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2′-Azido RNA, a Versatile Tool for Chemical Biology: Synthesis, X-ray Structure, siRNA Applications, Click Labeling

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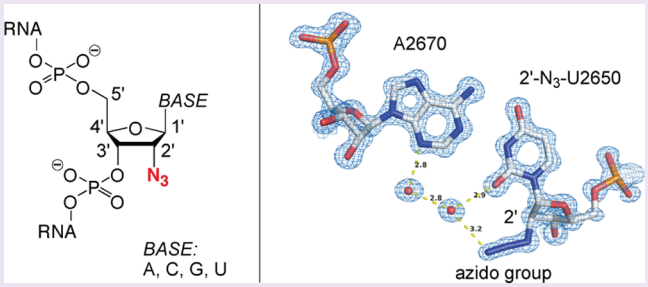
Supporting Information

ABSTRACT: Chemical modification can significantly enrich the structural and functional repertoire of ribonucleic acids and endow them with new outstanding properties. Here, we report the syntheses of novel 2′-azido cytidine and 2′-azido guanosine building blocks and demonstrate their efficient site-specific incorporation into RNA by mastering the synthetic challenge of using phosphoramidite chemistry in the presence of azido groups. Our study includes the detailed characterization of 2′-azido nucleoside containing RNA using UV-melting profile analysis and CD and NMR spectroscopy. Importantly, the X-ray crystallographic analysis of 2′-azido uridine and 2′-azido adenosine modified RNAs reveals crucial structural details of this modification within an A-form double helical environment. The 2′-azido group supports the C3′-*endo* ribose conformation and shows distinct water-bridged hydrogen bonding patterns in the minor groove. Additionally, siRNA induced silencing of the brain acid soluble protein (BASP1) encoding gene in chicken fibroblasts demonstrated that 2′-azido modifications are well tolerated in the guide strand, even directly at the cleavage site. Furthermore, the 2′-azido modifications are compatible with 2′-fluoro and/or 2′-*O*-methyl modifications to achieve siRNAs of rich modification patterns and tunable properties, such as increased nuclease resistance or additional chemical reactivity. The latter was demonstrated by the utilization of the 2′-azido groups for bioorthogonal Click reactions that allows efficient fluorescent labeling of the RNA. In summary, the present comprehensive investigation on site-specifically modified 2′-azido RNA including all four nucleosides provides a basic rationale behind the physico- and biochemical properties of this flexible and thus far neglected type of RNA modification.



The structural and functional repertoire of ribonucleic acids (RNA) can be significantly manipulated by chemical modifications. This is of particular importance for two major tools in current RNA research, namely, RNA interference (RNAi) and RNA bioconjugation. RNAi is a post-transcriptional gene silencing mechanism induced by small interfering RNA (siRNA) and micro-RNA (miRNA).[1,2] It has become one of the major tools for gene function analysis and has also been confronted with high expectations for the development of siRNA and miRNA as therapeutic agents to treat diseases.[3,4] However, for applications of siRNA as therapeutic agents, chemical modification is obligatory to enhance nuclease resistance, to prevent immune activation, to decrease off-target effects, and to improve pharmacokinetic and pharmacodynamic properties.[5−9] Additionally, the modifications should help these agents to penetrate cell membranes and improve siRNA delivery which remains one of the major challenges.[10] This latter issue closes the circle from siRNAs to bioconjugation since many approaches require an attachment of the nucleic acid portion to transporter units of diverse structure (such as peptides, lipids or cofactors) or labeling with fluorescence dyes to track the siRNAs in the cell. Both types of conjugates are accessible via modern bioorthogonal conjugation reactions.[11,12]

In a recent communication, we have originally reported on 2′-azido modified RNA.[13] Surprisingly, this modification had previously been neglected in the context of siRNA, most likely because of expected synthetic difficulties[14] with standard solid-phase RNA phosphoramidite chemistry based on the inherent reactivity between phosphor-III species and azides. Nevertheless, the prospect of possible siRNA applications,[6] but also of promising applications in modern bioconjugation chemistry,[11,12] prompted us to target 2′-azido RNA. We synthesized phosphodiester building blocks of 2′-azido-2′-deoxyuridine and 2′-azido-2′-deoxyadenosine and demonstrated that their coupling under standard conditions of RNA phosphotriester chemistry together with standard phosphoramidite chemistry

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for assembly of the other nucleotides works sufficiently well to achieve these RNA derivatives.[13]

Here, we present the synthesis of the novel phosphodiester building blocks of 2′-azido-2′-deoxycytidine **7** and 2′-azido-2′-deoxyguanosine **17** and expand the site-specific introduction of the 2′-azido modification into RNA to all four canonical nucleosides (Figure 1). Our study includes the detailed characterization of the 2′-azido modified RNA using UV, CD, and NMR spectroscopy. Importantly, we have solved the X-ray structures of 2′-azido containing model double helices at atomic resolution and we performed a series of experiments to evaluate their siRNA performance and potential for labeling using Click chemistry.

