Efficient Access to 3′-Terminal Azide-Modified RNA for Inverse Click-Labeling Patterns

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ABSTRACT: Labeled RNA becomes increasingly important for molecular diagnostics and biophysical studies on RNA with its diverse interaction partners, which range from small metabolites to large macromolecular assemblies, such as the ribosome. Here, we introduce a fast synthesis path to 3′-terminal 2′-O-(2-azidoethyl) modified oligoribonucleotides for subsequent bioconjugation, as exemplified by fluorescent labeling via Click chemistry for an siRNA targeting the brain acid-soluble protein 1 gene (BASP1). Importantly, the functional group pattern is inverse to commonly encountered alkyne-functionalized “click”-able RNA and offers increased flexibility with respect to multiple and stepwise labeling of the same RNA molecule. Additionally, our route opens up a minimal step synthesis of 2′-O-(2-aminoethyl) modified pyrimidine nucleoside phosphoramidites which are of widespread use to generate amino-modified RNA for N-hydroxysuccinimide (NHS) ester-based conjugations.

INTRODUCTION

Recently, azide-modified RNA1−5 has attracted considerable attention for being a valuable addition to the tool box of RNA bioconjugation.6,7 Of particular interest is the outreach for inverse Click labeling patterns that would create significantly more flexibility for complex labeling patterns as, for instance, needed for single-molecule fluorescence resonance energy transfer (FRET) studies.8,9 Also, azide-modified RNA will provide interesting alternatives to the existing RNA labeling concepts, such as expanding the range to Staudinger-type ligation.10 The prevalence of alkyne over azide-modified DNA and RNA stems from the straightforward integration of the alkyne functionality into the automated oligonucleotide solid-phase synthesis cycle using phosphoramidite building blocks.11−13 In contrast, azide-modified nucleoside phosphoramidites for solid-phase synthesis would encounter severe limitations because of the inherent reactivity of P(III) species with azides.14 Likewise, the rare encounter of, e.g., Staudinger ligation in the context with nucleic acids lies in the fact that neither the required azide nor the required P(III) moiety is easy to align with P(III) phosphoramidite chemistry for assembly.6,7,15−17 Hence, reported protocols for Staudinger-based conjugations on nucleic acids include inconvenient two-step procedures that attach the required N3 moiety postsynthetically onto amino group-functionalized RNA, employing N-hydroxysuccinimide (NHS) chemistry.18 Although efficient enzymatic prefunctionalization of DNA or RNA based on azide-modified nucleoside triphosphates has been reported,19−21 such a strategy would not be appropriate if single, site-specific azide modifications within nucleic acids are required.

Here, we describe the efficient preparation of a solid support for automated RNA synthesis using phosphoramidite building blocks that provides RNA with a 3′-terminal 2′-O-(2-azidoethyl) group (Figure 1). Efficient labeling with fluorescent dyes is evaluated for an siRNA application as well as the smooth transformation of the azido-labeled RNA into the corresponding amine derivative for NHS ester bioconjugation. Furthermore, potential strategies for diverse multiple label attachments are discussed. Additionally, our synthetic route opens up a minimal step synthesis of 2′-O-(2-aminoethyl) derivatized RNA. The modification allows for inverse Click labeling and selective, stepwise label attachment to RNA with diverse functional group patterns.

Figure 1. Chemical structure of 3′-end 2′-O-(2-azidoethyl) derivatized RNA. The modification allows for inverse Click labeling and selective, stepwise label attachment to RNA with diverse functional group patterns.

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modified pyrimidine nucleoside phosphoramidites which are widely used to prepare amino-functionalized RNA.

