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

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Ⓢ Supporting Information

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.
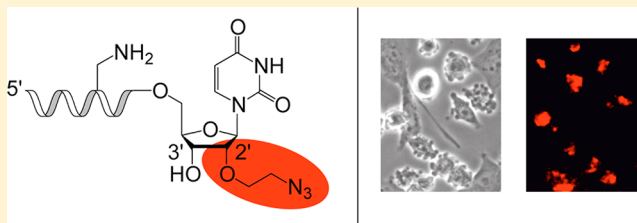
■ INTRODUCTION

Recently, azide-modified RNA[1−5] 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 ligations.[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 ligations 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 N$_3$ 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)
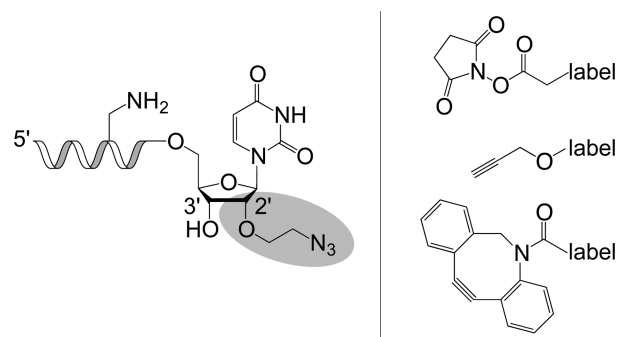

Figure 1. Chemical structure of 3′-end 2′-O-(2-azidoethyl) derivatized RNA. The modification allows for inverse Click labeling and selective, stepwise label attachment to RNA with diverse functional group patterns.

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.[22] 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.[7,11,16,17] 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 alkynes,[23] in the presence of both of the other attachment sites. Interestingly, not many types of chemically synthesized, azide-functionalized RNAs have been described in the literature, and for their assembly, the majority requires either phosphonate (e.g., 2′-O-[(2-azidoethoxy)methyl] RNA)[3] or phosphortriester chemistry (e.g., 2′-azido RNA).[4,5] 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.[24] 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[25,26] or RNA,[8,9,12] 3′-end azide anchors would be a major asset, provided the approach is facile and applicable to standard phosphoramidite chemistry.

We recall a previous report by Morvan and co-workers on a universal solid support for 3′-end azide labeling of DNA[27] and our own studies on 3′-deoxy-3′-azido RNA[28] that are compatible with the usage of nucleoside phosphoramidites. However, for the present study we aimed at an approach that keeps the 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.[29] Hence, we focused on the ribose 2′-O 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.[30,31] However, applying such pathways would involve multiple steps. Here, we aimed at a one-step protecting group-free synthesis using the substrates 2,2′-anhydrouridine **1** and 2-azidoethanol (which are commercially available or can be prepared by a single transformation from the precursors uridine[32] and 2-chloroethanol,[33] respectively) in the presence of boron trifluoride diethyl etherate (Scheme 1). The procedure was eleborated based on reports by Egli[34] and Sekine[35] 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) adipic 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[a]
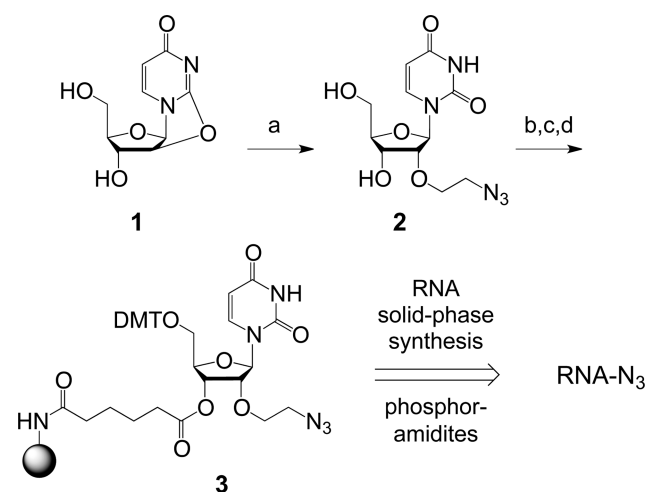
[a]Reaction conditions: (a) 5 equiv $HOCH_2CH_2N_3$, 2.5 equiv $BF_3 \cdot OEt_2$ in dimethylacetamide, 120 °C, 16 h, 55%; (b) 1.1 equiv DMT-Cl, in pyridine, 16 h, RT, 75%; (c) 3.5 equiv $PfpOOC(CH_2)_4COOPfp$, 1.2 equiv DMAP, in DMF/pyridine (1:1), room temperature, 1 h, 47%; (d) 3 equiv (w/w) amino-functionalized support (GE Healthcare, Custom Primer Support 200 Amino), 2 equiv pyridine, in DMF, room temperature, 48 h, loading: 60 mmol g^{-1}.

