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Diazirine-containing tag-free RNA probes for efficient RISC-loading and photoaffinity labeling of microRNA targets

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ABSTRACT

We designed and synthesized a photo-reactive and tag-free RNA probe for the identification of microRNA (miRNA) targets. To synthesize the RNA probe, we designed a novel nucleoside analog 1-*O*-[3-ethynyl-5-(3-trifluoromethyl-3*H*-diazirine-3-yl)]benzyl-β-D-ribofuranose containing aryl trifluoromethyl diazirine and ethynyl moieties. The RNA probe containing this analog was observed to form crosslinks with complementary RNA by UV irradiation and was rapidly tagged by Cu-catalyzed azide alkyne cycloaddition (CuAAC). In addition, the tag-free and photo-reactive miRNA-145 probe showed comparable gene silencing activity to that of unmodified miRNA-145. Therefore, miRNA probes containing the nucleoside analog are promising candidates for the identification of target mRNAs of miRNAs.

MicroRNAs (miRNAs) are small double-stranded RNAs composed of 20–23 base pairs that regulate gene expression by sequence-specific binding to target mRNAs.[1] During biosynthesis of miRNAs, initially, the primary-miRNA (pri-miRNA) is transcribed in the nucleus and subsequently cleaved by DROSHA/DGCR8 to generate a precursor-miRNA (pre-miRNA) having a stem-loop structure comprising ~70 bases.[2] The pre-miRNA is then transported to the cytoplasm via Exportin-5 and processed by Dicer to create a mature miRNA duplex.[3] The miRNA forms a RNA-induced silencing complex (RISC) with Argonaute protein, and one strand of the miRNA duplex is dissociated from RISC. This mature RISC binds to the target mRNA via the sequence complementary to the antisense (guide) strand remaining in RISC, thereby promoting the degradation and/or inhibition of translation of the target mRNA.[4] In recent years, it has been reported that abnormal expression of miRNAs is involved in various diseases, and thus the functions of miRNAs have been well-studied in the medical field.[5–10] However, as miRNAs tolerate mismatched base pairs and bind to multiple target mRNAs, the identification of target mRNAs of a miRNA is time-consuming and often costly. Several groups have reported methods for purifying target mRNAs of each miRNA using miRNA probes modified with a biotin tag.[11–14] We also succeeded in photoaffinity labeling of target mRNAs of miRNA-145 using a miRNA probe containing a photo-reactive nucleoside analog **1** and biotin tag at the 3′-end of the antisense strand (Fig. 1).[15,16] The miRNA probes modified with analog **1** can be expected to comprehensively label target mRNAs consisting of unknown sequences because of the sequence-independent high reactivity of the trifluoromethyl aryl diazirine moiety. In contrast, it has been reported that the introduction of biotin at the 3′-end of the antisense strand of miRNAs disturbs the formation of RISC.[17] Therefore, miRNA probes biotinylated at the 3′-end of antisense strands are considered to be unsuitable for the identification of target mRNAs of a miRNA.

Based on these findings, here, we designed and synthesized a novel nucleoside analog **2** for the development of a tag-free photo-cross-linking miRNA probe (Fig. 1). As analog **2** has an ethynyl group in addition to the photo-reactive diazirine moiety, the biotin tag can be introduced by Cu-catalyzed azide alkyne cycloaddition (CuAAC) after a photo-crosslinking reaction. Thus, we considered that by using the analog **2**-containing tag-free miRNA probe, the efficient photoaffinity labeling of target mRNAs would proceed without disturbing the RISC formation.

The synthetic route of analog **2** and its phosphoramidite **9** is shown in Scheme 1. 3-Ethynyl-5-[3-(trifluoromethyl)-3*H*-diazirine-3-yl]benzyl alcohol **4** was synthesized according to a previously reported method.[18] A β-anomer **5** was synthesized by glycosydation of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**3**), which was commercially available, with **4**. The benzoyl group of **5** was deprotected using a catalytic amount of $NaOCH_3$ in CH_3OH to produce **2** in 86% yield, and the primary hydroxyl group was protected by 4,4′-dimethoxytrityl (DMTr)

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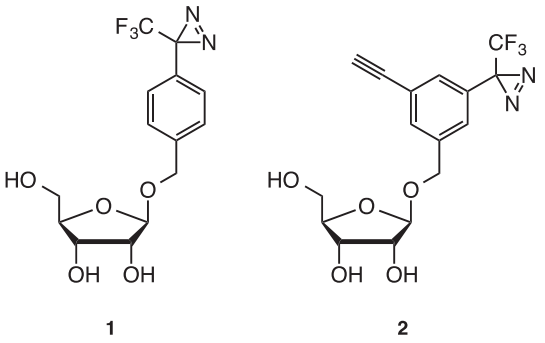