## RESULTS AND DISCUSSION

Chemical synthesis is the method of choice to prepare functionalized RNA with tailored properties. Frequently, this undertaking demands labeling with moieties that are incompatible with RNA solid-phase synthesis and, therefore, prefunctionalized RNA with tethers carrying, e.g., amino or alkyne groups is required. These anchors can then be transformed by using the classical NHS ester approach and the more recent Click conjugations, respectively. Our original efforts were driven by the motivation to equip the same RNA with an additional orthogonal anchor besides amine and alkyne groups. This goal would be amenable through azide modification that allows for selective labeling with strained cyclic alkyne groups, in the presence of both of the other attachment sites. Interestingly, not many types of chemically synthesized, alkyne-functionalized RNAs have been described in the literature, and for their assembly, the majority requires either phosphonate (e.g., 2′-O{(2-azidothoxy)methyl} RNA) or phosphotriester chemistry (e.g., 2′-azido RNA). Although these approaches are powerful and enable labeling of internal sequence positions, they require adjustments of standard RNA synthesis procedures which can represent a handicap for broader applications. Another recent promising approach to generate 2′-O{(2-azidoethyl) modified nucleic acids involves a convertible nucleoside, but this approach has been demonstrated thus far for DNA only. Here, we intended to create a fast and simple access to azide labeled RNA even if restrictions with respect to positioning of the azide group were encountered. For many applications, in particular, for multiple, specific labeling of DNA and RNA, 3′-OH of the oligoribonucleotide available to retain the possibility for ligations to construct larger RNA, e.g., by using in vitro selected DNA ligation enzymes. Hence, we focused on the ribose 2′-OH position for derivatization and favored the 2′-O{(2-azidoethyl) group. Nucleosides of this type and with defined protecting group patterns have been reported as intermediates for the synthesis of 2′-O{(2-aminoethyl) modified DNA and RNA. However, applying such pathways would involve multiple steps. Here, we aimed at a one-step protecting group-free synthesis using the substrates 2,2′-anhydouridine 1 and 2-azidoethanol (which are commercially available or can be prepared by a single transformation from the precursors uridine and 2-chloroethanol, respectively) in the presence of boron trifluoride diethyl etherate (Scheme 1). The procedure was elaborated based on reports by Egli and Sekine who demonstrated the corresponding transformation with a series of other alcohol derivatives. After careful optimization, the desired 2′-O{(2-azidoethyl) uridine 2 was achieved in acceptable yields. Compound 2 was then readily tritylated, then transformed into the corresponding pentafluorophenyl (Pfp) adic acid ester, and finally into the functionalized solid support 3.

### Scheme 1. Synthesis of the Solid Support 3 for 3′-End 2′-O{(2-azidoethyl) Modified RNA

![Scheme 1](image)

**Table 1. Selection of Synthesized 3′-End 2′-O{(2-azidoethyl) RNAs and Corresponding Dye Label Derivatives**

| no | sequence | amount [nmol] | m.w. [amu] | m.w. [amu] |
|----|----------|---------------|-------------|-------------|
| S1 | S′-ACG UU-2′-OCH2CH2N3 | 1300 | 1599.9 | 1598.9 |
| S2 | S′-UGU CUU AUU GCC AGA GAC CTU-2′-OCH2CH2N3 | 185 | 6724.1 | 6725.0 |
| S3 | S′-GGU CUC UGC CAA UAA GAC ATU-2′-OCH2CH2N3 | 176 | 6717.0 | 6718.6 |
| S4 | S′-UGU CUU AUU GCC AGA GAC CTU-2′-az-F545 | 23 | 7368.0 | 7368.8 |
| S5 | S′-GGU CUC UGC CAA UAA GAC ATU-2′-az-F545 | 28 | 7361.7 | 7361.9 |
| S6 | S′-AGA UGU GCC AGG AAA ACC A(Cy3–Sall-U)C UUU AAA AAA CUG GUU-2′-az-ADIBO-Cy5 | 5.6 | 12826.8 | 12827.0 |
| S7 | S′-AGA UGU GCC AGC AAA ACC A(Cy3–Sall-U)C UUU AAA AAA CUG GUU-2′-az-ADIBO-Cy5 | 4.3 | 12825.8 | 12825.8 |

