3P)_4$ (464 mg, 0.4 mmol, 0.1 eq.) was added. The solution was again kept under vacuum and then saturated with argon. DIPEA (816 μL, 4.8 mmol, 1.2 eq., freshly

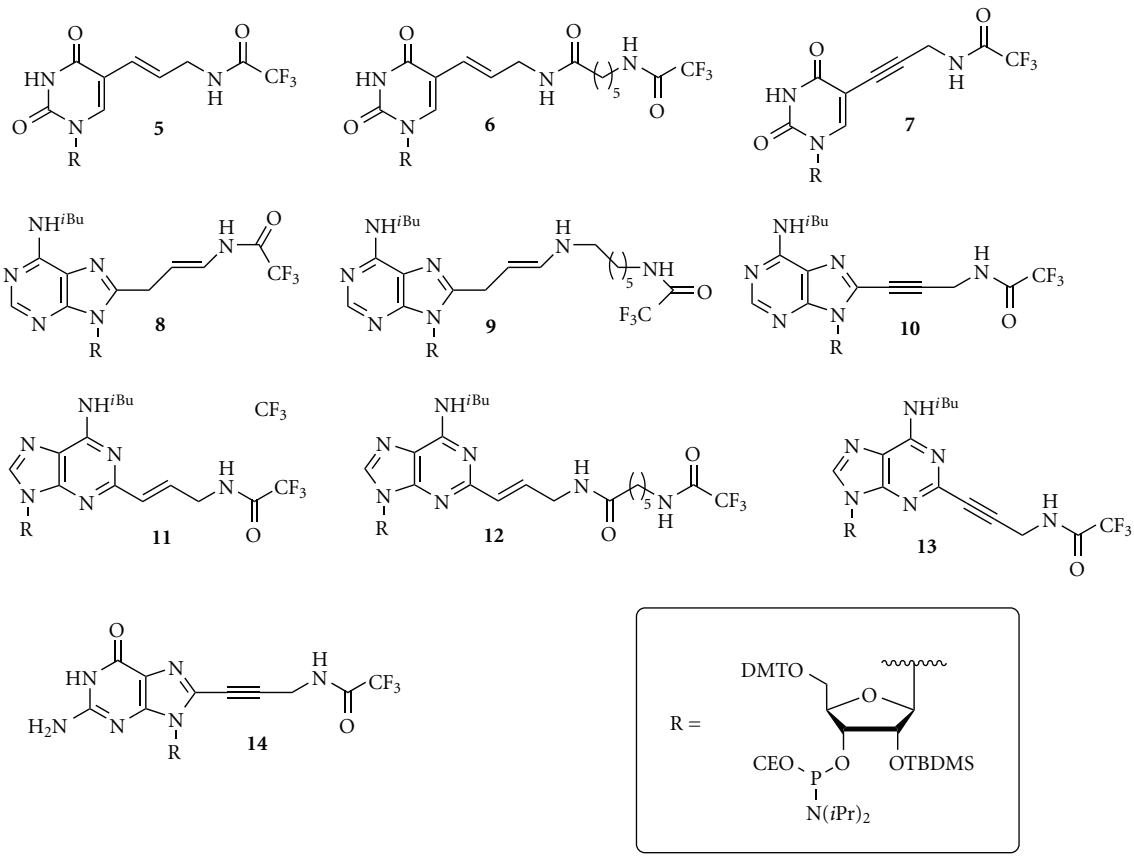


Figure 2: Amino-modified monomer building blocks used for RNA synthesis and functionalization.

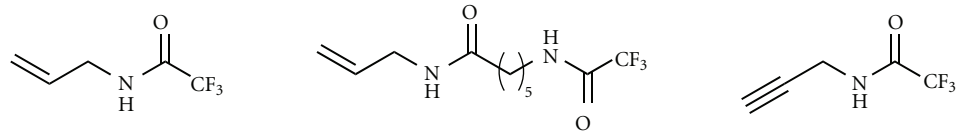


Figure 3: Amino linkers used for preparation of amino-modified nucleosides.

distilled over CaH_2) was added via a syringe, followed by the addition of *N*-propynyltrifluoroacetamide L3 (1208 mg, 8.0 mmol, 2.0 eq.) and CuI (152 mg, 0.8 mmol, 0.2 eq.). The mixture was stirred at 40°C for 20 hours. The solvent was then removed to give a brown viscous oil which was dissolved in DCM/MeOH 1 : 1 (v/v) and filtered to remove the precipitate. The solid residue was purified by silica gel column chromatography (DCM/MeOH 90 : 10 to 84 : 16 (v/v)) to give a pale yellow solid. Yield: 2765.4 mg (6.4 mmol, 80%). R_f (SiO_2; DCM/MeOH 60 : 40 (v/v)): 0.80. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.50 (1 H, m, H-5′), 3.64 (1 H, m, H-5″), 3.84 (1 H, m, H-4′), 4.12 (1 H, m, H-3′), 4.36 (2 H, d, J 5.4, CH_2), 4.88 (2 H, m, H-2′ and OH), 5.04 (1 H, d, J 4.8, OH), 5.40 (1 H, d, J 6.0, OH), 5.76 (1 H, d, J 6.3, H-1′), 6.57 (2 H, br s, NH_2), 10.20 (1 H, t, J 5.4, NH), 10.90 (1 H, br s, NH). ^{13}C NMR (300 MHz, DMSO-d_6): δ (ppm) = 29.40, 62.06, 70.47, 70.85, 72.75, 85.77, 88.38, 89.12, (110.03, 113.84, 117.65 and 121.47, q, J 288.02, CF_3), 117.24, 129.04, 150.90, 154.12, (155.54, 156.03, 156.51 and 157.00, q, J 36.76, (C=O)CF_3), 156.24. MALDI-TOF: $C_{15}H_{15}F_3N_6O_6$, calculated 432.10, found 432.82 $[M+H]^+$.

2.5. N-2-Isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosine 17. 8-(3-Trifluoroacetamido-prop-1-ynyl)guanosine **16** (216.5 mg, 0.50 mmol) was coevaporated with anhydrous pyridine (3 × 4 mL), and then dissolved in anhydrous pyridine (2 mL). The resulting solution was protected from moisture (drying tube), purged with argon and placed on ice. To the ice cold solution, TMS-Cl (0.55 mL, 4.1 mmol, 8.2 eq.) was added dropwise via a syringe. The ice bath was then removed and the mixture was stirred for 2 hours. The solution was cooled on ice and isobutyric anhydride (0.13 mL, 1.1 mmol, 2.2 eq.) was added dropwise via a syringe and the

ice bath was removed. After stirring for another 2 hours at room temperature, the reaction was placed again on ice and ice cold water (20 mL) was slowly added, followed after 15 minutes by concentrated ammonia solution (1.5 mL) to get a final 2.5 M concentration of ammonia. The mixture was kept on ice for 30 minutes, and then evaporated to dryness. The residue was coevaporated with toluene (3 × 5 mL) to remove traces of water, resuspended in MeOH and filtered to remove the precipitate. The filtrate was then concentrated, dissolved in a small amount of MeOH, absorbed on silica gel and purified by column chromatography (DCM/MeOH 95 : 5 to 91 : 9 (v/v)) to give *N*-2-isobutyryl-8-(3trifluoroacetamidoprop-1-ynyl)guanosine as a yellow solid. Yield: 183.3 mg (0.37 mmol; 73%). R_f (SiO_2; DCM/MeOH 80 : 20 (v/v)): 0.63, ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm)= 1.13 (6 H, d, J 6.9, $CH(CH_3)_2$), 2.79 (1 H, sept, J 6.9, $CH(CH_3)_2$), 3.53 (1 H, m, H-5′), 3.68 (1 H, m, H-5′′), 3.84 (1 H, m, H-4′), 4.17 (1 H, br s, H-3′), 4.40 (2 H, s, CH_2), 4.81 (1 H, br s, H-2′), 4.91 (1 H, br s, OH), 5.09 (1 H, br s, OH), 5.50 (1 H, br s, OH), 5.86 (1 H, d, J 6.0, H-1′), 10.21 (1 H, br s, NH), 11.62 (1 H, br s, NH), 12.16 (1 H, br s, NH). MALDI-TOF: $C_{19}H_{21}F_3N_6O_7$, calculated 502.14, found 502.84 $[M+H]^+$.

2.6. 5′-O-(4,4′-Dimethoxytrityl)-N-6-isobutyryl-8-(3-trifluoroacetamidoprop1ynyl)guanosine 18. *N*-2-Isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosine **17** (1174.4 mg, 2.4 mmol) was coevaporated with anhydrous pyridine (3 × 3 mL) and dissolved in anhydrous pyridine (10.0 mL). DMT-Cl (1219.8 mg, 3.6 mmol, 1.5 eq.) was dissolved in anhydrous pyridine (3.0 mL) and added dropwise to the ice cold solution of the nucleoside over 2.5 hours. After 6 hours at ice cold temperature, TLC analysis (DCM/MeOH 95 : 5(v/v)) showed that the starting material was completely consumed. Methanol (1.0 mL) was added to quench the unreacted DMT-Cl, and after 10 minutes, the solution was concentrated to dryness *in vacuo*. The residue was dissolved in DCM (100 mL), washed with a 5% aqueous solution of $NaHCO_3$ (2 × 20 mL) and water (2 × 20 mL). The organic layer was dried over Na_2SO_4 and concentrated to give a yellow gum which was purified by column chromatography (DCM/MeOH 97 : 3 to 95 : 5 (v/v) containing 1% triethylamine) to give a pale yellow foam. Yield: 1671.6 mg (2.08 mmol, 87%). R_f (DCM/MeOH 90 : 10 (v/v)): 0.44.^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 1.11 (6 H, 2 × d, J 6.9 and 6.6, $CH(CH_3)_2$), 2.72 (1 H, sept, J 6.9, $CH(CH_3)_2$), 3.11 and 3.45 (2 H, m, H-5′ and H-5′′), 3.69 and 3.71 (6 H, 2 × s, 2 × OCH_3), 4.04 (1 H, m, H-4′), 4.31 (3 H, br s, H-3′ and CH_2), 4.91 (1 H, m, H-2′), 5.04 (1 H, br s, OH), 5.61 (1 H, br s, OH), 5.94 (1 H, d, J 4.5, H-1′), 6.73 (4 H, 2 × d, J 8.7 and 9.0, aromatic), 7.23 (9 H, m, aromatic), 10.85 (3 H, br s, 3 × NH). ^{13}C NMR (300 MHz, DMSO-d_6): δ (ppm) = 18.71, 18.94, 29.33, 34.80, 54.86, 54.91, 64.72, 70.49, 71.36, 72.06, 84.21, 85.21, 89.91, 90.49, (110.02, 113.82, 117.65 and 121.47, q, J 288.5, CF_3), 112.73, 112.82, 120.83, 126.45, 127.47, 127.78, 129.64, 129.80, 131.52, 135.58, 144.87, 148.24, 154.33, (155.55, 156.05, 156.53 and 157.03, q, J 37.0, (C=O)CF_3), 157.88, 157.95, 180.09. MALDI-TOF: $C_{40}H_{39}F_3N_6O_9$, calculated 804.27, found 805.24 $[M+H]^+$.