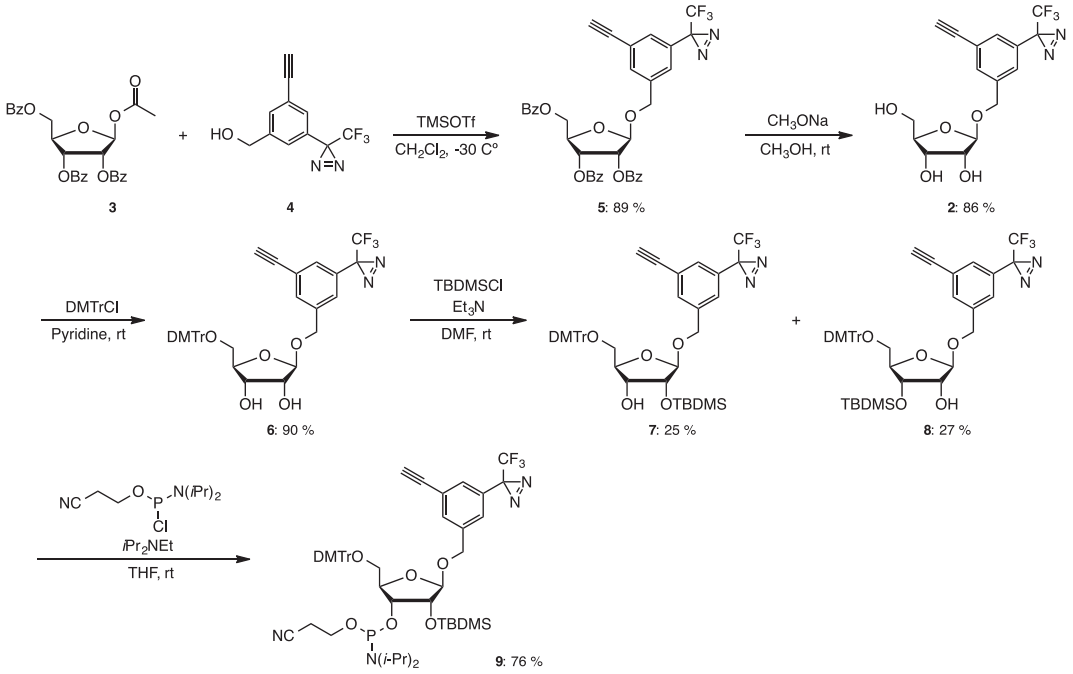
Fig. 1. The structures of nucleoside analogs.

group to obtain the 5-*O*-DMTr derivative **6** in 90% yield. Compound **6** was reacted with TBDMSCl in DMF, resulting in 2-*O*-protected **7** and 3-*O*-protected **8** in yields of 25 and 27%, respectively. After purifying 2-*O*-protected **7** by column chromatography, the remaining hydroxyl group was phosphitylated by a standard procedure, thereby producing the corresponding phosphoramidite **9** in 76% yield.

All the RNAs were synthesized using a DNA/RNA synthesizer (Table 1). RNA 1 and 9, which contain analog **2**, were synthesized using phosphoramidite **9**. The 3′-biotinylated RNA 7 and RNA 8 were synthesized according to our previous report.[16] RNA 10, which is the sense strand of miRNA-145, was annealed with RNA 6–9 to prepare the four miRNA probes 1–4 (Table 2).

First, we evaluated the photo-crosslinking ability of the RNA probe containing analog **2**. Analog **2**-containing RNA 1 was annealed with fluorescently labeled target RNA 2–5, which contains 4 different bases at the complementary site of analog **2**. The mixtures were irradiated with 365 nm UV-A for 30 min and then 302 nm UV-B for 10 min at 0 °C. Subsequently, the reaction mixtures were separated by 20% denaturing polyacrylamide gel electrophoresis (PAGE), and then the photo-crosslinking reaction was evaluated (Fig. 2). The band with slower mobility observed in the lane combining RNA 1 and 4 was extracted and analyzed by MALDI-TOF/MS (Fig. S17). As a result, the observed mass was in agreement with the calculated crosslinked structure. Furthermore, as the bands with slower mobility were observed in all four lanes, it was found that analog **2** can photo-crosslink to all four natural nucleosides of its complementary site. Therefore, we concluded that analog **2** can also be applied to identify target mRNAs consisting of unknown sequence.

Next, we verified the introduction of a biotin tag to the analog **2**-containing RNA probe by CuAAC (Scheme 2). To RNA 1, 80 equivalents of Biotin-TEG-azide (**10**)[19] was added in the presence of Na ascorbate,



Scheme 1. Synthesis of a photo-reactive nucleoside analog.

Table 1
Sequences of RNA probes.

Abbreviation of RNA	[a]Sequence	Calcd. MS	Observed MS
RNA 1	5′-UGAGGUU**2**UAGGUUG-3′	4963.99	4962.79
RNA 2	5′-**F**-CAACCUA**A**UACCUCA-3′	5178.79	5178.80
RNA 3	5′-**F**-CAACCUA**G**UACCUCA-3′	5194.28	5193.99
RNA 4	5′-**F**-CAACCUA**C**UACCUCA-3′	5154.25	5154.83
RNA 5	5′-**F**-CAACCUA**U**UACCUCA-3′	5155.24	5154.96
RNA 6	5′-GUCCAGUUUUCCCAGGAAUCCCU-3′	7350.41	7349.52
RNA 7	5′-GUCCAGUUUUCCCAGGAAUCCCU-**B**-3′	7789.91	7786.17
RNA 8	5′-GUCCAGUU**1**UCCCAGGAAUCCCU-**B**-3′	7893.98	7893.70
RNA 9	5′-GUCCAGUU**2**UCCCAGGAAUCCCU-3′	7350.41	7349.52
RNA 10	5′-GGAUUCCUGGAAAUUCUGUUCU-3′	6980.15	6976.12

[a] F indicates Fluorescein, B denotes Biotin.