**Tether abbreviations refer to 2′-OCH2CH2N3 (2′-az), 5-aminoallyl (Sall), dibenzocyclooctyne (ADIBO).**

The solid support 3 was efficiently used for automated RNA strand assembly using nucleoside phosphoramidite building blocks (Table 1). Standard cleavage and deprotection procedures resulted in high-quality crude products as exemplified in Figure 2A (top). The integrity of the azido-modified RNA was confirmed by LC-ESI mass spectrometry (Figure 2A, bottom). We also note that 2′-O{(2-azidoethyl) modified RNAs were efficiently reduced to the 2′-O{(2-aminoethyl) modified counterparts by incubation with tris(2-carboxyethyl)phosphine hydrochloride (TECP) in aqueous solution (Figure S1). Thus, the azidoethyl moiety can be used as a temporarily masked amino anchor for sequential
labeling of RNA that is functionalized together with an internal 2′-O-(2-aminoethyl) or 5-aminoallyl pyrimidine modification, using NHS ester conjugation reactions only.

Furthermore, we demonstrated the convenience of the 2′-O-(2-azidoethyl) RNA label in a typical azide−alkyne 1,3-dipolar cycloaddition reaction (Click chemistry)6,11 (Figure 2B, Table 1). We applied the copper-catalyzed version with acetonitrile acting as ligand of the CuI complex, stabilizing the oxidation state.36 The labeled RNA strand at 1 mM concentration was efficiently reacted with a commercially available, alkyne-modified 5-carboxytetramethylrhodamine dye (F545) (2 mM) in the presence of sodium ascorbate, and analyzed by anion exchange chromatography (Figure 2B). For reasons of comparability, we chose the siRNA sequence system used previously to knock down the brain acid-soluble protein 1 gene (BASP1) by transient siRNA nucleofection in the chicken DF-1 cell line.4,5,37 Expression of the BASP1 gene is specifically suppressed by Myc, an evolutionary conserved oncoprotein;38 conversely, the BASP1 protein is an efficient inhibitor of Myc-induced cell transformation.37

Three dye-labeled siRNAs were annealed, one labeled at the 3′-end of the antisense strand, the second labeled at the 3′-end of the sense strand, and the third labeled at both 3′-ends (Figure 3A). All three siRNA were efficiently nucleofected into chicken DF1 cells and localized by fluorescence microscopy (Figure 3B). Not unexpectedly, due to the stringent structural requirements for antisense strand recognition within the RISC complex,39,40 efficient silencing (comparable to the unmodified reference duplex) was only observed for the sense labeled siRNA duplex, while both siRNAs with 3′-labeled antisense strands were inactive, as analyzed by Northern blot hybridization (Figure 3C). The finding that the activity of the siRNA carrying a large chemical moiety is well tolerated only when it is placed at the 3-terminus of the sense strand is in accordance with our own previous findings4 and those by others.41−43

To further demonstrate the usefulness of 2′-O-(2-azidoethyl) RNA, we performed efficient dual fluorescent labeling of strands that additionally contained 5-aminoallyl uridine modifications, using NHS-chemistry and strain-promoted alkyne−azide conjugation (SPAAC).21 The sequence represents a preQ₁ class-I riboswitch aptamer,44 and the obtained cyanine dye pattern is applicable for bulk FRET investigations (Table 1, Figure 4, Figure S2).

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The efficient approach to 2′-O-(2-azidoethyl) labeled RNA and their applications can be mainly attributed to the one-step synthesis of the key compound 2′-O-(2-azidoethyl) uridine 2. This derivative additionally opens up a convenient route with minimal steps to 2′-O-(2-aminoethyl) uridine phosphoramidites (Scheme 2). 2′-O-(2-Aminoethyl) modified nucleic acids have been extensively studied for various purposes.45−50 and
interestingly, the reported syntheses of the building blocks usually entail initial alkylation of the ribose 2′-OH by methyl bromoacetate followed by a series of transformation reactions or involve extended protecting group concepts. The route presented here relies on tritylation of the azide, followed by azide to amine reduction under Staudinger conditions and trifluoroacetylation to give derivative. After phosphitylation, the corresponding uridine building block was obtained in excellent overall yield in only five steps from uridine.