The solid support **3** was efficiently used for automated RNA strand assembly using nucleoside phosphoramidite building blocks (Table 1). Standard cleavage and deprotection

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

no	sequence[a]	amount[b] [nmol]	m.w._calcd [amu]	m.w._found [amu]
S1	5′-ACG UU-2′-$OCH_2CH_2N_3$	1300	1599.9	1598.9
S2	5′-UGU CUU AUU GGC AGA GAC CTU-2′-$OCH_2CH_2N_3$	185	6724.1	6725.0
S3	5′-GGU CUC UGC CAA UAA GAC ATU-2′-$OCH_2CH_2N_3$	176	6717.0	6718.6
S4	5′-UGU CUU AUU GGC AGA GAC CTU-2′-az-F545	23	7368.0	7368.8
S5	5′-GGU CUC UGC CAA UAA GAC ATU-2′-az-F545	28	7361.7	7361.9
S6	5′-AGA UGU GCC AGC AAA ACC A(Cy3−5aall-U)C UUU AAA AAA CUG GU-2′-az-ADIBO-Cy5	5.6	12826.8	12827.0
S7	5′-AGA UGU GC(Cy3−5aall-U) AGC AAA ACC AUC UUU AAA AAA CUA GU-2′-az-ADIBO-Cy5	4.3	12825.8	12825.8

[a]Tether abbreviations refer to 2′-$OCH_2CH_2N_3$ (2′-az), 5-aminoallyl (5aall), dibenzocyclooctyne (ADIBO). [b]Isolated yields. For dye structures, see Figure 2 and Figure S2.

procedures resulted in high-quality crude products as exemplified in Figure 2A (top). The integrity of the azide-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 (TCEP) in aqueous solution (Figure Figure S1). Thus, the azidoethyl moiety can be used as a temporarily masked amino anchor for sequential
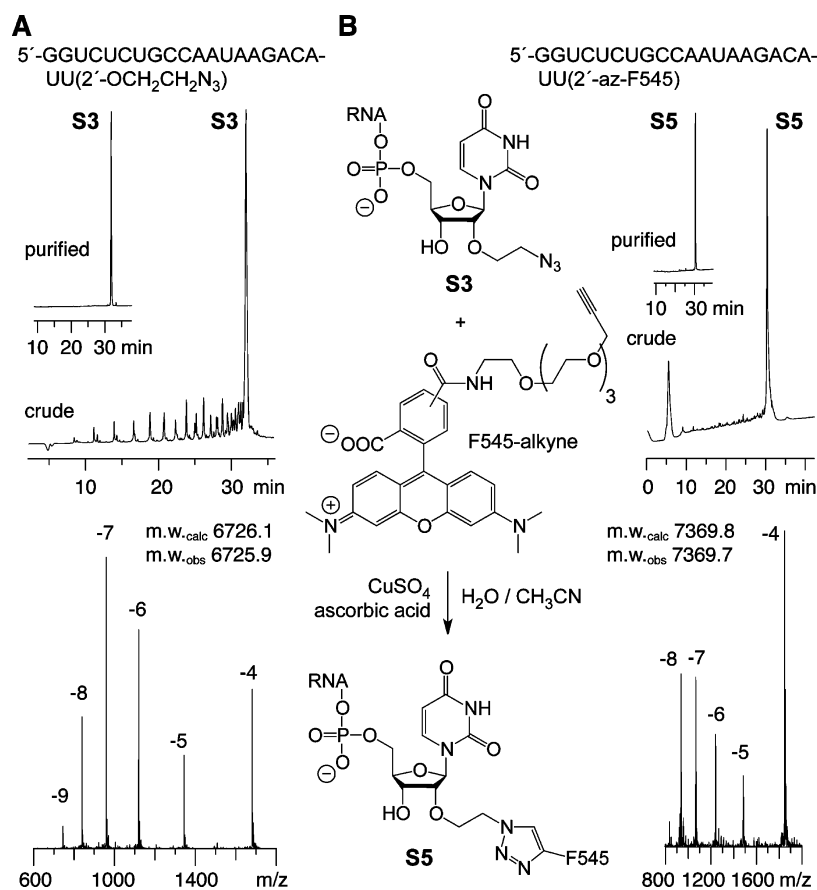
Figure 2. Synthesis, labeling, and analysis of an exemplary 2′-*O*-(2-azidoethyl) modified RNA based on the solid support **3**. (A) Anion exchange HPLC profiles of deprotected, crude (top) and purified (inset) RNA, and LC-ESI mass spectrum (bottom). (B) Reaction scheme of Click labeling with alkyne functionalized fluorescence dye (left); conditions: 5 mM $CuSO_4$, 10 mM sodium ascorbate, 50 °C, 3 h; c_{RNA} = 1 mM, c_{Dye} = 2 mM, H_2O/CH_3CN = 4/1; 60 μL total reaction volume. HPLC profiles of crude (top right) and purified (inset) labeled RNA, and LC-ESI mass spectrum (bottom); HPLC conditions: Dionex DNAPac column (4 × 250 mm), 80 °C, 1 mL min^{-1}, 0–60% buffer B in 45 min; buffer A: Tris-HCl (25 mm), urea (6 M), pH 8.0; buffer B: Tris-HCl (25 mM), urea (6 M), $NaClO_4$ (0.5 M), pH 8.0. For LC-ESI MS conditions, see the Experimental Procedures.