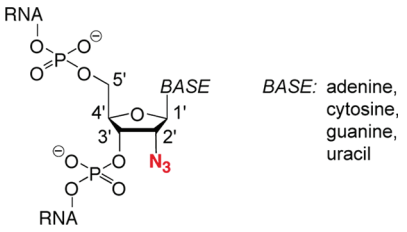


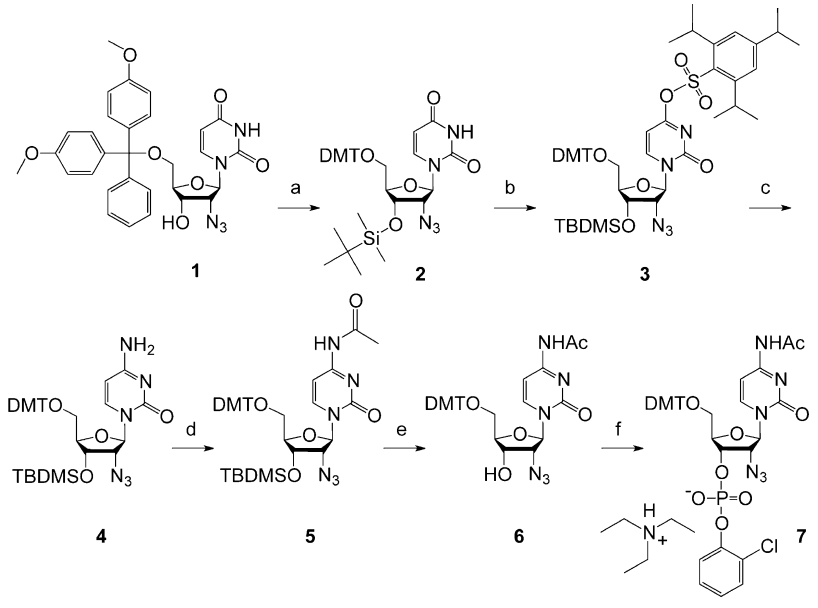
Figure 1. Structure of RNA with site-specifically 2′-azido-modified nucleosides. Such derivatives carry high potential for bioconjugations and applications in RNA interference.

RESULTS AND DISCUSSION

Synthesis of 2′-Azido Nucleoside Building Blocks. For building block **7** (Scheme 1), we started the synthesis from the 2′-azido-2′-deoxyuridine derivative **1**,[13] which was readily obtained from uridine. The 3′-OH of compound **1** was protected applying TBDMS chloride and imidazole in DMF to furnish derivative **2**. Then, reaction of **2** with 2,4,6-triisopropylbenzenesulfonyl chloride in the presence of triethylamine and DMAP in dichloromethane resulted in regioselective O^4-trisylation. After workup, the trisylated derivative **3** can be used without further purification and directly converted into **4** upon treatment with aqueous ammonium hydroxide in THF in 80% yield over the two steps (which is significantly higher compared to when compound **3** is isolated and purified by column chromatography). We also note that we applied aqueous ammonium hydroxide conditions based on our experience from a previous study where treatment of the 2′-methylseleno analog of **3** with 7 M NH_3 in anhydrous methanol resulted in the corresponding O^4-methyluridine derivative.[15] Acetylation of the amino function was then achieved with acetic anhydride in pyridine to provide **5**, followed by cleavage of the 3′-*O*-TBDMS group with 1 M TBAF and 0.5 M acetic acid in THF to give **6**. Finally, conversion into the corresponding phosphodiester **7** was achieved in good yields by reaction with in situ generated 2-chlorophenyl chlorophosphorotriazolide in analogy to a general procedure in the literature.[17] Starting with compound **1**, our route provides **7** in a 43% overall yield in six steps with four chromatographic purifications; in total, 2.1 g of **7** was obtained in the course of this study.

For building block **17** (Scheme 2), our route began with the tetraisopropyldisiloxane (TIPDS) 3′- and 5′-protected guanosine derivative **8**,[16] followed by protection of the exocyclic guanine 2-amino group using *N*,*N*-dimethylformamide dimethyl acetal to furnish derivative **9** and protection of the guanine lactam moiety with a O^6-(4-nitrophenyl)ethyl group introduced under Mitsunobu conditions to give **10**. Then, triflation of the ribose 2′-OH resulted in intermediate **11**, which was converted into the arabino nucleoside **12** in diastereoselective manner by treatment with potassium trifluoroacetate and 18-crown-6-ether. After triflation of the arabinose 2′-OH, compound **13** was reacted with lithium azide, producing key derivative **14** in high yields. Deprotection of the TIPDS moiety proceeded

Scheme 1. Synthesis of 2′-Azido Cytidine Building Block 7[a]



[a]Reaction conditions: (a) 2.0 equiv TBDMSCl, 4.0 equiv imidazole, in DMF, RT, 16 h, 95%; (b) 1.5 equiv 2,4,6-triisopropylbenzenesulfonyl chloride, 10.0 equiv NEt_3, 0.12 equiv DMAP, in CH_2Cl_2, RT, 1 h, 60%; (c) 32% aqueous NH_3, in THF, RT, 16 h, 95%; (d) 2.5 equiv acetic anhydride, in pyridine, 0°C to RT, 90 min, 97%; (e) 1 M TBAF/0.5 M acetic acid, in THF, RT, 2.5 h, 100%; (f) 5.5 equiv 1,2,4-triazole, 5.0 equiv NEt_3, 2.5 equiv 2-chlorophenyl phosphorodichloridate, 4.0 equiv 1-methylimidazole in THF, RT, 1 h, 82%.