2.7. 5′-O-(4,4′-Dimethoxytrityl)-2′-(tert-butyldimethylsilyl)-N-2-isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosine 19. 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-2-isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosine **18** (1623 mg, 2.02 mmol) was coevaporated with anhydrous pyridine (2 mL × 2), dry DCM (2 × 5 mL), kept under vacuum overnight, and then dissolved in anhydrous THF (20 mL). Anhydrous pyridine (606 μL, 7.47 mmol, 3.7 eq.) and $AgNO_3$ (505 mg, 3.0 mmol, 1.5 eq.) were added. The reaction mixture was stirred for 15 minutes to give a white emulsion, and TBDMS-Cl (528 mg, 3.43 mmol, 1.7 eq.) was added. The reaction was protected from moisture and stirred at room temperature until TLC (SiO_2, hexane/ethyl acetate 1 : 1 (v/v)) showed no starting material left (8.5 hours). The reaction mixture was diluted with ethyl acetate (50 mL), filtered to remove the precipitate and washed with a saturated solution of $NaHCO_3$ (2 × 20 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a white gum which was then purified by silica gel column chromatography (hexane/ethyl acetate 85 : 15 to 40 : 60 (v/v)) to give a pale yellow foam. Yield: 482.3 mg (0.53 mmol, 26%). R_f (SiO_2; hexane/ethyl acetate 1 : 1 (v/v) containing 4% MeOH): 0.41. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = −0.14 and −0.02 (6 H, 2 × s, $Si(CH_3)_2$), 0.76 (9 H, s, $SiC(CH_3)_3$), 1.09 (6 H, 2 × d, J 6.6 and 6.9, $CH(CH_3)_2$), 2.70 (1 H, sept, J 6.6 and 6.9, $CH(CH_3)_2$), 3.19 (1 H, m, H-5′), 3.51 (1 H, m, H-5′′), 3.67 and 3.69 (6 H, 2 × s, 2 × OCH_3), 4.09 (1 H, m, H-4′), 4.20 (1 H, br m, H-3′), 4.38 (2 H, br d, J 5.1, CH_2), 4.79 (1 H, m, H-2′), 5.95 (1 H, d, J 4.8, H-1′), 6.75 (4 H, 2 × d, J 8.7, aromatic), 7.27 (9 H, m, aromatic), 10.16 (1 H, t, J 5.1 and 5.4, NH), 11.38 (1 H, br s, NH), 12.14 (1 H, br s, NH). 13C NMR (300 MHz, DMSO-d_6): δ (ppm) = −5.40, −4.91, 17.81, 18.63, 18.88, 25.47, 29.24, 34.77, 54.89, 54.93, 64.49, 70.16, 71.95, 73.74, 84.26, 85.30, 90.66, (109.97, 113.79, 117.61 and 121.43, q, J 288.40, CF_3), 112.82, 112.87, 126.51, 127.55, 127.76, 129.70, 129.78, 135.50, 135.58, 144.86, 148.52, 154.10, (155.54, 156.03, 156.52 and 157.01, q, J 37.0, (C=O)CF_3), 157.93, 157.98, 180.07. MALDI-TOF: $C_{46}H_{53}F_3N_6O_9Si$, calculated 918.36, found 919.19 $[M+H]^+$.

2.8. 5′-O-(4,4′-Dimethoxytrityl)-2′-(tert-butyldimethylsilyl)-N-2-isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosinephosphoramidite 14. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-(*tert*-butyldimethylsilyl)-*N*-2-isobutyryl-8-(3-trifluoroacetamidoprop-1-ynyl)guanosine **19** (67.96 mg, 0.11 mmol) was coevaporated with anhydrous pyridine (2 × 5 mL), dry DCM (2 × 5 mL), kept under vacuum overnight, and then dissolved in anhydrous DCM (2 mL). To the resulting solution, DIPEA (75 μL, 0.44 mmol, 4 eq.) was added, followed by 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (37 μL, 0.16 mmol, 1.5 eq). The solution was stirred at room temperature. The reaction was followed by TLC (EtOAc : hexane 1 : 1 containing 1% TEA). After 2.5 hours, the reaction was stopped and washed with a saturated solution of $NaHCO_3$ (10 mL). The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography. The column was

packed with DCM/TEA (98 : 2), the residue was dissolved in 1-2 mL DCM/TEA (98 : 2), and the column was loaded. After 30 mL of DCM/TEA (98 : 2), the solvent was changed to DCM/TEA/MeCN (49 : 1 : 50, 50 mL). The fractions containing the desired product were collected, and the solvent was removed under reduced pressure. The product was lyophilized overnight. ^{31}P-NMR (300 MHz, DMSO-d_6), two diastereomers: δ (ppm) = 148.98, 149.71.

2.9. RNA Synthesis. Oligoribonucleotides were synthesized by the phosphoramidite method on a Pharmacia Gene Assembler Plus, at 1 μmol scale as described elsewhere [15]. Standard PAC-phosphoramidites as well as CPG supports were obtained from ChemGenes or Link Technologies. BMT (emp Biotech) was used as activator [16]. The modified nucleoside phosphoramidites were coevaporated three times with dry dichloromethane, kept under vacuum for 2 hours to remove traces of solvents, stored in vacuum over P_2O_5 overnight, and used in oligo synthesis as 0.1–0.15 M solution in acetonitrile. The solutions of phosphoramidites, BMT, and MeCN were kept over molecular sieve 0.3 nm. The coupling time for all natural and modified building blocks was 5 min. All syntheses were carried out "trityl-off". The obtained RNA was cleaved from the support and deprotected using NH_3 (30%) and ethanolic methylamine in a 1 : 1 mixture and TEAx3HF as described [17] and purified by gel electrophoresis using 10% denaturing polyacrylamide gels. Elution was carried out using 0.5 M LiOAc followed by EtOH precipitation. Oligonucleotides were analyzed by PAGE and MALDI MS.

2.10. Postsynthetic Labeling of Oligoribonucleotides with Alexa488, Cy5 and ATTO647N. 5 to 10 nmol amino-modified RNA were dissolved in 15–25 μL 0.1 M Borax buffer, at pH 9.4 for Alexa488 coupling reactions, and at pH 8.7 for Cy5 and ATTO647N coupling reactions. The mixture was added to a solution of 100 μg dye in 5 μL DMF (the solution of the dye in DMF was freshly prepared just before use). The coupling reaction was carried out at room temperature overnight in the dark. Excess of dye was removed via gel filtration (Sephadex G25 fine, GE Healthcare). The Alexa488 and Cy5 labeled RNAs were purified by denaturing PAGE. The sample was denatured at 90°C for 3 minutes, immediately subjected onto a 15% polyacrylamide gel (200 × 150 × 1.5 mm), and electrophoresis was run at 400 V for 5.5 hours in the dark. The band corresponding to the labeled RNA was cleaved out; the RNA was eluted with 0.5 M lithium acetate and precipitated from ethanol at −20°C (yield 15–37% for Alexa488, 26–54% for Cy5). The ATTO647N labeled RNA was purified by reverse-phase HPLC. The RNA was solved in 1 mL water and filtered through a 0.45 μL filter before subjecting onto the column. Column EC 250/4 Nucleodur 100-5 C18 ec (Macherey-Nagel), flow rate 0.5 mL/min, Buffer A 0.1 M TEAAc (pH 7), 5% MeCN, Buffer B 0.1 M TEAAc (pH 7), 70% MeCN, Gradient 0% B for 5 min, 10–55% B in 76 min, 55–100% B in 19 minutes(yield 40–50%).

2.11. Hybridization of Single-Stranded RNAs for Structural Studies on RNA Thermometer

Condition A. For the hybridization of unlabeled single-stranded oligonucleotides, D1L3 (300 pmol) and A1L3 (300 pmol) were dissolved in 50 mM Tris buffer, pH 7.4 in the presence of 10 mM $MgCl_2$ in a total volume of 100 μL. The solution was then well mixed, incubated at 78°C for 3 minutes, centrifuged to collect the vapor on the cap of the eppendorf tube, wrapped in aluminium film, and slowly cooled down to room temperature. The hybrid was analyzed by 15% native polyacrylamide gel.

Condition B. Alexa Fluor 488-labeled D1L3 (25 pmol) and Cy5-labeled A1L3 (25 pmol), were dissolved in 20 mM KH_2PO_4-K_2HPO_4 buffer, pH 6.5 in the presence of 10 mM $MgCl_2$ and 100 mM KCl in a total volume of 50 μL. The components were then mixed, centrifuged and incubated at 78°C for 3 minutes. The mixture was slowly cooled down to room temperature in aluminium film. The hybridization was checked by 15% native polyacrylamide gel.

2.12. 5′-O-(4,4′-Dimethoxytrityl)riboflavin 21. To a suspension of riboflavin **20** (1.5 g, 4 mmol) in dry pyridine TEA (2 mL, 14.4 mmol), DMAP (24 mg, 0.2 mmol), and 4,4′-dimethoxytritylchloride (1.7 g, 5.2 mmol) were added under argon. After 4 hours of stirring at room temperature in the dark, the reaction was quenched with dry methanol (5 mL). The solvent was evaporated under reduced pressure. The residue was dissolved in DCM (100 mL) and filtered. The clear yellow filtrate was dried with Na_2SO_4 and the solvent was removed. Purification of the crude product by column chromatography with DCM/MeOH (98 : 2) gave the tritylated product **21** as an orange solid. Yield: 1.4 g (2.1 mmol, 53%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.39 (s, 3 H, CH_3), 2.41 (s, 3 H, CH_3), 3.20–3.17 (m, 2 H), 3.72 and 3.73 (2 × s, 2 × 3 H, 2 × OCH_3), 3.92 (br s, 1 H), 4.23 (br s, 1 H), 4.62 (m 1 H), 4.98 (m, 1 H), 6.85 (m, 4 H, DMT), 7.46–7.16 (m, 9 H, DMT), 7.83 (s, 1 H), 7.90 (s, 1 H), 11.34 (s, 1 H, NH). ESI-MS: $C_{38}H_{38}N_4O_8$, calculated 678.27, found 677.26 $[M-H]^-$.

2.13. 2′,3′,4′-Tri-O-acetyl-5′-O-(4,4′-dimethoxytrityl)riboflavin 22. 5′-*O*-(4, 4′-Dimethoxytrityl)-riboflavin **21** (1.4 g, 2.06 mmol) in dry pyridine (200 mL) was cooled in an ice bath under argon atmosphere. After dropwise addition of acetic anhydride (7.6 mL, 80 mmol), the solution was stirred in the dark at room temperature overnight. Then, methanol (5 mL) was added, and the solvent was evaporated. Column chromatography with DCM/MeOH (98 : 2) gave the title compound **22** as orange foam. Yield: 1.5 g (1.86 mmol, 90%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 1.49 (s, 3 H, acetyl-CH_3), 2.02 (s, 3 H, acetyl-CH_3), 2.27 (s, 3 H, acetyl-CH_3), 2.39 (s, 3 H, CH_3), 2.46 (s, 3 H, CH_3), 3.12–3.05 (m, 1 H), 3.29–3.22 (m, 1 H), 3.73 (2 × s, 2 × 3 H, 2 × OCH_3), 5.16–4.79 (br m, 2 H), 5.24 (m, 1 H), 5.54 (m, 2 H), 6.88 (m, 4 H, DMT), 6.37–7.20 (m, 9 H, DMT), 7.64 (s, 1 H), 7.89 (s, 1 H), 11.40 (s, 1 H, NH), ESI-MS: $C_{44}H_{44}N_4O_{11}$, calculated 804.30, found 803.29 $[M-H]^-$.