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 as cosolvent 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 findings[4] 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 preQ$_1$ class-I riboswitch aptamer,[44] and the obtained cyanine dye pattern is applicable for bulk FRET investigations (Table 1, Figure 4, Figure S2).

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
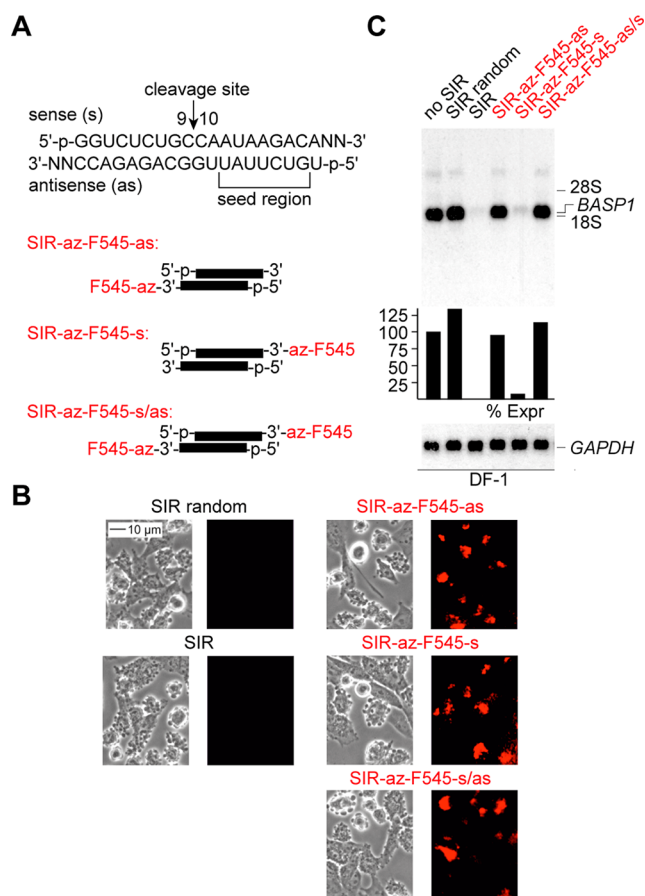
Figure 3. Silencing of the brain acid-soluble protein 1 gene (*BASP1*) by siRNA duplexes with fluorescent labels (F545) clicked to 3′-terminal 2′-*O*-(2-azidoethyl) anchors. (A) General organization (top) and labeling pattern of the siRNA duplex (bottom); for detailed RNA sequences see Table S1. (B) *BASP1* siRNAs show cytoplasmic localization in DF1 cells visualized by fluorescence microscopy. The amounts of nucleofected siRNAs were 0.24 nmol. (C) Activities of 2′-az-F545 labeled *BASP1* siRNAs and corresponding controls (random siRNA and unmodified siRNA) monitored by Northern analysis of *BASP1* expression in DF1 cells. Expression of *GAPDH* served as loading control.