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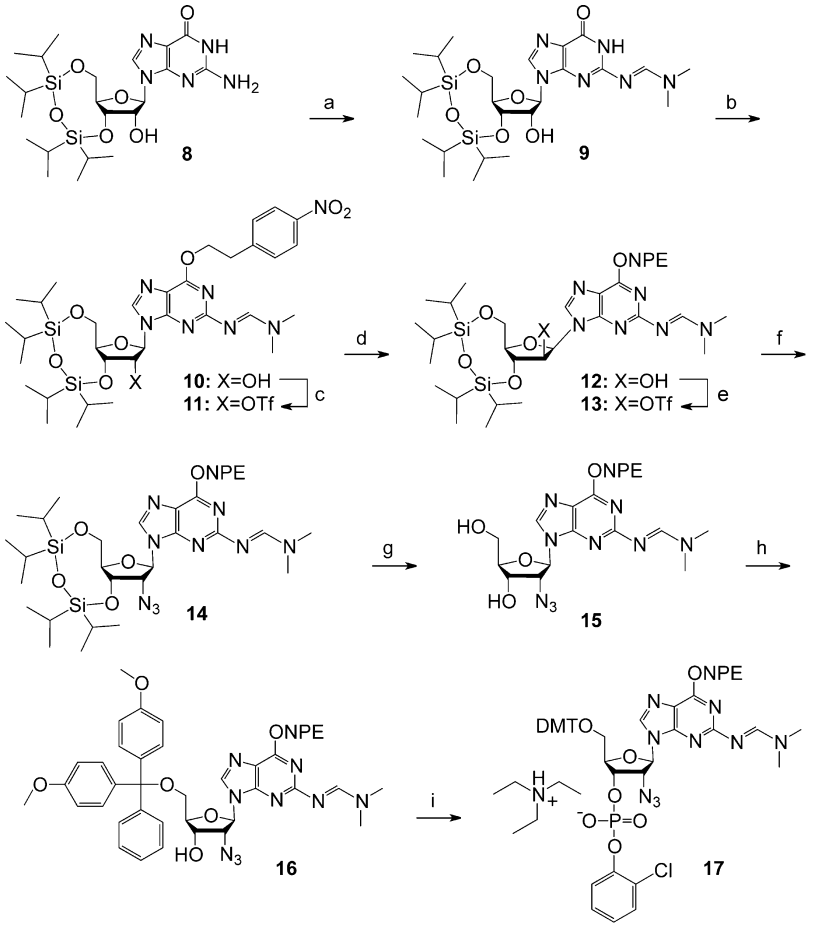
Scheme 2. Synthesis of 2′-Azido Guanosine Building Block 17[a]



[a]Reaction conditions: (a) 2.5 equiv *N*,*N*-dimethylformamide dimethyl acetal, in DMF, 6 h, RT, 84%; (b) 1.3 equiv NPE-OH, 1.4 equiv PPh_3, 1.3 equiv DIAD, in dioxane, 16 h, RT, 40%; (c) 1.5 equiv trifluoromethanesulfonyl chloride, 3.0 equiv DMAP, in CH_2Cl_2, 30 min, 0°C, 44%; (d) 5.0 equiv $CF_3COO^-K^+$, 2.0 equiv 18-crown-6, 1.5 equiv $(iPr)_2NEt_3$ in toluene, 16 h, 80°C, 99%; (e) 1.5 equiv trifluoromethanesulfonyl chloride, 2.5 equiv NEt_3, 1.5 equiv DMAP, in CH_2Cl_2, 15 min, 0°C, 82%; (f) 5.0 equiv LiN_3, in DMF, 16 h, RT, 71%; (g) 1 M TBAF/0.5 M AcOH, in THF, 2.5 h, RT, 93%; (h) 1.4 equiv DMT-Cl, in pyridine, 16 h, RT, 91%; (i) 5.5 equiv 1,2,4-triazole, 5.0 equiv NEt_3, 2.5 equiv 2-chlorophenyl phosphorodichloridate, 4.0 equiv 1-methylimidazole in THF, RT, 1 h, 70%.

straightforward using tetrabutylammonium fluoride (TBAF) and acetic acid. Finally, derivative **15** was transformed into the dimethoxytritylated compound **16**, and conversion into the corresponding phosphodiester **17** was achieved in good yields by reaction with in situ generated 2-chlorophenyl chlorophosphorotriazolide in analogy to a general procedure in the literature.[17] Starting with compound **8**, our route provides **17** in a 5% overall yield in nine steps with seven chromatographic purifications; in total, 0.5 g of **17** was obtained in the course of this study. A recent report on the synthesis of 2′-methylselenoguanosine[18] suggested that protection of O^6 could be omitted to shorthen the synthesis of **17**; however, in our hands, we observed significant triflation at O^6 when the lactam moiety was unprotected.

Synthesis of 2′-Azido Modified RNA. For the incorporation of the 2′-azido-modified nucleoside phosphodiester building blocks **7** and **17** into RNA, we conducted strand assembly by automated standard RNA solid-phase synthesis using 2′-*O*-TOM protected nucleoside phosphoramidites[19] up to the position of the intended azide modification. The synthesis was interrupted after the detritylation step that liberated the terminal 5′-hydroxyl group. Then, coupling of the phosphodiester building block **7** or **17** was achieved manually by activation with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT)[20] in yields up to 95%. After capping, strand elongation was continued by automated RNA phosphoramidite chemistry. Up to an elongation of about 25 nucleotides we had no indication (from inspection of oligonucleotide byproduct by LC−ESI mass spectrometry) that the ribose 2′-azido group reacted with the phosphoramidite moiety under the coupling conditions used. For deprotection, the oligoribonucleotides containing 2′-azido groups were first treated with *syn*-2-pyridine aldoxime/tetramethylguanidine in dioxane/water to cleave the 2-chlorophenyl phosphate protecting groups.[20] Then, standard deprotection conditions using CH_3NH_2 in ethanol/water followed by treatment with 1 M TBAF in THF were applied. We point out that the O^6-(4-nitrophenyl)ethyl groups are stable during the first deprotection step (CH_3NH_2 in ethanol/water) but become cleaved during the second step of deprotection (TBAF in THF) when fluorine anions induce β-elimination to release the lactam moieties of the guanine nucleobases.[21−23] After purification by anion-exchange HPLC (Supporting Figure 1), the expected molecular weights were confirmed for all RNAs by LC−ESI mass spectrometry (Table 1, Supporting Figure 1).