■ CONCLUSIONS
The presented approach to 3′-terminal azide-modified RNA is significant for diverse applications in RNA biochemistry and RNA chemical biology as exemplified here for fluorescently labeled siRNAs. Another potential of this type of modification lies in the combined prefunctionalization together with amine (and, in principle, also with alkyne) moieties of the same RNA to allow for selective and stepwise attachment of sensitive moieties that cannot be directly incorporated into RNA. Efficient generation of complex labeling patterns is, e.g., required for multicolor single-molecule FRET studies and is currently undertaken in our laboratory.

■ EXPERIMENTAL PROCEDURES

General Remarks. 1H and 13C NMR spectra were recorded on a Bruker DRX 300 MHz or Avance II+ 600 MHz instrument. The chemical shifts are referenced to the residual proton signal of the deuterated solvents: CDCl3 (7.26 ppm), d6-DMSO (2.49 ppm) for 1H NMR spectra; CDCl3 (77.0 ppm) or d6-DMSO (39.5 ppm) for 13C NMR spectra (see also Figures S3–S6). 1H- and 13C-assignments were based on COSY and HSQC experiments. MS experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrument. Analytical thin-layer chromatography (TLC) was carried out on Marchery-Nagel Polygram SIL G/UV254 plates. Flash column chromatography was carried out on silica gel 60 (70–230 mesh). All reactions were carried out under argon.
atmosphere. Chemical reagents and solvents were purchased from commercial suppliers and used without further purification. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4 Å).

2′″-O-(2-Azidoethyl)uridine (2). 2″-Anhydrouridine (1) (565 mg, 2.5 mmol) was coevaporated with dry pyridine three times and stored over P2O5 in a desiccator for four hours before use. Then, compound 1 was suspended in DMA (4 mL) and BF3·OEt2 (785 μL, 6.25 mmol) was added under argon and heated to 120 °C. 2-Azidoethanol (1250 mg, 14.3 mmol) was injected into the solution and the mixture was refluxed for 16 h. After the reaction was finished solvents were removed in vacuo, and the oily residue was redissolved in methanol and adsorbed on silica gel. Compound 2 was purified by column chromatography on SiO2 with CH2Cl2/CH3OH, 100:0 to 98:2. Yield: 549 mg of (CH2Cl2/CH3OH = 92/8):

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\delta = 0.56. \text{H NMR (300 MHz, CDCl3):} \delta = 8.19 \text{ (m, 4H, RO}_{2}\text{CH}_{2}\text{CH}_{2}\text{CH}_{2} \text{C}=(\text{O})_{2}\text{C}_{2}F_{5} \text{);} \ 2.58 \text{ (m, 2H, RO}_{2}\text{CH}_{2}\text{CH}_{2}\text{CH}_{2} \text{CO}_{2}\text{C}_{2}F_{5} \text{);} \ 2.84 \text{ (m, 2H, RO}_{2}\text{CH}_{2}\text{CH}_{2} \text{CO}_{2}\text{C}_{2}F_{5} \text{);} \ 3.51 \text{ (m, 2H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 3.58 \text{ (m, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 3.86 \text{ (m, 1H, H}^1\text{C}=(\text{C})_{2} \text{);} \ 3.93 \text{ (s, 6H, H}_2\text{CO}_3 \text{);} \ 4.11 \text{ (m, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 5.34 \text{ (dd, } j = 7.0^\text{,} j = 5.3 \text{, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 5.51 \text{ (d, } j = 8.1 \text{ Hz, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 5.93 \text{ (d, } j = 2.55 \text{ Hz, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 7.04 \text{ (m, 4H, H}_2\text{CO}_3 \text{);} \ 8.14 \text{ (d, } j = 8.1 \text{ Hz, 1H, H}^2\text{C}=(\text{C})_{2} \text{);} \ 9.38 \text{ (s, 1H, N-H) ppm. 13C NMR (150 MHz, CDCl3):} \delta = 24.01, 24.20, 33.00, 33.44 (4C, RO}_{2}\text{CH}_{2}\text{CH}_{2}\text{CH}_{2} \text{CO}_{2}\text{C}_{2}F_{5} \text{);} \ 50.91 (C(2)″); 55.39 (2C, CH}_3\text{O); 61.18 (C(5)); 65.89 (C(3)″); 70.14 (C(1)″); 80.90 (C(4)″); 81.23 (C(2)″); 87.56 (C(3)); 102.60 (C(5)); 113.49 (C(ar)); 127.40 (C(ar)); 130.31 (C(ar)); 130.55; 138.97 (C(6)); 144.24; 150.42; 158.95; 163.32; 169.16; 172.40 ppm. ESI-MS (m/z): [M+Na]⁺ calc for C_{44}H_{40}N_{5}F_{5}O_{11}Na, 932.24; found 932.32.