2.14. 2′,3′,4′-Tri-O-acetylriboflavin 23. 2′,3′,4′-Tri-*O*-acetyl-5′-*O*-(4,4′-dimethoxytrityl)riboflavin **22** (500 mg, 0.6 mmol) was dissolved in 20 mL nitromethane. To this solution, anhydrous $ZnBr_2$ (1.25 g, 5.6 mmol) was added. After 2 minutes, the reaction was quenched by addition of ammonium acetate (50 mL, 1 M in water). DCM (50 mL) were added, the organic layer was separated and washed with brine (2 × 20 mL), followed by evaporation under reduced pressure. The crude product was extracted several times with ice cold diethyl ether to remove residual DMT. In this way, compound **23** (250 mg, 0.5 mmol) were obtained as a yellow solid. The product was used for the next reaction without further purification. MALDI-TOF: $C_{23}H_{26}N_4O_9$, calculated 502.17, found 503.23 $[M+H]^+$.

2.15. 3-(Tetraethylene glycol)propionic Acid tert-Butyl Ester 25. Tetraethylene glycol **24** (38.8 g, 200 mmol) was dissolved in dry THF (80 mL). Under argon atmosphere, sodium (0.2 g, 8.7 mmol) was added. After all of the sodium had dissolved, *tert*-butylacrylate (7.7 g, 60 mmol) were added and the mixture was stirred at room temperature for 23 hours. The slightly yellow solution was neutralized with 1 M HCl and the solvents were evaporated under reduced pressure. The residue was redissolved in brine (80 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water and dried with sodium sulfate. After evaporation of the solvents, the oil was subjected to column chromatography on silica gel (DCM/MeOH 98 : 2) to give **25** as colorless oil. Yield: 6.65 g (20.6 mmol, 34%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.45 (s, 9 H, *tert*-butyl), 2.50 (t, 2 H, CH_2-COO-*tert*-Bu), 3.74–3.60 (m, 18 H, OCH_2CH_2O). MALDI-TOF: $C_{15}H_{30}O_7$, calculated 322.20, found 345.16 $[M+Na]^+$.

2.16. 3-(Tetraethylene glycol)propionic Acid tert-Butyl Ester Phosphoramidite 26. 3-(Tetraethylene glycol)propionic acid *tert*-butyl ester **25** (1 g, 3.1 mmol) was coevaporated three times with dichloromethane and then dissolved in 25 mL DCM under argon. To this solution, freshly distilled DIPEA (2.5 mL) and 2-cyanoethyl-*N*,*N*-diisopropropylchlorophosphoramidite (0.85 ml, 3.6 mmol) were added. After 1.5 hours, the reaction was stopped by addition of 1 mL methanol. The mixture was diluted with ethylacetate (20 mL) and TEA (1.5 mL) and washed with a saturated solution of $NaHCO_3$ (25 mL) and brine (25 mL). The organic layer was dried with Na_2SO_4, and the solvents were removed under reduced pressure. The crude product was coevaporated three times with dry DCM and stored overnight in an evacuated desiccator with calcium chloride. According to TLC analysis, the phosphoramidite was sufficiently pure to be used for the next reaction without further purification. ^{31}P NMR (300 MHz, $CDCl_3$), two diastereomers: δ (ppm) = 148.45, 149.37.

2.17. 5′-O-(3-(Tetraethylene glycol)propionic Acid tert-Butyl Ester-β-cyanoethylphosphoryl)-2′,3′,4′-tri-O-Acetylriboflavin 27. All starting materials were coevaporated separately with dry DCM. The riboflavine derivative **23** (100 mg, 0.2 mmol) was dissolved in dry MeCN (15 mL). To this solution, phosphoramidite **26** (136 mg, 0.26 mmol) in 2 mL dry MeCN and BMT (192 mg, 1 mmol) in 3 mL MeCN were added. The reaction mixture was stirred for 1 hour at room temperature. A solution of 0.2 M iodine in a mixture of THF/pyridine/H_2O (2 : 1 : 1) was added. After ten minutes, sodium bisulfite (5% in water) was added until the brown color had vanished. The mixture was diluted with DCM (40 mL). The organic layer was washed with sodium bicarbonate and brine and finally dried over sodium sulfate. The solvents were evaporated under reduced pressure. The residue was purified by reversed phase HPLC. Column VP125 Nucleosil 120-10 C18 ec (Macherey-Nagel), flow rate 0.5 mL/min, Eluent A 10% MeOH, Eluent B 70% MeOH, Gradient 0–70% B in 5 CV, 70–100% B in 3 CV, 100% B 3 CV. Yield: 59.5 mg (63 nmol, 32%). ^{1}H NMR (300 MHz, $CDCl_3$), two diastereomers: δ (ppm) = 1.44 (s, 9 H, *tert*-butyl), 1.79 (s, 3 H), 2.21 (2 × s, 3 H), 2.35 and 2.33 (2 × s, 3 H), 2.44 (s, 3 H), 2.50 (t, 2 H, CH_2-COO-*tert*-Bu), 2.57 (s, 3 H), 2.81 (m, 2 H, CH_2-CN), 3.74–3.60 (m, 18 H, OCH_2CH_2O), 4.40–4.21 (m, 6 H, P-O-CH_2), 5.33–4.89 (br, 2 H), 5.41 (m, 1 H), 5.50 (m, 1 H), 5.65 (m, 1 H), 7.60 and 7.59 (2 × s, 1 H), 8.02 (s, 1 H), 8.80 and 8.76 (2 × s, 1 H, NH). MALDI-TOF: $C_{41}H_{58}N_5O_{18}P$, calculated 939.35, found 962.62 $[M+Na]^+$.

2.18. 5′-O-(3-(Tetraethylene glycol)propionic Acid-β-cyanoethylphosphoryl)-2′, 3′, 4′-tri-O-Acetylriboflavin 28. The fully protected coupling compound **27** (25 mg, 26.6 nmol) was treated with 1 mL DCM/TFA (1 : 1) for 1 hour at room temperature. Subsequently, the solvents were evaporated under reduced pressure, and the residue was purified by reversed phase HPLC to give the free acid as an orange solid. Column VP125 Nucleosil 120-10 C18 ec (Macherey-Nagel), flow rate 0.5 mL/min, Eluent A 10% MeOH, Eluent B 70% MeOH, Gradient 0–70% B in 5 CV, 70–100% B in 3 CV, 100% B 3 CV. Yield 18.8 mg (21.3 nmol, 80%). ^{1}H NMR (300 MHz, DMSO-d_6), two diastereomers: δ (ppm) = 1.61 (s, 3 H), 2.20 (s, 3 H), 2.25 (s, 3 H), 2.41 (s, 3 H), 2.43 (t, 2 H, CH_2-COOH), 2.51 (s, 3 H), 2.93 (m, 2 H, CH_2-CN), 3.63–3.48 (m, 18 H, OCH_2CH_2O), 4.41–4.10 (m, 6 H, P-O-CH_2), 4.83 (d, 1 H), 5.18–5.00 (br, 1 H), 5.32 (m, 1 H), 5.48 (m, 2 H), 7.73 (s, 1 H), 7.90 (s, 1 H), 11.40 (s, 1 H, NH). MALDI-TOF: $C_{37}H_{50}N_5O_{18}P$, calculated 883.29, found 906.43 $[M+Na]^+$.

2.19. 5′-O-(3-(Tetraethylene glycol)propionic Acid Phosphoryl)riboflavin 29. In 4 mL of a 30% ammonia solution in methanol, the riboflavin derivative **28** (15 mg, 17 nmol) was dissolved and stirred at room temperature. After 30 min, MALDI analysis showed complete conversion to product. The solvents were evaporated, and the solid was taken up in 1 mL deionized water and subjected onto a short DOWEX column (H^+ form, 4 × 1.5 cm). The product was eluted with water and dried *in vacuo*. Yield: 11 mg (15.6 nmol, 92%). ^{1}H NMR: (600 MHz, DMSO-d_6): δ (ppm) = 2.40 (s, 3 H), 2.43 (t, 2 H, CH_2-COOH), 2.49 (s, 3 H), 3.61–3.47 (m, 16 H, OCH_2CH_2O), 3.64 (m, 1 H), 3.86 (m, 1 H), 3.90 (m, 1 H), 4.00 (m, 2 H), 4.11 (m, 1 H), 4.24 (m, 1 H), 4.65 (d, 1 H),

4.89 (m, 1 H), 7.90 (s, 2 H), 11.35 (s, 1 H, NH). MALDI-TOF: $C_{28}H_{41}N_4O_{15}P$, calculated 704.23, found 705.32 $[M+H]^+$.

2.20. Introduction of Spin Labels to RNA Containing 4-Thiouridine. The modified nucleotide 4-thiouridine was incorporated at preselected positions of RNAs, to be used for postsynthetic spin labeling with 1-oxyl- 2,2,5,5-tetramethylpyrroline-3-methyl)methane-thiosulfonate or *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide. RNA samples were incubated for 2 hours at room temperature with a 200-fold excess of dithiothreitol (DTT) in 50 μL of 100 mM sodium phosphate puffer (pH 8.0). The reducing agent was removed by demineralization using centrifugal filters, followed by lyophilization of the RNA. Spin labeling was carried out overnight by incubation of a 100 μM solution of the respective RNA in 90 mM sodium phosphate buffer (90% v/v) (pH 8.0) and DMF (10% v/v) with a 10-fold excess of 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate or *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, respectively. Unbound nitroxide reagent was removed by demineralization and lyophilization as described above. The spin label efficiency ranged from 10% to 100%.

2.21. Introduction of Spin Labels to RNA Containing 2′-Aminouridine. RNAs were synthesized with 2′-aminouridine at predefined positions and subsequently spin labeled.

2.21.1. Reaction with (1-Oxyl-2,2,5,5-tetramethylpyrrolin-3-carboxylat)-N-Hydroxysuccinimidyl Ester. A solution of the RNA in 80 mM borax buffer (pH 8.5) (90% v/v), DMF (10% v/v) containing 10 mM NaCl was incubated for 5 hours at 37°C with a 500-fold excess of (1-oxyl-2,2,5,5-tetramethylpyrrolin-3-carboxylat)-*N*-hydroxysuccinimidyl ester. Side products and unreacted reagents were extracted with 300 μL chloroform at room temperature followed by precipitation of the labeled RNA from ethanol. The pellet was resolved in water, the solution was demineralized using centrifugal filters, and finally, the sample was lyophilized. Yields were between 70% and 80%.

2.21.2. Reaction with 4-Isocyanato-2,6-tetramethylpiperidyl-N-oxid. A solution of the RNA in 50 mM borax buffer (pH 8.5) (50% v/v), DMF (20% v/v) and formamide (30% v/v) was cooled to −8°C and incubated for 2 hours with a 15-fold excess of 4-isocyanato-2,6-tetramethylpiperidyl-*N*-oxid. Side products and unreacted reagent were extracted with 300 μL chloroform at room temperature followed by precipitation of the labeled RNA from ethanol. The pellet was resolved in water, the solution was demineralized using centrifugal filters, and finally, the sample was lyophilized. Yields were between 75% and 100%.