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Table 1. Selection of 2′-Azido Modified RNAs[a]

sequence (5′→3′)	yield [nmol]	molecular weight [amu] calcd	obsd
GGC^{N3}UAGCC	90	2549.6	2550.0
GAAGGGCAACC^{N3}UUCG	159	4838.9	4838.9
GGUCUCUGCC^{N3}AAUAAGACATT	26	6678.1	6677.8
GGUCUCUGC^{N3}CAAUAAGACATT	15	6678.1	6677.4
UGUCUUAUUGGC^{N3}AGAGACCTdG	332	6697.1	6696.7
UGUCUUAUUGGCAGAGACC^{N3}TdG	394	6697.1	6697.2
GGCUAG^{N3}CC	110	2549.6	2549.6
UGUCUUAUUG^{N3}GCAGAGACCTdG	90	6697.1	6696.5
UGUCUUAUUGG^{N3}CAGAGACCTdG	42	6697.1	6696.5
UGUCUUAUUGGCAG^{N3}AGACCTdG	10	6697.1	6696.6

[a] C^{N3}, 2′-N_3 cytidine; G^{N3}, 2′-N_3 guanosine

Pairing Stability of 2′-Azido Modified RNA. Next, we investigated the influence of the 2′-azido group on the thermal stability of RNA double helices by temperature-dependent UV spectroscopy (Supporting Figure 2). At 150 mM NaCl and 10 mM Na_2HPO_4 (pH 7.0), the non-modified, self-complementary RNA 5′-GGCUAGCC-3′ melted at 62.2 ± 0.5 °C (c_{RNA} = 16 μM), while the 2′-azido guanosine modified counterpart 5′-GGCUAG^{N3}CC-3′ displayed a T_m value of 59.3 ± 0.5 °C (c_{RNA} = 16 μM) (Supporting Figure 2A). This demonstrated that the 2′-azido group causes a slight decrease in thermal stability, about −1.4 °C per modification since the influence stems from *two* isolated 2′-N_3-G/C base pairs (bp) within the palindromic duplex. A detailed determination of the thermodynamic stabilities based on the concentration dependence of their melting points[24] provided a $\Delta G°$ of −13.7 ± 0.3 kcal/mol for the unmodified duplex and −13.3 ± 0.3 kcal/mol for the 2′-N_3-G containing duplex (Supporting Figure 3). Additionally, we evaluated the influence on the thermal stability of the corresponding 2′-azido cytidine nucleoside and therefore compared the hairpin forming RNA 5′-GAAGGGCAAC-CUUCG to its 2′-azido cytidine modified counterpart 5′-GAAGGGCAACC^{N3}UUCG. While the non-modified RNA melted at 71.7 ± 0.5 °C, the modified hairpin showed a minimally increased T_m value of 72.3 ± 0.5 °C (Supporting Figure 2C). As expected for a monomolecular melting transition, the melting points did not change in response to varying concentration (c_{RNA} = 2−32 μM). Shape analysis of the melting curves[24] provided the thermodynamic stabilities of a $\Delta G°$ of −6.6 ± 0.3 kcal/mol for the unmodified duplex and −6.5 ± 0.3 kcal/mol for the 2′-N_3-C containing duplex (Supporting Figure 4).

CD and NMR Spectroscopic Analysis. We applied CD spectroscopy to the duplex and hairpin systems, and the spectra clearly indicated that the overall conformation of a typical A-form double helical architecture was retained for the RNA double helices with 2′-azido-C or 2′-azido-G containing base pairs (Supporting Figure 2B,D).

The ^{1}H NMR spectra of the duplex and hairpin systems described above were recorded, and we observed that the chemical shift as well as the line shape of the imino proton resonances were again hardly affected by the modification (Figure 2). As these resonances reflect the integrity of Watson−Crick base pairs, the ^{1}H NMR data suggests that 2′-azido-C or 2′-azido-G containing base pairs are well tolerated within RNA double helices in aqueous buffer solution.

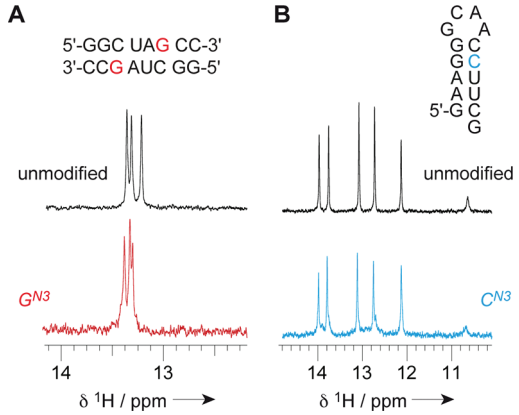

Figure 2. ^{1}H NMR imino proton spectra of 2′-azido-modified oligoribonucleotides. (A) GGCUAGCC (top) and GGCUAG^{N3}CC (bottom). (B) GAAGGGCAACCUUCG (top) and GAAGGG-CAACC^{N3}UUCG (bottom); conditions: c_{RNA} = 0.1 mM, 25 mM Na_2HAsO_4, pH 7.0. C^{N3}, 2′-N_3 cytidine; G^{N3}, 2′-N_3 guanosine.