2′″-O-(2-Azidoethyl)uridine Modified Solid Support (3). Compound 2b (70 mg, 77 μmol) was dissolved in DMF (1.7 mL) and pyridine (12 mg [12 μL], 154 μmol). Then, amino-functionalized solid support (GE Healthcare, Custom Primer Support 200 Amino, 323 mg) was added. The suspension was agitated for 48 h at room temperature and the beads were collected on a Büchner funnel. The beads were washed with N,N-dimethylformamide, methanol, and dichloromethane and dried. Capping was performed by treatment of the beads with a mixture of 3.0 mL of solution A (acetic anhydride/2,4,6-trimethylpyridine/acetonitrile, 2/3/5) and 3.0 mL of solution B (4-(N,N-dimethylylamino)pyridine/acetonitrile, 0.5 M) for 5 min at room temperature. The suspension was filtered again and the beads were washed extensively with acetonitrile, methanol, and dichloromethane and dried under vacuum. Loading of the support 3 was 60 μmol/g.

2′″-O-[(N-Trifluoracetyl)-2-aminoethyl]−5′″-O-(4,4″-dimethoxytrityl)uridine (4). Compound 2a (460 mg, 0.75 mmol) was dissolved in THF (7.25 mL). Water (69 μL, 3.8 mmol) and triphenylphosphine (392 mg, 1.5 mmol) was added and the solution was stirred for 5 h at room temperature. Then, ethyl trifluoracetate (1065 mg [0.89 mL], 7.5 mmol) and triethylamine (770 mg [1.06 mL], 7.6 mmol) were added and stirring was continued overnight. The reaction mixture was evaporated and the crude product was purified by column chromatography on SiO2 with CH2Cl2/CH3OH, 100:0 to 95:5.