3. Results and Discussion

3.1. Synthesis of Natural and Modified RNA with Commercially Available Building Blocks. The coupling reaction of RNA phosphoramidites is less facile than that of DNA phosphoramidite building blocks; hence, the coupling yields normally are slightly poorer. Thus, chemical synthesis of RNA is limited by the length of the oligomers. Up to now, we, therefore, preferred to synthesize nonmodified RNA molecules which are longer than 70 bases by *in vitro* transcription techniques as described [17]. Shorter nonmodified and modified RNAs in our laboratory are prepared by solid phase synthesis. Our procedure is based on 2′-*O*-*tert*-butyldimethylsilyl (TBDMS) nucleoside protection and involves sequential couplings of β-cyanoethyl-(*N*,*N*′-diisopropyl)phosphoramidites of 5′-*O*-dimethoxytrityl-2′-*O*-TBDMS nucleosides essentially by the method as described [17]. For assembly of the oligoribonucleotides, we predominantly use CPG or polystyrene beads to which the first ribonucleoside derivative is attached via its 3′-OH group as a succinate linkage. The standard synthesis is on the micromole scale, and for coupling of phosphoramidites, we use 5-benzylmercaptotetrazole activation [16] and coupling times of 5 min. Under these conditions, the yields in couplings are usually in the 99% range. We have synthesized a variety of amino- and 4-thiouridine-modified RNAs (Tables 1, 2, and 3) that were postsynthetically functionalized with fluorescent dyes, spin labels, or flavine mononucleotide as described below.

The 5′- and 3′-amino modifiers, as well as 4-thiouridine were introduced during RNA solid phase synthesis using commercially available building blocks (Figure 1). While for the 5′-amino modifier, the standard coupling conditions were used, 4-thiouridine was introduced by two consecutive couplings of 5 minutes each using fresh amidite solution. After chain assembly was completed, cleavage from the solid support and removal of base-protecting groups were accomplished by treatment with aqueous NH_3 (30%) and ethanolic methylamine in a 1 : 1 mixture for 2 h at room temperature. We found this protocol advantageous over the usage of NH_3/MeOH, as we had applied previously [18]. Finally, 2′-*O*-TBDMS groups were removed by fluoride ion treatment. While 1 M tetrabutylammonium fluoride was conventionally used for this purpose, we prefer the use of triethylamine trihydrofluoride first suggested by Gasparutto et al. [19]. In combination with polar aprotic solvents and at elevated temperatures, this reagent offers advantages in speed and reliability [20]. Therefore, we use triethylamine trihydrofluoride/DMF (3 : 1) at 55°C for 1.5 hours for efficient removal of the 2′-*O*-TBDMS groups. Following deprotection, oligonucleotides were desalted by precipitation with *n*-butanol and purified by electrophoresis on denaturing polyacrylamide gels or by HPLC. The yields of oligonucleotides shown in Tables 1–3 were in the range of 1 to 400 nmol after purification.

3.2. Synthesis of Amino-Modified RNA Building Blocks and Incorporation in RNA. Within a collaborative research project, the three-dimensional architecture of structured RNAs, for example, RNA thermometers [7] or RNA four-way junctions [8], is investigated by quantitative fluorescence resonance energy transfer (FRET) measurements. For absolute distance measurements by FRET, the microenvironment of the probe attached to the sample is very important. However,

Table 1: Sequences of synthesized linker-modified RNA oligonucleotides. The bold letters (U/A) mark the position of the modification (see Figure 2).

Name	Length	Sequence	Mod.	Dye
A1L1	25 nts	UGG CAA GC**U** CGC AGU CGG CAC CGC C	5	Cy5
A1L2	25 nts	UGG CAA GC**U** CGC AGU CGG CAC CGC C	6	Cy5, ATTO 647N
A1L3	25 nts	UGG CAA GC**U** CGC AGU CGG CAC CGC C	7	Cy5
A2L1	25 nts	**U**GG CAA GCU CGC AGU CGG CAC CGC C	5	Cy5
A2L2	25 nts	**U**GG CAA GCU CGC AGU CGG CAC CGC C	6	Cy5, ATTO 647N
A2L3	25 nts	**U**GG CAA GCU CGC AGU CGG CAC CGC C	7	Cy5
D1L1	25 nts	GGC GG**U** GCC GAC UGC GAG CUU GCC A	5	Alexa 488
D1L2	25 nts	GGC GG**U** GCC GAC UGC GAG CUU GCC A	6	Alexa 488
D1L3	25 nts	GGC GG**U** GCC GAC UGC GAG CUU GCC A	7	Alexa 488
L2-TEST	8 nts	GGA A**U**U CC	6	—
2-L3-A	8 nts	GGA A**U**U CC	7	ATTO 647N
8-L1-A	8 nts	UU**A** GUA CU	8	—
2-L3-A	8 nts	GGA A**A**U CC	10	ATTO 647N
RNA-25	32 nts	CCC CAC G**U**C AAG GCG UGG UGG CCG AAG GUC GG	6	Cy5
RNA-26	32 nts	CCG ACC **U**UC GGC CAC CUG ACA GUC CUG UGG GG	6	Alexa 488
4U-Ther.	60 nts	GGC GG**U** GCC GAC UGC GAG CUU GCC AUG UUG AAC UUU UGA AUA GUG AUU CAG GAG GUU AAU	6	Alexa 488
4U-Ther.	60 nts	GGC GGU GCC GAC UGC GAG CUU GCC AUG UUG AAC **U**UU UGA AUA GUG AUU CAG GAG GUU AAU	6	Alexa 488
4U-Ther.	60 nts	GGC GGU GCC GAC UGC GAG CUU GCC AUG UUG AAC U**U**U UGA AUA GUG AUU CAG GAG GUU AAU	6	Alexa 488
4U-Ther.	60 nts	GGC GGU GCC GAC UGC GAG CUU GCC AUG UUG AAC UUU UGA AUA GUG AU**U** CAG GAG GUU AAU	6	Alexa 488
4U-Ther.	60 nts	GGC GGU GCC GAC UGC GAG CUU GCC AUG UUG AAC UUU UGA AUA GUG AUU CAG GAG GUU AA**U**	6	Alexa 488

the commercial availability of building blocks for internal labeling of RNA is rare. Therefore, often molecules with a deoxy sugar moiety at the position of the internal label are applied for structural studies. In order to provide a set of nucleotides that upon incorporation in RNA can be used for RNA functionalization at a desired internal site, we started an effort to synthesize various ribonucleoside phosphoramidite building blocks with amino linkers of different lengths and flexibility attached to the heterocyclic base. We choose one short (L1) and one long (L2) rather flexible linker (Figure 3) coupled to the nucleobases by Heck chemistry [12, 13] and a short alkynyl linker (L3) (Figure 3) that was introduced by Sonogashira coupling [11].

In a first row of experiments, we have attached the three different linkers at C5 of uridine by palladium catalyzed cross-coupling reactions as described [14] (see **5**, **6**, and **7** in Figure 2).

Furthermore, adenosine derivatives with alkenyl linkers L1 and L2 at C8 or C2 were prepared under analogous conditions as used for uridine derivatives **5** and **6**. However, a striking difference was the formation of double bond isomers in the Heck coupling reaction. Even though this phenomenon is well known in the field of palladium catalyzed cross-coupling reactions, we were surprised observing a strong influence of the nature of the catalysts on formation of either isomer. Thorough investigation of a variety of catalysts and reaction conditions allowed us to tune reaction towards one of the two possible isomers by the choice of the catalysts (H. Nguyen, B. Appel, and S. Müller, manuscript in preparation). The specific amino-modified adenosine derivatives shown in Figure 2 were obtained with $Na_2[PdCl_4]$ (**8**, **9**, and **12**) or with the catalyst palladacycle (**11**) [21].

The phosphoramidite building blocks **7**, **10**, **13**, and **14** carrying the propargylamino linker (Figure 2) were prepared by Pd-catalyzed Sonogashira cross-coupling. Due to the milder conditions compared with Heck chemistry, successful Sonogashira reaction could be carried out at fully protected nucleosides. Protection is not essential; however, we found introduction of sugar and base protecting groups prior to the cross-coupling reaction advantageous, since the overall yield in this case was higher. The synthesis starts with the introduction of the DMT- and TBDMS protecting group via standard procedures [22, 23]. Then, the propargylamino linker L3 was coupled to the nucleobase by Sonogashira

Table 2: Sequences of spin labelled RNAs. The bold U marks the position of 4-thiouridine, and the underlined U the position of 2′-aminouridine.

Name	Sequence	Spin label	Yield (%)
VW1	GGG CUA AAA CA**U** ACC AGA UUUCGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG CUC	TPM-MTS	25
		TMP-IAA	23
VW2	GGG CUA AAA CAU ACC AGA UUUCGA UCU GGA GAG GUG AAG AA**U** ACG ACC ACC UAG CUC	TPM-MTS	10
		TMP-IAA	63
VW3	GGG CUA AAA CA**U** ACC AGA UUUCGA UCU GGA GAG GUG AAG AA**U** ACG ACC ACC UAG CUC	TPM-MTS	20
		TMP-IAA	80
VW4	GGG CUA AAA CAU ACC AGA UU**U**CGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG CUC	TPM-MTS	77
VW5	GGG CUA AAA CAU ACC AGA UUUCGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG C**U**C	TPM-MTS	25
VW6	GGG CUA AAA CAU ACC AGA UU**U**CGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG C**U**C	TPM-MTS	100
VW7	GGG CUA AAA CAU ACC AGA UUUCGA UCU GGA GAG GUG AAG AA**U** ACG ACC ACC UAG C**U**C	TPM-MTS	61
VW8	GGG CUA AAA CAU ACC AGA UUUCGA UCU GGA GAG G**U**G AAG AAU ACG ACC ACC UAG CUC	TPM-MTS	13
VW9	GGG CUA AAA CAU ACC AGA UUU CGA UCU GGA GAG GUG AAG AAU ACG ACC ACC**U**AG CUC	TPM-MTS	45
VW10	GGG CUA AAA CA**U** ACC AGA UU**U**CGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG CUC	TPM-MTS	89
VW11	GGG CUA AAA CA**U** ACC AGA UUUCGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG C**U**C	TPM-MTS	89
VW12	GGG CUA AAA CAU ACC AGA UU**U**CGA UCU GGA GAG GUG AAG AAU ACG ACC ACC UAG C**U**C	TPM-MTS	10
HPAS11	ACC AGA GAA ACA GCC U$\underline{U}$A GGA UAU GCU GG	TMC-NHS	81
		TEMPO-NCO	83
HPAS12	CCA GCA GAA GGA CG$\underline{U}$ CGU AUA UUA CCU GGU	TMC-NHS	80
		TEMPO-NCO	77
HPAS13	ACC AGA GAA ACA ACC U$\underline{U}$G GC	TMC-NHS	82
		TEMPO-NCO	97
HPAS14	GGC AAG G$\underline{U}$C GUA UAU UAC CUG GU	TMC-NHS	85
		TEMPO-NCO	75
HPAS15	ACC AGA GAA ACA GAC U$\underline{U}$G GC	TMC-NHS	72
		TEMPO-NCO	100
HPAS16	GCC AAG $\underline{U}$CG UAU AUU ACC UGG U	TMC-NHS	80
		TEMPO-NCO	82

reaction, and finally, the phosphoramidite was prepared as described above for the C5-propargylamino uridine derivative.