X-ray Analysis of a 2′-Azido Containing RNA. Encouraged by the NMR spectroscopic results, we set out for the X-ray analysis of a 2′-azido modified RNA. We focused on the 27 nt fragment of *E. coli* 23 S rRNA sarcin-ricin loop (SRL) region[25,26] as a crystallization scaffold. The modification of interest was placed in the double helical region of this scaffold to obtain insights on the impact of a 2′-azido group within an A-form RNA double helix. Analysis of the SRL structure (Protein Data Bank [PDB] identification no. 483D) revealed that the 2′-OH groups of U2650 and A2670 are not involved in crystal contacts and hence should be available for modifications. Moreover, these two nucleotides are forming a Watson−Crick base pair in a double helical region. Three SRL sequences were therefore synthesized: an unmodified SRL (used as a control for crystallization and diffraction) and two modified sequences with the introduction of a 2′-azido group into U2650 or A2670 (Figure 3, Supporting Figure 5 and Supporting Figure 6). Crystallization trials revealed that both 2′-azido modified RNAs crystallized, providing crystals diffracting to atomic resolution (Supporting Table 2). X-ray structure determinations showed that the azido groups are well-defined in the electron density maps for both 2′-azido modified RNAs (Figure 3). Superimpositions of both azido-modified RNA structures with the unmodified RNA revealed a root-mean-square deviation (rmsd) of 0.07 and 0.14 Å. These values were within the errors on coordinates (0.13 and 0.11 Å), thus showing that the 2′-azido group does not affect the RNA structure (Figure 4). However, detailed analysis of the RNA hydration pattern disclosed a displacement of several water molecules from the RNA major groove in presence of the 2′-azido group (Figure 4B,C). In addition, a water molecule appears to be involved into an intraresidue U(O2)/water/2′-azido interaction in the 2′-N_3-U2650 structure (Figure 3B). These modifications of the RNA hydration shell might be responsible for the slight changes in melting temperatures compared to the unmodified RNA. Additionally, another possible contribution of the decrease in thermal stability could also be a small, but significant, influence of the azido group on the nucleobase polarization, as recently postulated for a 2′-F-modified RNA.[27]

RNA Interference of 2′-Azido Modified siRNA. We have recently pointed at the promising potential of 2′-azido modified RNA for RNA interference, exemplified by using siRNA duplexes with single and double 2′-azido uridine and/or 2′-

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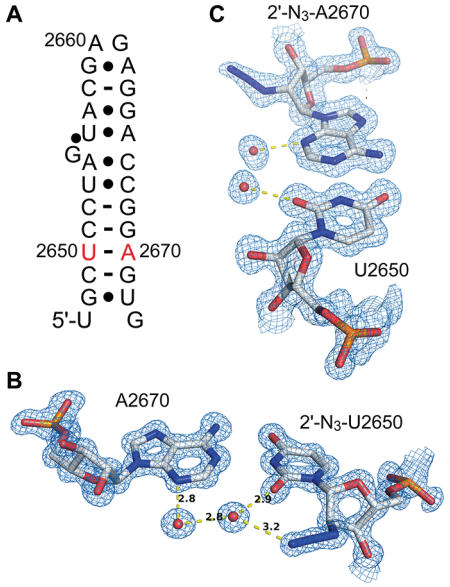

Figure 3. X-ray structure of 2′-azido modified RNA at atomic resolution. (A) *E. coli* Sarcin-ricin stem-loop (SRL) RNA used for crystallization; secondary structure. (B) $2F_{obs} - F_{calc}$ electron density map showing the 2′-N_3-U2650/A2670 base pair. Water molecules are shown as red spheres. (C) $2F_{obs} - F_{calc}$ electron density map showing the U2650/2′-N_3-A2670 base pair.

azido adenosine derivatizations.[13] Here, we expand these investigations toward 2′-azido cytidine and 2′-azido guanosine to further evaluate 2′-azido-modified oligoribonucleotides for siRNA applications. For reasons of comparability, we employed the same model system used previously to knock down the brain acid soluble protein 1 (BASP1) encoding gene by transient siRNA nucleofection in the chicken DF-1 cell line.[13,28] Expression of the *BASP1* gene is specifically suppressed by Myc, an evolutionary conserved oncoprotein;[29] conversely, the BASP1 protein is an efficient inhibitor of *Myc*-induced cell transformation.[28] First, we synthesized six siRNA duplexes for the *BASP1* target gene with the sequence organization depicted in Figure 5A (see also Supporting Table 1), three of them with a single 2′-azido-2′-deoxycytidine and three of them with a single 2′-azido-2′-deoxyguanosine modification, all of the modifications in either very close vicinity, or directly at the cleavage site. The modified siRNAs caused significant gene silencing as observed for the non-modified reference duplex (Figure 5B), with the highest remaining level of expression of about 10% observed for "SIR Az-G14 as". Remarkably, the 2′-azido group is very well tolerated in the guide strand, even when the site of modification is directly at the cleavage site (positions 10, 11). As expected, the modification caused increased nuclease resistance (Supporting Figure 7). Moreover, we compared the 2′-azido modified siRNAs to their 2′-fluoro and 2′-*O*-methyl counterparts and found comparable silencing efficiencies (Figure 5C). Additionally, we mixed 2′-azido, 2′-fluoro and 2′-*O*-methyl modifications up to a number of seven within the antisense strand (Figure 5C). Some of these modified duplexes showed more reduced efficiencies and remaining expression levels of about 25−35%, even for non-azido siRNA species. Reduced efficiency was also observed for a siRNA type with a total of two 2′-fluoro (positions 9, 11) and three 2′-*O*-methyl groups (positions 3, 7, 15). When we added two 2′-azido modifications to either positions 8 and 12 or 8 and 13, the expression levels were slightly increased, thereby less for the first mentioned substitution pattern. In a further experiment, we demonstrated that the level of suppression of these highly modified siRNAs was dose-dependent (Supporting Figure 8). Moreover, time-course experiments showed a significant level of suppression even after 2 days of incubation for the unmodified as well as the highly modified siRNA duplexes, whereas after 9 days full expression levels were reached again for all four duplexes tested (Supporting Figure 9). Taken together, our siRNA data demonstrates that the 2′-azido group represents an interesting and powerful alternative that complements the existing repertoire of 2′-modifications for siRNA applications.