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[29,30] or involve extended protecting group concepts.[48−50] The route presented here relies on tritylation of the azide **2**, followed by azide to amine reduction under Staudinger conditions and trifluoroacetylation to give derivative **4**. After phosphitylation,[30] 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 amino (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.,
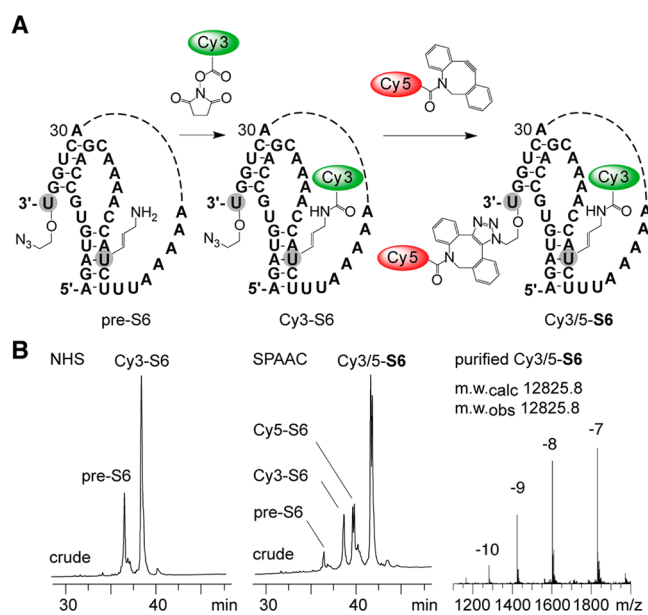

Figure 4. Example for double labeling of 3′-terminal 2′-*O*-(2-azidoethyl) modified RNA. (A) Labeling scheme for the preQ₁ riboswitch RNA from *Fusobacterium nucleatum*.[44] (B) HPLC profiles of crude reaction mixture after *N*-hydroxysuccinimide (NHS) ester based Cy3 conjugation (left) and subsequent strain-promoted alkyne azide conjugation (SPAAC) of Cy5 (middle), LC-ESI mass spectrum (right). For HPLC and LC-ESI mass specrometry conditions, see Figure 2 caption; for dye structures, see Figure S2.

Scheme 2. Short Synthesis of a 2′-*O*-(2-Aminoethyl) Uridine Phosphoramidite[a]
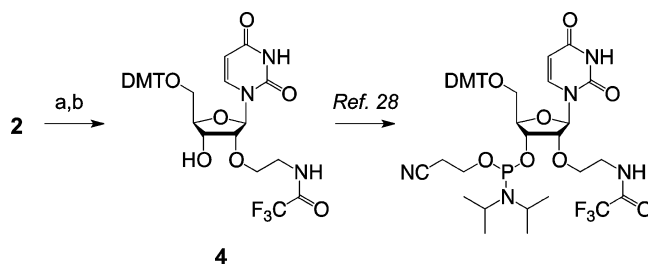
[a]Reaction conditions: (a) 1.1 equiv DMT-Cl, in pyridine, 16 h, RT, 75%; (b) i. 2 equiv PPh₃, 5 equiv H₂O, in tetrahydrofurane, room temperature, 5 h, ii. 10 equiv CF₃COOEt, 10 equiv NEt₃, CH₃OH, 0 °C, 14 h, 61% (over 2 steps).

required for multicolor single-molecule FRET studies and is currently undertaken in our laboratory.