Adenosine derivatives with the propargyl amino linker at C8 and C2 were prepared following the same route. However, as an additional step, the exocyclic amino group of the base was protected prior to introduction of the other protecting groups and of the linker unit.

The propargylamino linker was also attached to C8 of guanosine starting from 8-bromoguanosine. The exocyclic amino group was protected as an amide using isobutyric anhydride. The DMT and the TBDMS group were introduced according to standard methods [22, 23] as described above, and finally, the phosphoramidite was prepared with 4 eq. of ethyl diisopropyl amine and 1.5 eq. of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (Figure 4).

Table 3: Sequences of two-stranded or three-stranded amino modified aptazymes. APU: 5-(3-Aminoprop-1-enyl)uridine. AMU: 2′-amino-uridine.

Name	Length	Sequence	Mod. X
HPAT2-L12	72 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GAU A**X**G CUU CGG CAG AAG GAC GUC GUA UAU UAC CUG GUA	APU
HPAT2-L14	72 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GA**X** AUG CUU CGG CAG AAG GAC GUC GUA UAU UAC CUG GUA	APU
HPAT2-U12	72 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GAU A**X**G CUU CGG CAG AAG GAC GUC GUA UAU UAC CUG GUA	AMU
HPAT2-U14	72 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GA**X** AUG CUU CGG CAG AAG GAC GUC GUA UAU UAC CUG GUA	AMU
HPAT1-AAM	43 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GA**X** AUG CUG G	AMU
HPAT1-AHI	43 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GA**X** AUG CUG G	APU
HPAT1-BAM	43 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GAU A**X**G CUG G	AMU
HPAT1-BHI	43 nts	AAA GAG AGA AGU GAA CCA GAG AAA CAG CCU UAG GAU A**X**G CUG G	APU
HPAT2	31 nts	CCA GCA GAA GGA CGU CGU AUA UUA CCU GGU A	—

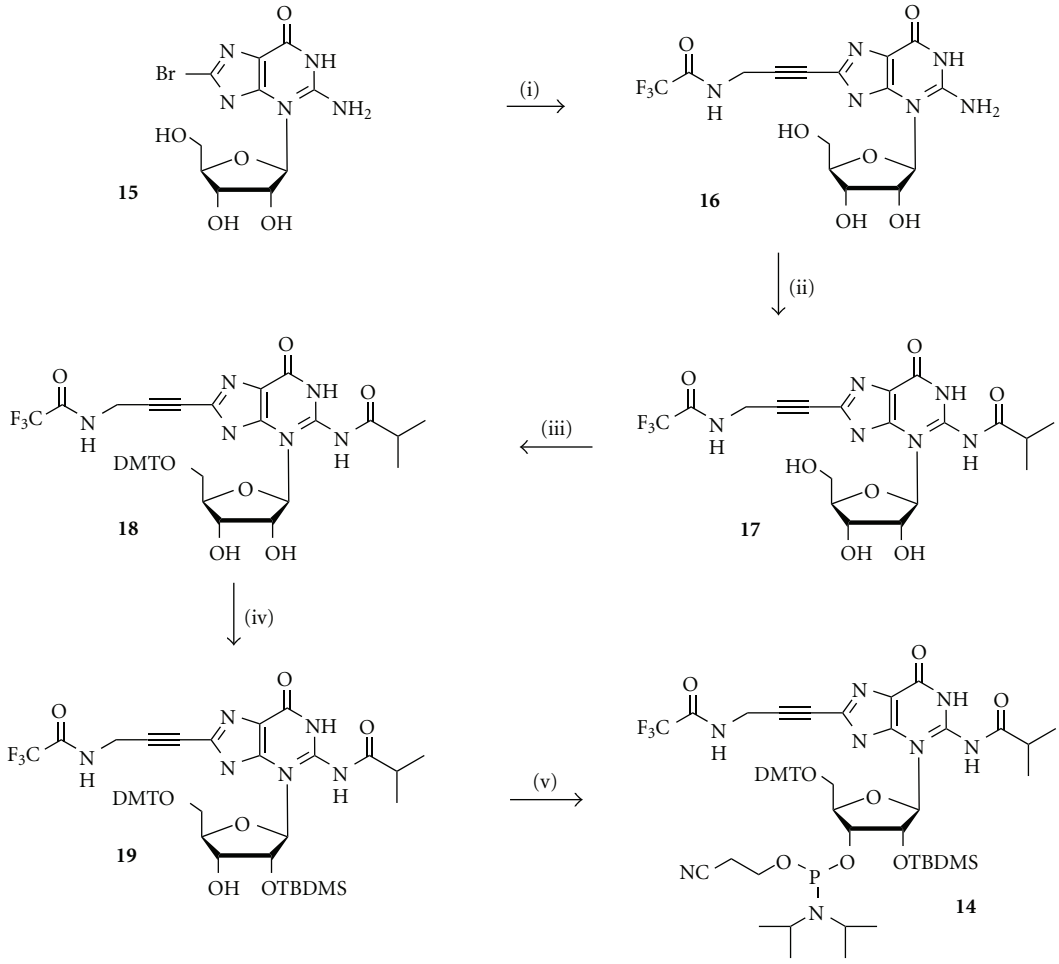


Figure 4: Synthesis of the guanosine-phosphoramidite. (i) 8-bromoguanosine **15**, 2.0 eq. L3, 0.1 eq. $Pd(PPh_3)_4$, 0.2 eq. CuI, 1.2 eq. DIPEA, 40°C, 20 h (80%); (ii) **16**, pyridine, 8.2 eq. TMS-Cl, 0°C, 2 h, 2.2 eq. isobutyric anhydride, rt, 2 h, NH_3 up to 2.5 M, 0°C; (iii) **17**, pyridine,1.5 eq. DMT-Cl, 0°C, 2.5 h; (iv) **18**, THF, 3.7 eq. pyridine, 1.5 eq. $AgNO_3$, 15 min, 1.7 eq. TBDMS-Cl, rt, 8.5 h; (v) **19**, DCM, 4 eq. DIPEA, 1.5 eq. 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, rt, 2.5 h.

Several of the prepared phosphoramidite building blocks were successfully incorporated into oligonucleotides using standard solid phase chemistry (Table 1) [17]. The modified phosphoramidites were thoroughly dried by repetitive coevaporating with dichloromethane. After solving the amidite in dry acetonitrile to a concentration of 0.1 to 0.15 M, molecular sieves were added and the solution was kept at room temperature for one hour prior to use. Amino-modified phosphoramidites were coupled for 5 min, as were the standard building blocks. The coupling yield was in the 99% range, comparable with those of the unmodified phosphoramidites. After synthesis, oligonucleotides were deprotected under standard conditions [17], leading also to removal of the TFA group protecting the aliphatic amine of the linker. Purification was carried out by gel electrophoresis, followed by MALDI-TOF MS analysis. Table 4 shows some example masses of MALDI-TOF-MS determination for some synthesized RNA oligonucleotides.

3.3. Postsynthetic Labeling. The amino-modified RNAs were subsequently used for attachment of various dyes. Due to selective reaction of activated carboxylic acids with aliphatic amines, the fully deprotected RNA was used without the danger of significant side reactions at the sugar hydroxyl groups or aromatic amino groups of the bases. In majority, activate esters of Cy5, ATTO647N and Alexa488 were used for postsynthetic labeling (Figure 5).

For Cy5 and ATTO647N, *N*-hydroxy-succinimidyl (NHS) esters were used. For Alexa488, the NHS-ester is only available as anisomeric mixture of 5- and 6-esters (Figure 6). After reaction with the dye, RNAs labeled with either isomer can be separated by gelelectrophoresis. However, we found it being more efficient using the tetrafluorophenyl (TFP) ester of Alexa488, which is available as the pure 5-isomer (Figure 6).

For each coupling reaction, an amount of 5 to 10 nmol RNA was dissolved in borax buffer at pH 8.7 for labeling with Cy5 and ATTO647N and pH 9.4 for reaction with Alexa488. The dyes were added dissolved in DMF, and the reaction was carried out at room temperature overnight in the dark. Afterwards, the excess of dye was removed by gel filtration. Purification of dye-labeled RNAs was carried out by PAGE (Cy5- and Alexa488-labeled strands) or by RP-HPLC (ATTO647N-labeled oligonucleotides). Successful labeling was confirmed by MALDI-TOF MS analysis. The data shown in Table 5 are representative examples of MS analyzed dye labeled oligonucleotides.

Figure 7 shows the MALDI-TOF-MS spectra of the oligonucleotide 2-L3-A prior to and after labeling with ATTO647N, as an example for the superior quality of the labeled oligonucleotides.

3.4. Hybridization of Single-Stranded RNAs for Structural Studies. To demonstrate that the amino linkers as well as the fluorescent labels do not intervene in the hybridization of single-stranded oligonucleotides, the hybrid of Alexa Fluor 488-labeled D1L3 and the complementary Cy5-labeled A1L3 was prepared (Figure 8). The oligonucleotides and their hybrid were visualized by illumination with UV-light at 254 and 365 nm. As can be seen from the gel, the two dye-labeled single-stranded oligonucleotides were fully hybridized.

In a parallel experiment, Alexa488-labeled D1L3 and Cy5-labeled A1L3 were dissolved in KH_2PO_4-K_2HPO_4 buffer containing $MgCl_2$ and KCl, according to the required buffer conditions in the FRET experiments to be conducted. The mixture was mixed, incubated at 78°C for 3 minutes, and slowly cooled down to room temperature. Reaction analysis by native polyacrylamide gel electrophoresis (data not shown) confirmed quantitative hybridization. Several of the synthesized RNAs upon hybridization to their complementary strands were used in fluorescence measurements, in particular in single molecule detection (SMD) and time correlated single photon counting (TCSPC) bulk experiments [14].

3.5. Synthesis of an Activated FMN Derivative for Postsynthetic Labeling of Amino-Functionalized RNA. In previous work, we have engineered an FMN responsive hairpin aptazyme that can be switched on and off in a reversible mode [10]. As we have shown, FMN can be removed from its binding pocket by reduction of the isoalloxazine ring, which is associated with a change in geometry and subsequently with loss of binding. In the original system, FMN was externally added, requiring a 400-fold excess to saturate its binding site in the aptazyme and to obtain a functional response. Current work is focused on engineering a hairpin aptazyme with FMN being covalently attached via a suitable linker. This small molecular device is supposed to be advantageous in terms of entropic costs for FMN binding to the aptamer and iterative cycles of reversible switching. For this purpose, a suitable FMN derivative for postsynthetic labeling of amino-modified RNA was synthesized. Recent experiments with different FMN derivatives have shown that only the isoalloxazine ring system is required for interaction with the aptazyme, while the ribose unit as well as the phosphate do not seem to play a major role (T. Marschall and S. Müller, unpublished observation). Thus, we decided to attach a carboxyl group containing linker to the ribose chain of riboflavin which subsequently could be postsynthetically attached to amino-functionalized RNA as described in Figures 5, 9, and 10.