Labeling of 2′-Azido RNA: Click Chemistry. To further underline the high chemical flexibility of 2′-azido modified RNA strands, we demonstrated their amenability to one of the most widely used bioconjugation reaction, namely ,the azide−alkyne 1,3-dipolar cycloaddition reaction, commonly referred to as Click chemistry.[11,12] We chose the copper-catalyzed version with acetonitrile as cosolvent acting as ligand of the Cu^{I} complex, thereby stabilizing the oxidation state.[30] Applying this setup, 2′-azido modified *BASP1* siRNA 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 6A,B).

While the siRNA labeled in standard fashion at the 3′-end of the *sense* strand was of the same activity compared to the

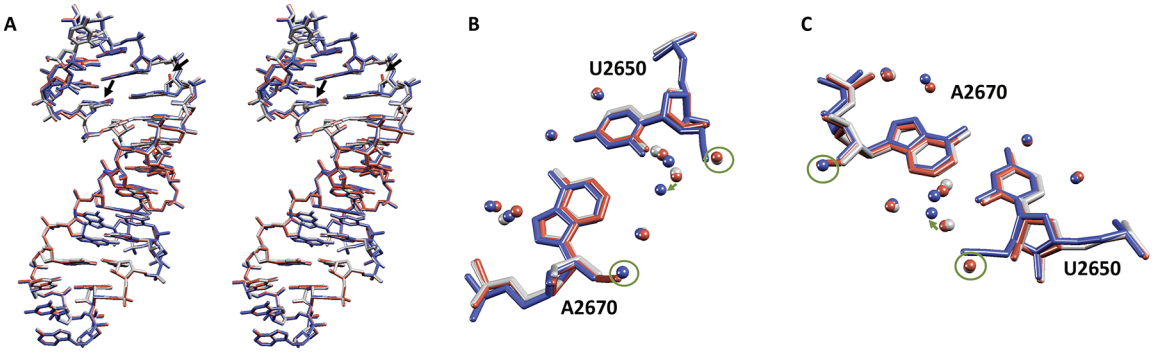

Figure 4. Structure comparison of 2′-azido modified and unmodified SRL RNA. (A) Stereoview showing a superposition of unmodified (gray), 2′-N_3-A2670 (red), and 2′-N_3-U2650 (blue) RNA; arrows indicate positions of 2′-N_3 groups. (B, C) Detailed views (two different orientations) of the U2650-A2670 base pair and hydration in superposed RNA structures with and without 2′-N_3 modification. A water molecule hydrogen-bonded with 2′-OH and O2 of U2650 is shifted in the 2′-N_3-U2650 SRL structure (green arrow).

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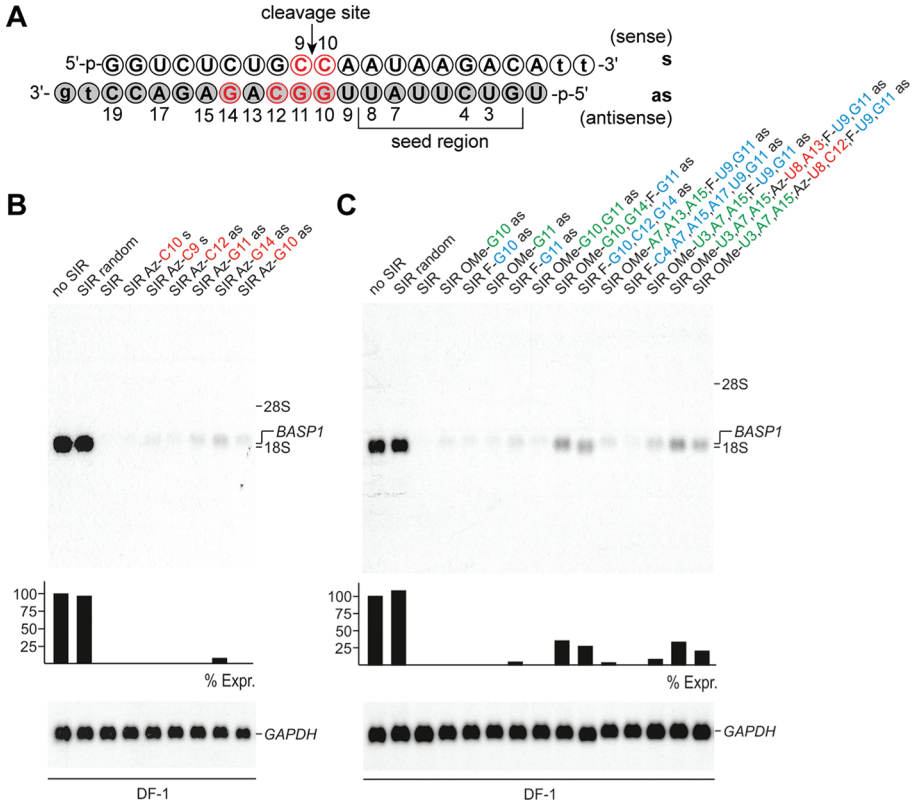