■ EXPERIMENTAL PROCEDURES

General Remarks. ^{1}H and ^{13}C 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), d_6-DMSO (2.49 ppm) for ^{1}H NMR spectra; CDCl₃ (77.0 ppm) or d_6-DMSO (39.5 ppm) for ^{13}C NMR spectra (see also Figures S3−S6). ^{1}H- and ^{13}C-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). 2,2′-Anhydrouridine 1 (565 mg, 2.5 mmol) was coevaporated with dry pyridine three times and stored over P_2O_5 in a desiccator for four hours before use. Then, compound **1** was suspended in DMA (4 mL) and BF$_3$·OEt$_2$ (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 SiO$_2$ with CHCl$_3$/CH$_3$OH, 95:5. Yield: 431 mg of **2** as a white solid (55%). TLC (CH$_2$Cl$_2$/CH$_3$OH = 85:15): R_f = 0.51. ^{1}H NMR (300 MHz, DMSO): δ 3.17 (m, 2H, H^1−C(2″) H^2−C(2″)); 3.58 (m, 2H; H^1−C(5′) H^2−C(5′)); 3.86 (m, 2H, H^1−C(1″) H^2−C(1″)); 3.88 (m, 1H, H−C(4′)); 4.04 (m, 1H, H−C(2′)); 4.60 (dd, J = 4.8 Hz, J = 9.8 Hz, 1H, H−C(3′)); 5.14 (m, 2H, HO-C(3′), HO-C(5′)); 5.72 (d, J = 8.0 Hz, 1H, H−C(5)); 5.88 (d, J = 4.8 Hz, 1H, H−C(1′)); 7.94 (d, J = 8.0 Hz, 1H, H−C(6)); 11.29 (s, 1H, NH) ppm. ^{13}C NMR (150 MHz, DMSO): δ 49.93 (C(2″)); 60.39 (C(5′)); 68.2 (C(3′)); 68.86 (C(1″); 81.31 (C(2′); 84.93 (C(4′)); 86.15 (C(1′)); 101.79 (C(5)); 140.32 (C(6)); 150.56; 163.10 ppm. ESI-MS (m/z): [M-H]$^-$ calcd for C$_{11}$H$_{15}$N$_5$O$_6$, 312.11; found 312.46.

2′-O-(2-Azidoethyl)-5′-O-(4,4′-dimethoxytrityl)uridine (2a). Compound **2** (372 mg, 1.19 mmol) was coevaporated with dry pyridine three times and dissolved in pyridine at room temperature and under argon atmosphere. 4,4′-Dimethoxytrityl chloride (443 mg, 1.31 mmol) was added in two portions over a period of 2 h. Stirring was continued overnight, and when TLC showed complete reaction, methanol was added and the solution was evaporated in vacuo. The residue was dissolved in CH$_2$Cl$_2$ and extracted with 5% citric acid, water, and saturated NaHCO$_3$. The organic layer was dried over Na$_2$SO$_4$ and evaporated. The crude product was purified by column chromatography on SiO$_2$ with CH$_2$Cl$_2$/CH$_3$OH, 100:0 to 98:2. Yield: 549 mg of **2a** as a white foam (75%). TLC (CH$_2$Cl$_2$/CH$_3$OH = 92/8): R_f = 0.54. ^{1}H NMR: (300 MHz, CDCl$_3$): δ 2.58 (d, J = 9.7 Hz, 1H, HO-C(3′)); 3.49 (m, 2H, H^1−C(2″), H^2−C(2″)); 3.58 (m, 2H; H^1−C(5′), H^2−C(5′)); 3.80 (s, 6H, H$_3$CO) 3.96 (m, 1H, H^2−C(1″)); 3.96 (m, 1H, H−C(2′)); 4.04 (m,1H, H−C(4′)); 4.19 (m, 1H, H^1−C(1″)); 4.51 (m, 1H, H−C(3′)); 5.30 (d, J = 8.1 Hz, 1H, H−C(5)); 5.93 (s, 1H, H−C(1′)); 6.85 (m, 4H, H−C(ar)); 7.31 (m, 9H, H−C(ar)); 8.09 (d, J = 8.1 Hz, 1H, H−C(6)); 9.16 (s, 1H, N−H) ppm. ^{13}C NMR (150 MHz, CDCl$_3$): δ 50.99 (C(2″)); 55.40 (CH$_3$O); 61.03 (C(5′)); 68.43 (C(3′)); 70.09 (C(1″); 83.16 (C(2′); 87.27 (C(4′)); 87.73 (C(1′)); 102.27 (C(5)); 113.47 (C(ar)); 127.33 (C(ar)); 130.25 (C(ar));140.32 (C(6)); 144.50; 150.33; 158.91; 163.39 ppm. ESI-MS (m/z): [M+Na]$^+$ calcd for C$_{32}$H$_{33}$N$_5$O$_8$Na, 638.21; found 638.40.