Starting from riboflavin, first a fully protected riboflavin derivative was synthesized. Protection of the 5′-OH group was achieved by chemoselective tritylation of the primary hydroxyl group with DMT-Cl in pyridine. The remaining secondary hydroxyl groups were reacted with acetic anhydride to give the fully protected derivative **22** in good yields (83–90%). The selective deprotection of the 5′-OH group under acidic conditions (2% DCA in DCM) led to the formation of a side product with the same molecular mass as the main product. This suggested the migration of one of the acetyl groups under DMT deprotection conditions. It has been shown before that under acidic conditions, the acetyl group of the C4′-OH group can move to the neighbouring C5′-OH group [24]. Separation of the two isomers by column chromatography on silica gel was not successful. Therefore, we looked for alternative procedures. Alternatively to acids, also $ZnBr_2$ has been suggested as superior reagent

Table 4: Masses of the synthetic RNAs determined by MALDI-TOF-MS (linear negative-ion mode, found M = $[M-H]^-$).

Sequence	Cal. mass	Found mass	Δm	Error (%)
L2-TEST	2677.73	2675.99	0.77	0.027
2-L3-A	2585.63	2583.99	0.64	0.024
8-L1-A	2526.53	2525.96	0.43	0.017
A1L1	8041.95	8040.41	0.53	0.010
A1L2	8155.03	8153.15	0.88.	0.011
A1L3	8039.93	8039.23	0.30	0.003
A2L1	8041.95	8044.29	3.36	0.040
A2L3	8039.93	8041.17	2.24	0.030
D1L1	8098.93	8098.62	0.69	0.010
D1L2	8212.03	8211.46	0.43	0.005
D1L3	8096.93	8099.73	3.80	0.040

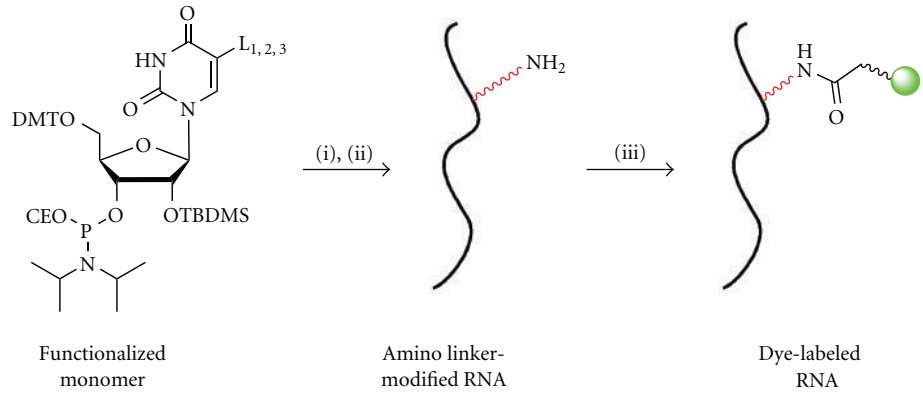


Figure 5: Postsynthetic labeling of amino-modified oligonucleotides: (i) Chemical synthesis of amino-modified RNA; (ii) Deprotection; (iii) Postsynthetic labeling with fluorescent dyes.

for removal of DMT groups [25, 26]. Indeed, using $ZnBr_2$ in dry nitromethane led to the formation of the desired product **23** within a few minutes, without acetyl migration. However, during purification of the detritylated riboflavin derivative by column chromatography, again migration of acetyl protecting groups was observed. Therefore, the solid crude product was just washed several times with ice cold diethyl ether, yielding compound **23** with satisfying purity.

For the synthesis of the carboxylic acid containing linker component, tetraethylene glycol was reacted with *tert*-butyl acrylate (Figure 10). The product of this Michael addition was converted to the corresponding phosphoramidite with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite by standard procedures [27]. The coupling reaction between the riboflavin derivative **23** and the linker phosphoramidite **26** was accomplished in acetonitrile with BMT activation followed by oxidation with an aqueous solution of iodine to give the flavinmononucleotide derivative **27**. The fully protected FMN derivative was treated with trifluoroacetic acid in dichloromethane to eliminate the acid labile *tert*-butyl protecting group (**28**). After the purification of the free acid form of the FMN derivative by RP-HPLC, cleavage of the *β*-cyanoethyl and the acetyl groups was achieved by stirring in 30% ammonia in methanol. Evaporation of the ammonia solution and subsequent treatment with DOWEX (H^+-form) yielded the desired product **29** that upon activation as NHS ester was used for postsynthetic RNA functionalization.

The FMN sensitive hairpin aptazyme was chemically synthesized as a three-stranded or two-stranded system using the phosphoramidite approach. In order to facilitate coupling of the FMN derivative with the oligonucleotides, 2′-aminouridine or 5-propargylaminouridine (Figure 11) were introduced in predefined positions of the RNA chain, providing the aliphatic amine for selective attachment of the FMN derivative (Table 3). Suitable positions for FMN attachment have been defined on the basis of the structure model of the FMN aptamer [28] with the help of PyMOL.

In order to couple the FMN derivative to the amino-modified RNA, an NHS-ester was generated *in situ* by reacting the unprotected FMN derivative **29** with 1.2 eq. TSTU and 2 eq. DIPEA for one hour at room temperature in DMF (Figure 12). The formation of the activated ester was monitored by MALDI mass spectrometry. After all of the free carboxylic acid had been converted to its NHS-form, the FMN derivative was coupled to the amino group bearing oligonucleotide. Successful coupling was confirmed by polyacrylamide gel electrophoresis (Figure 12). The functional characterization of the resulting FMN-RNA constructs in ribozyme activity assays is currently in progress.

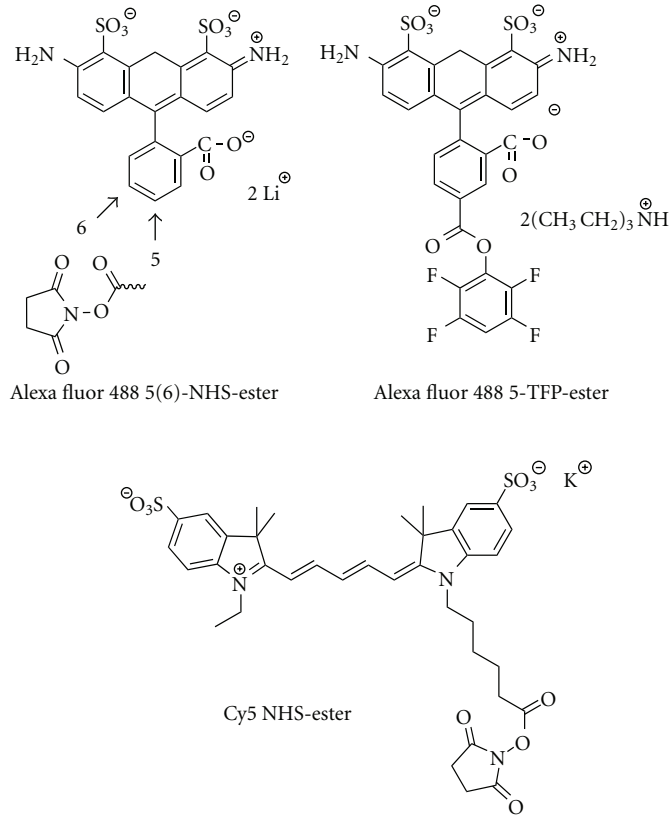


Figure 6: Activated derivatives of fluorescent dyes for postsynthetic labeling of RNA.

Table 5: Masses of dye-labeled oligonucleotides determined by MALDI-TOF-MS (linear negative-ion mode, found M = $[M+H]^-$).

Labeled sequence	Cal. mass	Found mass	Δm	Error (%)
2-L3-A-ATTO 647N	3213.63	3212.44	0.19	0.006
A1L2-ATTO 647N	8783.03	8782.28	0.25	0.003
A2L2-ATTO 647N	8783.03	8776.95	5.08	0.057
A2L2-Cy5	8793.25	8791.53	0.72	0.008

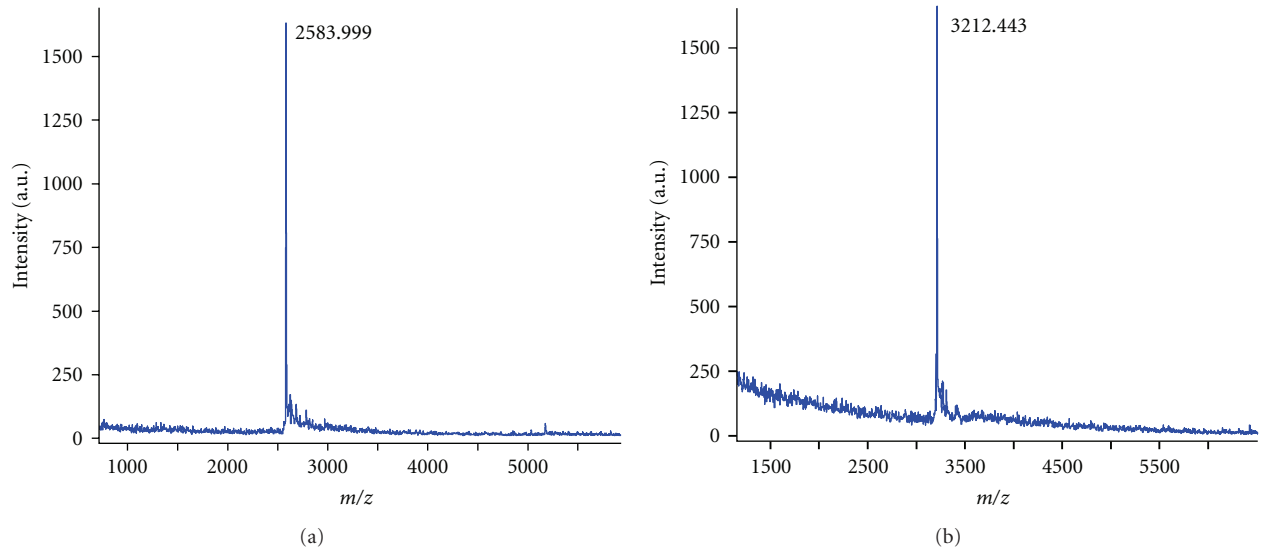


Figure 7: MALDI-TOF mass spectra of the amino-modified oligonucleotide 2-L3-A (a) and its corresponding conjugate with ATTO647N (b). For the sequence of 2-L3-A, compare Table 1.

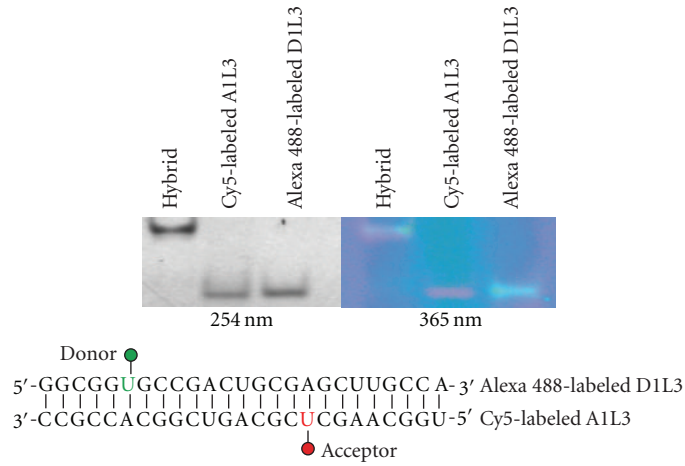


Figure 8: Hybridization of Alexa488-labeled D1L3 and Cy5-labeled A1L3: 3 μM Alexa488-labeled D1L3: 3 μM Cy5-labeled A1L3, 10 mM $MgCl_2$, 50 mM Tris buffer, pH 7.4, total volume 100 μL, 78°C 3 min, slowly cooling to rt. The hybrid was analyzed by 15% native PAA-gel: (left) illuminated at 254 nm, (right) illuminated at 365 nm.