Figure 5. Gene silencing by 2′-azido modified siRNAs. (A) Sequence of the brain acid soluble protein 1 (BASP1)[13,28] targeting siRNA duplex used in this study; nucleosides in red indicate positions for 2′-azido modification tested. (B) Biological activities of 2′-azido cytidine or 2′-azido guanosine modified siRNAs. (C) Biological activities of 2′-OMe (green), 2′-F (blue), and 2′-N_3 (red) modified siRNAs, directed against *BASP1* mRNA. Chicken DF-1 cells grown on 60 mm dishes were transiently nucleofected with 0.12 nmol (~1.5 μg) aliquots of unmodified (SIR) or modified siRNAs (SIR Az, SIR OMe, SIR F) containing azido, methoxy, or fluoro groups at the indicated nucleotides on sense (s) or antisense (as) strands. An equal aliquot of siRNA with a shuffled (random) nucleotide sequence was used as a control. Total RNAs were isolated 2 days after siRNA delivery, and 5-μg aliquots were analyzed by Northern hybridization using a DNA probe specific for the chicken *BASP1* gene, and subsequently a probe specific for the housekeeping quail *GAPDH* gene.[28] The levels (%) of *BASP1* expression were determined using a phosphorimager and are depicted as bars relative to mock transfections (no SIR, 100%). The electrophoretic positions of rRNAs are indicated in the margin. All siRNAs depicted contain overhangs of 2′-deoxynucleosides (lower case letters). SIR random: 5′-UCUGGGUCUAAGCCAAACAUT/5′-UGUUUGG-CUUAGACCCAGAUdG.

unmodified reference, siRNAs with the fluorophore attached to the *antisense* strand at internal nucleoside positions exhibited reduced activities (Figure 6C). The latter is not unexpected due to the stringent structural requirements for strand recognition within the RISC complex.[6] However, since a very recent study has reported on successful examples of fluorescent labeling of the antisense strand,[31] we also evaluated our type of labeling for three selected internal antisense positions. We observed significant but reduced efficiencies for two of the corresponding siRNAs (C19, G11), while the C12-labeled counterpart had less activity (Figure 6C). Nevertheless, the fluorescently labeled siRNAs allowed their reliable localization within the chicken DF1 cells by fluorescence microscopy (Figure 6D). Taken together, this set of experiments demonstrates that the 2′-azido group of our RNA derivatives can efficiently react under Click conditions and is therefore open to the wide field of bioconjugation and applications.

Summary, Reflection, and Conclusion. This study reports clear evidence of the accessibility of site-specifically 2′-azido modified RNA with respect to all four standard nucleosides. Importantly, the azido nucleosides, once incorporated into RNA by individual cycles of phosphotriester chemistry, are compatible with subsequent strand assembly using phosphoramidite chemistry. It is precisely this point that has caused confusion in the recent literature with reports explicitely claiming that phosphoramidite chemistry would not work in the presence of azide functionalities.[14,32] On the basis of our studies, we can confirm incompatibility only at the level of nucleosides, as experienced by unsuccessful trials to synthesize 2′-azido nucleoside phosphoramidite building blocks; however, we and others observed clear compatibility with strand elongation of azide containing DNA[33,34] and RNA.[35] We further consider that the combination of phosphotriester chemistry for azido nucleoside building blocks and phosphoramidite chemistry for strand elongation could, in principle, be replaced by a uniform phosphonate strategy (see also ref 33). Nevertheless, we believe that a combined approach (as used here) is highly attractive due to the widespread use of phosphoramidite chemistry in most synthetic nucleic acid laboratories, and therefore one may expect a more rapid dissemination. We also mention that for RNA targets carrying multiple azido groups a uniform phosphonate chemistry is expected to be of higher efficiency due to straightforward synthesis automatization.

With the synthetic tool in hands to generate 2′-azido modified RNA at any of the four nucleosides, we were able to study their structural features in detail. The crystal structure revealed that the modification is very well tolerated and

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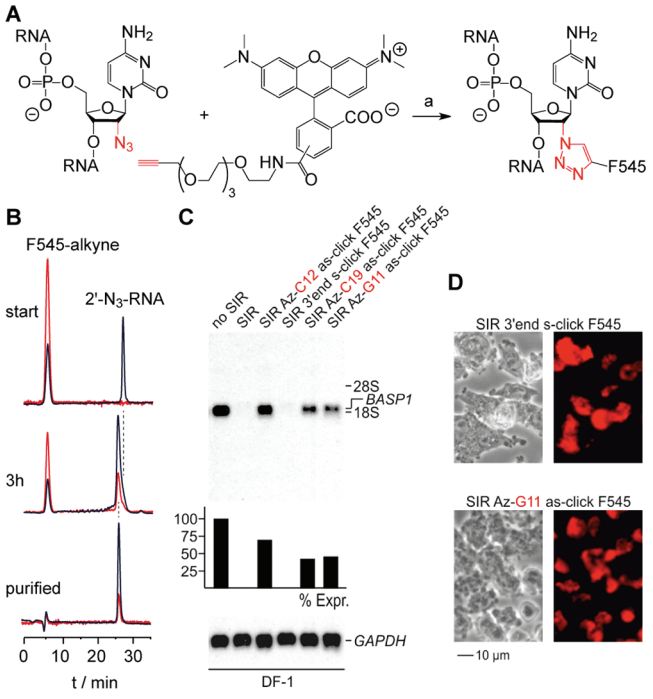