2′-O-(2-Azidoethyl)-5′-O-(4,4′-dimethoxytrityl)-3′-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]uridine (2b). Compound **2a** (100 mg, 162 μmol) was coevaporated with pyridine twice and dried over P_2O_5 for three hours, then it was dissolved in DMF/Pyr (=1/1; 4.6 mL) and DMAP (24 mg, 196 μmol), and adipinic acid pentafluorophenyl ester (275 mg, 575 μmol) was added. After one hour the reaction mixture was evaporated and coevaporated with acetone and CH$_2$Cl$_2$ two

times. The crude product was purified by column chromatography on SiO$_2$ with acetone/CH$_2$Cl$_2$, 95:5 to 85:15. Yield: 70 mg of **2b** as a white foam (47%). TLC (acetone/CH$_2$Cl$_2$ = 8:2): R_f = 0.56. ^{1}H NMR: (300 MHz, CDCl$_3$): δ 1.93 (m, 4H, RO$_2$CH$_2$(CH$_2$)$_2$CH$_2$−CO$_2$C$_6$F$_5$); 2.58 (m, 2H, RO$_2$CH$_2$(CH$_2$)$_2$CH$_2$CO$_2$C$_6$F$_5$); 2.84 (t, J = 6.8, 2H, RO$_2$CH$_2$(CH$_2$)$_2$CH$_2$CO$_2$C$_6$F$_5$); 3.51 (m, 2H, H^1−C(2″), H^2−C(2″)); 3.58 (m, 1H; H^1−C(5′)); 3.81 (m, 1H, H^2−C(5′)); 3.86 (m, 1H, H^2−C(1″)); 3.93 (s, 6H, H$_3$CO); 4.11 (m, 1H, H^1−C(1″)); 4.43 (m, 2H, H−C(4′)); 5.34 (dd, J^1 = 7.0, J^2 = 5.3, 1H, H−C(3′)); 5.51 (d, J = 8.1 Hz, 1H, H−C(5)); 5.93 (d, J = 2.55 Hz, 1H, H−C(1′), 7.00 (m, 4H, H−C(ar)); 7.40 (m, 9H, H−C(ar)); 8.14 (d, J = 8.1 Hz, 1H, H−C(6)); 9.38 (s, 1H, N−H) ppm. ^{13}C NMR (150 MHz, CDCl$_3$): δ 24.01, 24.20, 33.00, 33.44 (4C, RO$_2$CH$_2$(CH$_2$)$_2$CH$_2$−CO$_2$C$_6$F$_5$), 50.91 (C(2″)); 55.39 (2C, CH$_3$O); 61.18 (C(5′)); 69.89 (C(3′)); 70.14 (C(1″); 80.90 (C(4′)); 81.23 (C(2′); 87.56 (C(1′)); 102.60 (C(5)); 113.49 (C(ar)); 127.40 (C(ar)); 130.31 (C(ar)); 135.05; 139.87 (C(6)); 144.24; 150.42; 158.95; 163.32; 169.16; 172.40 ppm. ESI-MS (m/z): [M+Na]$^+$ calcd 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-dimethylamino)pyridine/acetonitrile, 0.5 M) for 5 min at room temperature. The suspension was filtrated 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 trifluoroacetate (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 SiO$_2$ with CH$_2$Cl$_2$/CH$_3$OH, 100:0 to 95:5. Yield: 315 mg of **4** as a white foam (= 61%). TLC (CH$_2$Cl$_2$/CH$_3$OH = 95/5): R_f = 0.4. ^{1}H NMR (300 MHz, CDCl$_3$): δ 2.85 (d, J =8.7 Hz, 1H, HO-C(3′)); 3.50−3.65 (m, 4H, H^1−C(5′), H^2−C(5′), H^1−C(2″), H^2−C(2″)); 3.79 (s, 6H, H$_3$CO); 3.93−4.05 (m, 4H, H−C(2′), H−C(4′), H^1−C(1″), H^2−C(1″)); 4.42 (m, 1H, H−C(3′)); 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, HNCOCF$_3$); 8.05 (d, J =8.1 Hz, 1H, H−C(6)); 9.95 (s, 1H, N−H) ppm. ^{13}C NMR (150 MHz, CDCl$_3$): δ 39.75 (C(2″)); 55.39 (CH$_3$O); 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(5)); 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]$^+$ calcd for C$_{32}$H$_{33}$N$_5$O$_8$Na, 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 a *ABI 392 Nucleic Acid Synthesizer* following standard methods: detritylation (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: Ac$_2$O/*sym*-collidine/acetonitrile (2/3/5); oxidation (1.0 min) with I$_2$ (20 mM) in THF/pyridine/H$_2$O (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 MeNH$_2$ in EtOH (33%, 0.5 mL) and MeNH$_2$ in water (40%, 0.5 mL) for 7 h at room temperature. (For RNA containing 5-aminoallyl uridines, the column was first treated with 10% diethylamine in acetonitrile (20 mL), washed with acetonitrile (20 mL) and dried. Then, the solid support was treated with MeNH$_2$ in EtOH (33%, 1 mL) and NH$_3$ in H$_2$O (28%, 1 mL) for 10 min at room temperature and 20 min at 65 °C.) The supernatant was removed from and the solid support was washed three times with ethanol/water (1/1, v/v). The supernatant and the washings were combined with the deprotection solution of the residue and the whole mixture was evaporated to dryness. To remove the 2′-silyl protecting groups, the resulting residue was treated with tetrabutylammonium fluoride trihydrate (TBAF·3H$_2$O) in THF (1 M, 1 mL) at 37 °C overnight. The reaction was quenched by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 1 mL). The volume of the solution was reduced and the solution was desalted with a size exclusion column (*GE Healthcare, HiPrep 26/10 Desalting*; 2.6 × 10 cm; Sephadex G25) eluating with H$_2$O; the collected fraction was evaporated to dryness and dissolved in 1 mL H$_2$O. Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a *Dionex DNAPac PA-100* column (4 mm × 250 mm) at 80 °C. Flow rate: 1 mL/min, eluant A: 25 mM Tris·HCl (pH 8.0), 6 M urea; eluant B: 25 mM Tris·HCl (pH 8.0), 0.5 M NaClO$_4$, 6 M urea; gradient: 0−60% B in A within 45 min or 0−40% B in 30 min for short sequences up to 15 nucleotides, UV-detection at 260 nm.