Yield: 315 mg of 4 as a white foam (= 61%). TLC (CH2Cl2/CH3OH = 95/5): Rf = 0.4. 1H NMR (300 MHz, CDCl3): δ = 2.85 (d, J = 8.7 Hz, 1H, HO-C(3)′); 3.50–3.65 (m, 4H, H^1-C(2)′, H^2-C(2)′, H^2-C(2)″); 3.79 (s, 6H, H2CO3); 3.93–4.05 (m, 4H, H=C(2)′, H=C(4)′, H^2-C(1)″, H^1-C(1)″); 4.42 (m, 1H, H=C(2)″); 5.33 (d, J = 8.1 Hz, 1H, H=C(5)′); 5.86 (s, 1H, H=C(1)″); 6.85 (m, 4H, H=C(ar)); 7.24–7.39 (m, 9H, H=C(ar)); 7.71 (m, 1H, HNCOCF3); 8.05 (d, J = 8.1 Hz, 1H, H=C(6)); 9.95 (s, 1H, N-H) ppm. 13C NMR (150 MHz, CDCl3): δ = 39.75 (C(2)′); 55.39 (CH3CO); 61.08 (C(5)); 68.55 (C(3)′); 69.37 (C(1)″); 83.36 (C(2)′); 83.49 (C(4)′); 87.30; 87.33 (C(1)″); 102.61 (C(ar)); 113.48 (C(ar)); 127.36 (C(ar)); 130.22 (C(ar)); 135.38; 135.36; 140.01 (C(6)); 144.43; 151.13; 158.87; 158.91; 163.48 ppm. ESI-MS (m/z): [M+Na]⁺ calc for C24H24N3O3Na, 708.28; found 708.21.
**RNA Solid-Phase Synthesis.** Standard phosphoramidite chemistry was applied for RNA strand elongation using solid support 3: for the synthesis 2′-O-TOM standard RNA nucleoside phosphoramidite building blocks were purchased from GlenResearch and ChemGenes, the polystyrene support from GE Healthcare (Custom Primer Support, 80 µmol/g; PS 200). All oligonucleotides were synthesized on an ABI 392 Nucleic Acid Synthesizer following standard methods: deprotection (80 s) with dichloroacetic acid/1,2-dichloroethane (4/96); coupling (2.0 min) with phosphoramidites/acetonitrile (0.1 M × 130 µL) and benzylthiotetrazole/acetonitrile (0.3 M × 360 µL); capping (3 × 0.4 min, Cap A/Cap B = 1/1) with Cap A: 4-(dimethylamino)pyridine in acetonitrile (0.5 M) and Cap B: Ac2O/sym-collidine/acetonitrile (2/3/5); oxidation (1.0 min) with I2 (20 mM) in THF/pyridine/H2O (35/10/5). The solutions of amidites and tetrazole, and acetonitrile were dried over activated molecular sieves (4 Å) overnight.

**Deprotection of 2′-O-(2-azidoethyl) Modified RNA.** The solid support was treated with MeNH2 in EtOH (33%, 0.5 mL) and MeNH2 in water (40%, 0.5 mL) for 7 h at room temperature. (For RNA containing 5-aminoallyl uridines, the solid support was treated with MeNH2 in EtOH (33%, 0.5 mL) and NH3 in H2O (28%, 1 mL) for 10 min at room temperature. (For RNA containing 5-aminoallyl uridines, the solid support was treated with MeNH2 in EtOH (33%, 0.5 mL) and NH3 in H2O (28%, 1 mL) for 10 min at room temperature. (For RNA containing 5-aminoallyl uridines, the solid support was treated with MeNH2 in EtOH (33%, 0.5 mL) and NH3 in H2O (28%, 1 mL) for 10 min at room temperature.)

**The solid support was treated with tetrabutylammonium fluoride (55% vol/vol) with a H2O/acetonitrile ratio of 4/1 (0.1 M (Et3NH)+HCO3−, H2O and eluted with H2O/CH3CN (1/1). Fractions containing RNA were desalted with a size exclusion LC system. RNA sequences were analyzed in the negative-ion mode with a potential of −4 kV applied to the spray needle. LC; Sample (200 pmol RNA dissolved in 30 µL of 20 mM EDTA solution; average injection volume: 30 µL); column (Waters XTerraMS, C18 2.5 µm; 1.0 × 50 mm) at 21 °C; flow rate: 30 µL/min; eluant A: 8.6 mM TEA, 100 mM 1,1,1,3,3,3-hexafluoroisopropanol in H2O (pH 8.0); eluant B: methanol; gradient: 0–100% B in A within 30 min; UV-detection at 254 nm.