Figure 6. Internal labeling of selectively 2′-azido modified RNAs with a fluorescent dye (F545) using Click chemistry. (A) Reaction scheme: (a) 5 mM $CuSO_4$, 10 mM sodium ascorbate, 50 °C, 3 h; c_{RNA} = 1 mM, c_{Dye} = 2 mM, H_2O/CH_3CN = 4/1; 60 μL total reaction volume. (B) Reaction analysis of F545-alkyne and UGUCUUAUUGGCAGAGAC-(C^{N3}*19*)TdG followed by anion exchange chromatography. (C) Activities of G11-, C12-, and C19-labeled *BASP1* siRNA and corresponding controls (unmodified siRNA and siRNA with 3′-end F545-labeled sense strand); for conditions see Figure 5. (D) *BASP1* siRNA with internal (G11) 2′-azido-clicked F545 dye at the antisense strand (bottom) and *BASP1* control siRNA with standard 3′-end sense strand labeling (top) show cytoplasmic localization in DF1 cells visualized by fluorescence microscopy. The amounts of nucleofected siRNAs were 0.24 nmol (C) or 0.48 nmol (D), respectively.

accommodated in the minor groove, with the formation of water-bridged hydrogen bonding networks to the azide. Compared to the structure of the unmodified RNA, the spine of hydration is altered slightly, in a way that the larger azide group replaces a water molecule. These minor structural changes are in line with the hardly affected biophysical characteristics obtained from CD and NMR spectroscopy. Also, the influence on thermodynamic stabilites (analyzed by UV melting curve analysis) were rather small.

To address the functional repertoire of 2′-azido modified RNA, we explored two aspects, namely, siRNA applications and bioconjugation. Concerning the first matter, the azide modification accounts for a structurally non-perturbing alteration, such as 2′-OCH_3 and 2′-F, and like these behaves in comparable manner with respect to its performance in the siRNA system tested. In particular, the 2′-azido group possesses the extraordinary property of being well accepted in the guide (antisense) strand, while most other modifications are much better tolerated in the passenger (sense) strand.[8,31,36−38] The 2′-azido modification hence complements the existing set of ribose modifications so far used in siRNA technologies, however, with the major advantage that it brings in additional chemical reactivity. This reactivity can be exploited for labeling of the RNA as exemplified here by introduction of fluorescent dyes.

So far, the use of the Click reaction for RNA labeling has relied predominantly on alkyne modified nucleic acids, while the labeling partner was modified with an azide.[39−45] The feasibility of the present synthetic approach now opens reverse attachment of the required functional groups, thereby increasing flexibility. This will be of high significance for selective and internal nucleic acid labeling with multiple dyes on the same RNA, as for example requested by multicolor single-molecule FRET techniques[46] or for nucleic acid cyclization,[47] lariat formation or branching.[48] The possibility of reverse attachments will be of further interest in the emerging field of RNA nanotechnologies.[49,50]

The 2′-azido modification accounts for the rare and exceptional type of RNA modification that on the one hand is well accommodated within the RNA, thereby leaving biological recognition processes in cellular systems largely unhindered, and on the other hand confers bioorthogonal reactivity to the system. Allocating this functional group and its properties at the ribose 2′ position that is equivalent in any of the four canonical nucleosides makes this modification a highly reliable and flexible tool for RNA chemical biologists.

METHODS

For the synthesis of 2′-azido building blocks **7** and **17** and for solid-phase synthesis, deprotection, purification, and mass spectrometry of 2′-azido modified RNA, see the Supporting Information.

X-ray Crystallography. The 27-nucleotide SRL hairpin was crystallized as described.[25] This sequence was chosen as a test case since crystallization conditions easily produce well-diffracting crystals. Crystals were grown for 3 days at 20 °C for unmodified SRL sequence, but 3 months were required for 2′-N_3-A2670-modified SRL and 1 year for 2′-N_3-U2650-modified SRL. Crystals were cryoprotected for about 5 min in a reservoir solution containing 15% (v/v) of glycerol and 3.5 M ammonium sulfate and flash-frozen in liquid ethane for data collection. The significant increase in time required for growing crystals of both 2′-azido modified SRL sequences led to an important non-merohedral crystal twinning that could be detected only during X-ray diffraction (splitting of diffraction spots). Reasonably good data could however be collected using the highly focused beam of the X06SA beamline at the SLS synchrotron. Data were processed with the XDS Package.[51] Structures were refined with PHENIX.[52] A significant twinning fraction (20.5%) was detected for 2′-N_3-U2650-modified SRL crystals. The structure was refined against twinned data using PHENIX.

Click Labeling. For labeling, 2′-azido 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[30] to reach final concentrations of 1 mM RNA, 2 mM dye, 5 mM $CuSO_4$, 10 mM sodium ascorbate, and a H_2O/acetonitrile ratio of 4:1 in a total reaction volume of 60 μL. The reaction mixture was degassed and stirred for 3−4 h under argon atmosphere at 50 °C. To monitor the reaction and to purify the reaction mixtures, anion exchange HPLC was used as described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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