Purification of 2′-O-(2-Azidoethyl) Modified RNA. Crude RNA products were purified on a semipreparative *Dionex DNAPac PA-100* column (9 mm × 250 mm) at 80 °C with flow rate 2 mL/min. Fractions containing RNA were loaded on a C18 *SepPak Plus* cartridge (*Waters/Millipore*), washed with 0.1−0.15 M (Et$_3$NH)$^+$HCO$_3^-$, H$_2$O and eluted with H$_2$O/CH$_3$CN (1/1). RNA containing fractions were lyophilized. Analysis of the quality of purified RNA was performed by anion-exchange chromatography with same conditions as for crude RNA; the molecular weight was confirmed by LC-ESI mass spectrometry. Yield determination was performed by UV photometrical analysis of oligonucleotide solutions.

Mass Spectrometry of 2′-O-(2-Azidoethyl) Modified RNA. All experiments were performed on a *Finnigan LCQ Advantage MAX* ion trap instrumentation connected to an *Amersham Ettan micro* 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 H$_2$O (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*), CuSO$_4$, and sodium ascorbate were added consecutively; acetonitrile was added as cosolvent[36] to reach final concentrations of 1 mM RNA, 2 mM dye, 5 mM CuSO$_4$, 10 mM sodium ascorbate, and a H$_2$O/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.[4,5,37] Lyophilized synthetic siRNA (for sequence see Figure 3 and Table S1) targeted against the chicken *BASP1* mRNA sequence 5′-CAGGUCUCUGCCAAU-AAGACA-3′, were dissolved in a buffer containing 100 mM potassium acetate, 30 mM Hepes-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 × 10^6 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 electroporation (*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 thiocycanate. After

addition of bromochloropropane, 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 *BASP1* or *GAPDH* mRNAs was performed as described previously.[37]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra for compounds **2**, **2a**, **2b**, and **4**; reduction of 2′-(2-azidoethyl) 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
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