**Copper-Catalyzed Azide−Alkyne Cycloaddition (CuAAC) Labeling.** 2′-O-(2-Azidoethyl) modified RNA (60 nmol) was lyophilized in a 1 mL Eppendorf tube. Then, aqueous solutions of F545 (Acetylene-Fluor 545, Click Chemistry Tools), CuSO4 and sodium ascorbate were added consecutively; acetonitrile was added as cosolvent to reach final concentrations of 1 mM RNA, 2 mM dye, 5 mM CuSO4, 10 mM sodium ascorbate, and a H2O/acetonitrile ratio of 4/1 in a total reaction volume of 60 µL. The reaction mixture was degassed and stirred for 3 to 4 h under argon atmosphere at 50 °C. To monitor the reaction and to purify the reaction mixtures, anion exchange HPLC as described above was used.

**Double Labeling Using N-Hydroxysuccinimide Ester (NHS) Chemistry and Strain-Promoted Alkyne–Azide Cycloadditions (SPAAC).** Lyophilized 3′-end 2′-O-(2-azidoethyl) RNA (25 nmol) containing a single 5′-(E-3-amino-prop-1-enyl)uridine (5′-aminoallyl uridine) was dissolved in labeling buffer (25 mM phosphate buffer, pH 8.0) and DMSO (55% vol/vol) with a final concentration of 225 µM RNA and 1.125 mM Sulfo-Cy3-NHS ester in a total volume of 110 µL. The reaction mixture was shaken for 5 h at room temperature in the dark. Then, the RNA was precipitated with absolute ethanol (2.5 volumes of labeling reaction) and a 1 M aqueous solution of sodium acetate (0.2 volumes of labeling reaction), for 4 h at −20 °C. The suspension was centrifuged for 30 min at 4 °C at 13 000 × g to remove the excess of unreacted and hydrolyzed dye. The pellets were dried under high vacuum and dissolved in nanopure water and DMSO (50% vol/vol) to reach final concentrations of 312 µM RNA and 686 µM ADIBO derivatized Cy5 dye in a total volume of 80 µL. The reaction mixture was shaken for 3 h at room temperature in the dark. To monitor the reaction and to purify the reaction mixtures, anion exchange HPLC as described above was used.

**RNA Interference and Northern Analysis.** Delivery of siRNAs into cells and analysis of gene silencing were done essentially as described.4,37 Lyophilized synthetic siRNA (for sequence see Figure 3 and Table S1) targeted against the chicken BASP1 mRNA sequence 5′-CAGGUCUCUGCCAAUCGAGAACA-3′, were dissolved in a buffer containing 100 mM potassium acetate, 30 mM Heps-KOH (pH 7.4), and 2 mM magnesium acetate, yielding a 40 µM siRNA solution. The solution was heated at 90 °C for 1 min, incubated at 37 °C for 1 h, and then stored at −80 °C. For transfection of siRNA, 5 × 105 cells of the chicken fibroblast line DF-1 were pelleted at 50 × g for 5 min at room temperature, suspended in 100 µL of nucleofector solution V (Lonza/Amaxa), and mixed with 12 µL of siRNA solution containing 0.24 nmol (~3.0 µg) of duplex RNA. The mixture was subjected to electroperoration (Lonza/ Amaxa) using the nucleofector program U-20, and then immediately diluted with 0.5 mL of culture medium. Transfected cells were seeded onto 60-mm dishes containing 4 mL of culture medium and cultivated at 37 °C. Medium was changed after one day, and total RNA was isolated after two days with the RiboPure Kit (Ambion). Briefly, cells were homogenized in a solution containing phenol and guanidine thiocyanate. After
addition of bromochloropropene, RNA was recovered from the aqueous phase by binding to a glass-fiber filter and subsequent elution using a low-salt buffer. Northern analysis using 5 μg of total RNA and specific DNA probes for detection of βASPI or GAPDH mRNAs was performed as described previously.37

■ ASSOCIATED CONTENT

2 Supporting Information

1H and 13C NMR spectra for compounds 2, 2a, 2b, and 4; reduction of 2′-(2-azidoethoxy) RNA; chemical structures of fluorescent dyes used; siRNA sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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