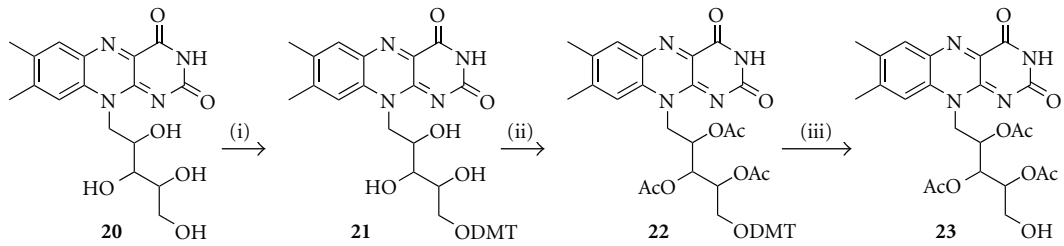
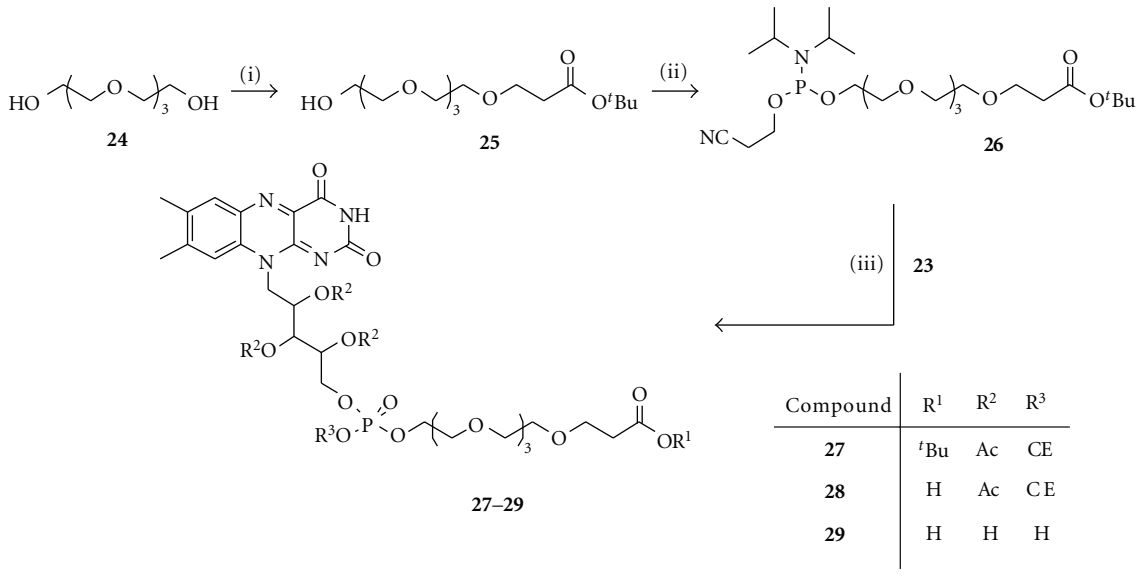


Figure 9: Synthesis of protected riboflavine derivatives: (i) DMT-Cl, DMAP, pyridine, rt, overnight (53%); (ii) acetic anhydride, pyridine, 0°C → rt, overnight (90%); (iii) $ZnBr_2$ in nitromethane, 2 min (83%).



Compound	R^1	R^2	R^3
27	^{t}Bu	Ac	CE
28	H	Ac	CE
29	H	H	H

Figure 10: Synthesis of the fully protected linker unit: (i) Na, *tert*-butyl acrylate, THF, rt, 1 d (34%); (ii) 2-Cyanoethyl-*N*,*N*-diisopropropyl-chlorophosphoramidite, DIPEA, DCM, rt, 1.5 h (quant.); (iii) (a) BMT, MeCN, rt, 1 h; (b) 0.2 M I_2 in THF/pyridine/H_2O (2 : 1 : 1) (32%).

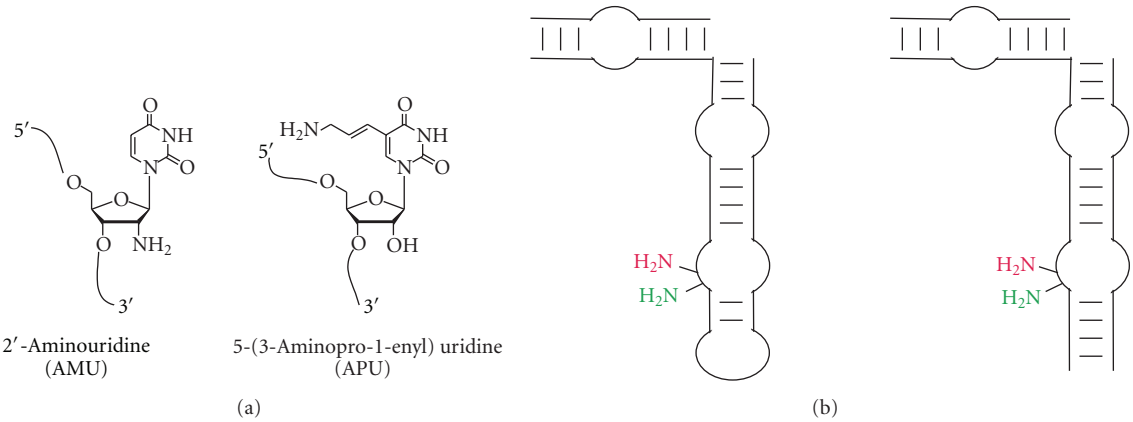


Figure 11: (a) Structure of the two nucleotide analogs used for the incorporation of an aliphatic amino group into the aptazyme RNA. (b) Two-stranded (left) and three-stranded (right) aptazyme. The modifications AMU and APU are located at position U12 (red) and U14 (green), respectively.

3.6. Synthesis of Specifically Spin-Labeled RNAs. Within a collaborative project on structural analysis of the tetracycline dependent riboswitch [9] and of the FMN responsive aptazyme described above [10] by electron paramagnetic resonance (EPR) spectroscopy, we have synthetically prepared RNAs carrying a suitable spin label at a predefined site. The basic strategy of site-directed spin-labeling originally has been developed with proteins and involves modification of the SH group of an appropriate cysteine with a selective paramagnetic nitroxide reagent. Applying the same technique to RNA, labeling initially was achieved by incorporating a guanosine monophosphorothioate at the 5′-end followed by spin-labeling at the sulfur [29]. The strategy was extended towards site-specific introduction of a phosphorothioate group at RNA backbone locations or of modified bases such as 4-thiouridine and subsequent attachment of a suitably activated nitroxide spin label [30–32]. More recent work employs aliphatic amino groups at the 2′-position of a specific sugar residue or attached at the heterocyclic base [33–35].

We have made use of two different strategies, incorporating either 4-thiouridine or 2′-aminouridine into RNA followed by postsynthetic reaction with the nitroxide spin label. For reaction with 4-thiouridine containing RNAs, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (TPM-MTS) and *N*-(1-oxyl- 2,2,5,5-tetramethyl-3-pyrrolidinyl) iodoacetamide (TMP-IAA) was used, for reaction with 2′-aminouridine containing RNA, (1oxyl-2,2,5,5-tetramethylpyrrolin-3-carboxylate)-*N*-hydroxy-succinimidyl ester (TMC-NHS) or 4-isocyanato-2,6-tetramethylpiperidyl*N*-oxid (TEMPO-NCO) (Figure 13). As mentioned already above, the 12 modified RNAs VW1 to VW12 shown in Table 2 were synthesized within a structural study of a tetracycline responsive RNA aptamer [9]. HPAS11 and HPAS16 were synthesized and spin labeled for an EPR study of an artificial flavin mononucleotide responsive riboswitch [10].

For RNAs labeled via 4-thiouridine, reaction with the TPM-MTS spin label was favored, because initial EPR measurements showed that the TMP-IAA label has suboptimal properties, presumably because it is too mobile due to the rather flexible CH_2-CO-NH-CH_2-spacer (Figure 13, lower part). Therefore, the majority of 4-thiouridine containing RNAs was reacted with TPM-MTS for introduction of the less mobile nitroxide spin label. The yields shown in Table 2 were reproduced in up to five labeling reactions for each individual RNA. Thus, the variation of labeling efficiency observed for individual RNAs suggests that the structure of the RNA aptamer significantly influences the labeling reaction, making spin labeling of individual uridines dependent on the specific location in the aptamer sequence.

A second point that may account for the observed low labeling efficiency is the rather unstable disulfide bond attaching the spin label at the uracil residue. Therefore, as an alternative, we have also studied conditions for spin labeling via the RNA sugar-phosphate backbone (Figure 14). To this end, RNAs containing 2′-aminouridine at a specific position (HPAS11 to HPAS16) were synthesized and labeled using either TEMPO-NCO or TMC-NHS. It has been described in the literature that the steric hindrance of the 2′-NH_2-group hampers reaction with NHS esters [36]. Therefore, we have extended the reaction time from 15 minutes to 4 hours and used a 500-fold excess of the NHS-reagent to overcome the problem of low reaction yields. On the contrary to NHS esters, isocyanates were shown to virtually quantitatively react with 2′-amino sugars [33]. However, being less selective, side reactions at the nucleobases were observed when a high excess of the isocyanate reagent was used [37]. The authors have optimized reaction conditions such that a 2′-amino group was selectively modified without concurrent side reaction. In that, they observed an increase of the yield of the modified oligonucleotide upon lowering the temperature, which presumably is the result of repression of competing isocyanate hydrolysis [37]. In parallel rows of labeling reactions under varying conditions, we have also looked at reaction conditions to deliver the spin-labeled RNA with high yield and high quality. Best results were obtained with an only 15-fold excess of the isocyanate reagent over RNA, and a reaction temperature of −8°C. Under these conditions, about 80% labeling efficiency were reproducibly

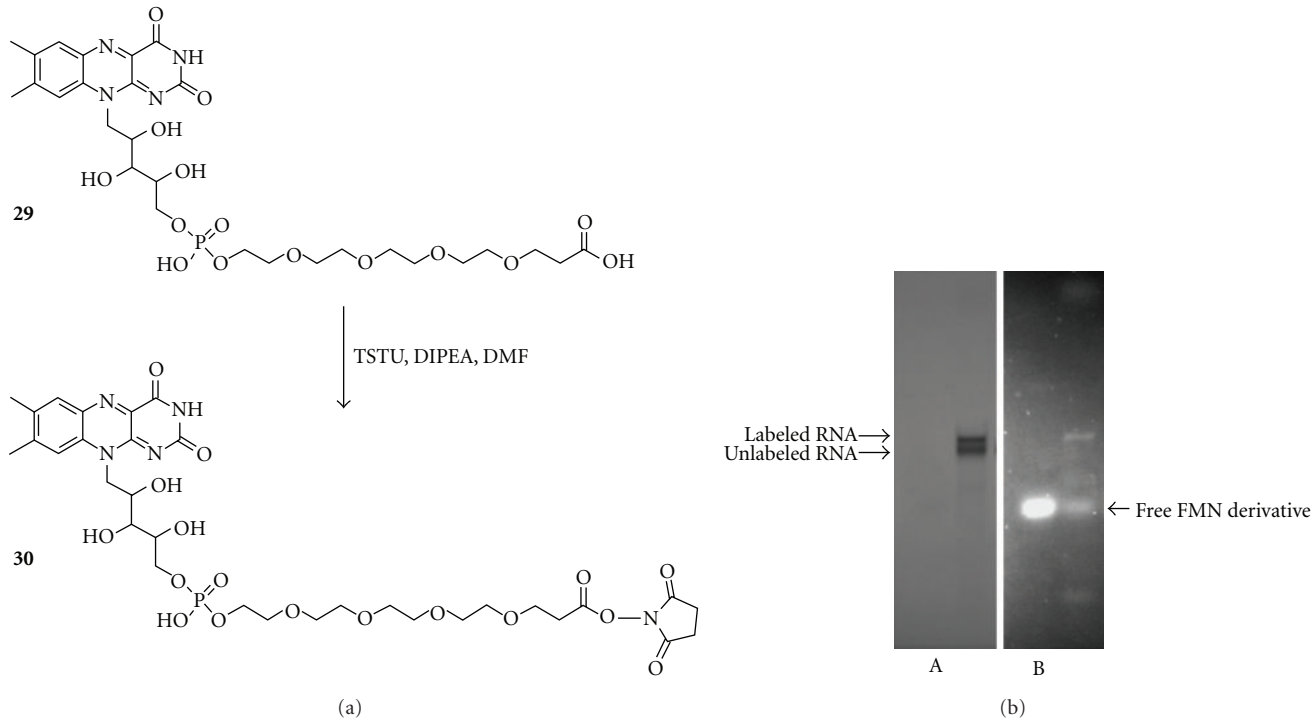


Figure 12: (a) Formation of the *N*-hydroxysuccinimidyl (NHS) ester. (b) PAGE analysis of the coupling reaction between amino-modified RNA HPAT1-BHI (for sequence see Table 3) and the FMN derivative. Visualization is at UV 254 nm (A) and 365 nm (B).

obtained for both labeling reagents with all RNA samples (Table 2). In order to avoid side reactions, we have added the spin label reagent only once in 15-fold excess even though it was originally described that repeated additions of the reagent give nearly quantitative results [33]. EPR spectra of RNAs showed that the spin labels conjugated via the 2′-amino group are rather immobile (H.-J. Steinhoff, University Osnabrück, oral information), making labeling via this position most favorable. It was somewhat surprising that mobility of TEMPO seemed to be even more limited than that of the TPC- label, because the 6-membered piperidyl ring is conformationally more flexible than the 5-membered pyrroline ring. Obviously, the nature of the spacer unit (urea versus amide bond) also has a strong influence. Overall, in the specific context of RNA sequences shown in Table 2, 2′-amino sugar labeling is advantageous over 4-thiouridine labeling in terms of efficiency and immobility.

4. Conclusion

As demonstrated above, the chemical synthesis of RNA offers the possibility of site-specific introduction of modified nucleosides. This is an important advantage over enzymatic procedures, making RNA synthesis an essential tool for a large number of studies in different fields. We have succeeded in the synthesis of a number of monomer building blocks that upon incorporation in RNA allow for postsynthetic attachment of spectroscopic reporter groups or other desired functionalities. The amino-modified monomers presented in this paper are a valuable addition to the RNA chemist's tool box. Due to the different length and flexibility of the linkers that carry the aliphatic amino groups for postsynthetic functionalization, molecular entities that become attached to the RNA have differing degrees of motional freedom. This is of particular interest for fluorescence spectroscopic studies, because systematic errors may result from fluorescence dyes that are sterically hindered, or on the contrary are too mobile, limiting the accuracy of the measurement. Therefore, using our amino linker building blocks of the U series (Figure 2), an extensive analysis of the influence of the linker lengths and rigidity on the accuracy of FRET measurements was carried out [14]. The results will pave the way for more sophisticated studies of RNA structure dynamics. Likewise, EPR spectroscopic analysis of RNA will profit from the new building blocks, since spin labels attached to the RNA of interest, are subjected to the same conditions and restrictions as the fluorescent dyes in fluorescence spectroscopy.

Lastly, we have synthesized an FMN-RNA conjugate to be used for functional studies of an FMN responsive aptazyme. This example shows that there is much room for RNA engineering and construction of sophisticated RNA devices. Organic chemistry offers a large number of possibilities to prepare suitably activated modifiers that can be postsynthetically attached to amino-modified RNA, or to RNA carrying other functionalities such as for example thiol groups, or alkynes and azides as used for labeling by Click chemistry [38]. Thus, in combination with the development of synthetic routes to appropriately activated modifiers, RNAs can be conjugated with a large variety of tags, making the nucleic acid not only visible in spectroscopic

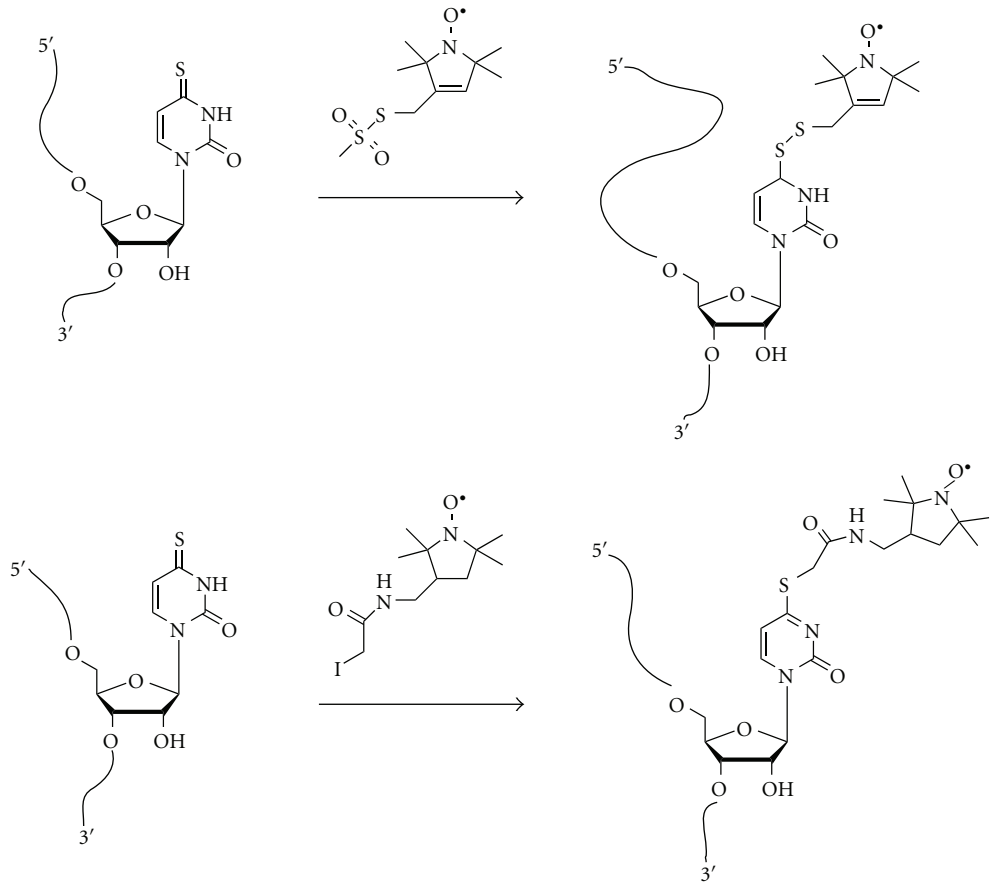


Figure 13: Strategy for postsynthetic introduction of spin labels to 4-thiouridine containing RNA.

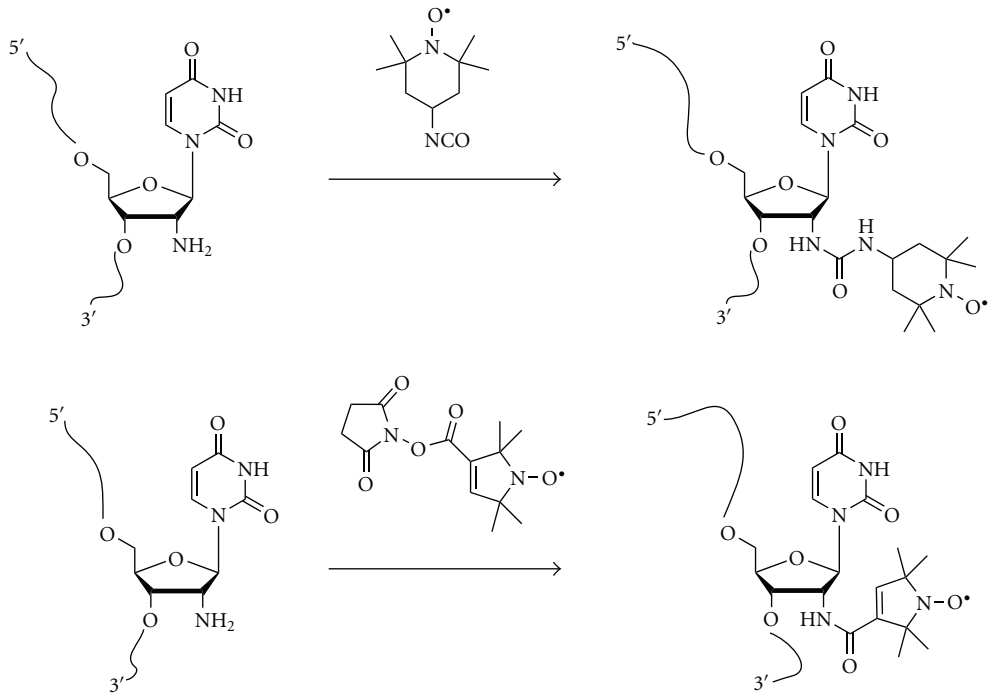


Figure 14: Labeling of the 2′-amino group of a model RNA.

experiments, but also adding new functionality as demonstrated with the RNA-FMN conjugate described herein.

Abbreviations

CE:	Cyanoethyl
DCM:	Dichloromethane
DIPEA:	Diisopropylethylamine
DMA:	Dimethylamine
DMAP:	Dimethylaminopyridin
DMF:	Dimethylformamide
DMT:	Dimethoxytrityl
MeCN:	Acetonitril
TBDMS:	*tert*-butyldimethylsilyl
TEA:	Triethylamine
BMT:	1-(Benzylmercapto)-1*H*-tetrazole
TEMPO-NCO:	4-isocyanato-2,6-tetramethylpiperidyl-*N*-oxyl
TFA:	Trifluoroacetic acid
TMC-NHS:	(1-oxyl-2,2,5,5-tetramethylpyrrolin-3-carboxylat)-*N*-hydroxysuccinimidyl ester
TMP-IAA:	*N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) iodoacetamide
TPM-MTS:	(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate.

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