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# Nucleobase azide–ethynylribose click chemistry contributes to stabilizing oligonucleotide duplexes and stem-loop structures

Yoshiaki Kitamura [a,b], Ryo Asakura [a], Koki Terazawa [a], Aya Shibata [a,b], Masato Ikeda [a,b,c,d], Yukio Kitade [a,b,e,*]

[a] Department of Biomolecular Science, Graduate School of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[d] Gifu Centre for Highly Advanced Integration of Nanosciences and Life Sciences (G-CHAIN), Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[e] Department of Applied Chemistry, Faculty of Engineering, Aichi Institute of Technology, 1247 Yachigusa, Yakusa-cho, Toyota, Aichi 470-0392, Japan

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ABSTRACT

The formation of 1,4-disubstituted 1,2,3-triazoles through copper-catalyzed azide–alkyne cycloaddition (CuAAC) in oligonucleotides bearing 1-deoxy-1-ethynyl-β-D-ribofuranose ($R^E$) can have a positive impact on the stability of oligonucleotide duplexes and stem-loop structures.



Copper-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) has been applied to the synthesis of functional molecules in a wide range of fields, including the biological and material sciences, owing to its selectivity, reliability, and versatility.[1,2] CuAAC has also been widely used for the post-modification of oligonucleotides (ONs).[3] To incorporate ethynyl residues into ONs by the standard phosphoramidite solid-phase method, various nucleic acid mimics bearing ethynyl groups and their phosphoramidite derivatives, as well as their solid phase-linked monomers, have been developed. We have also engaged in the development of versatile probes possessing an ethynyl unit and post-labeling methods using CuAAC.[4,5] As previously demonstrated by our group, ONs bearing aryl acetylene derivatives, namely, 5-ethynyl-1,3-benzenedimethanol ($B^E$) and (*S*)-4-ethynylmandelol ($M^E$), rapidly react at room temperature with azide compounds in the absence of copper ligands, and the corresponding labeled ONs are obtained in excellent yield.[4,5] Double-stranded oligonucleotides containing $B^E$ or $M^E$ inside the sequence were less stable than that of the corresponding standard oligonucleotide duplexes.[5]

We have recently developed an efficient stereoselective synthesis of 1-deoxy-1-ethynyl-β-D-ribofuranose ($R^E$), and demonstrated that it reacts with azide compounds several times faster than $B^E$ under CuAAC conditions.[6] $R^E$ is the simplest acetylenic β-*C*-nucleoside, and its phosphoramidite derivative (**1**) can be used for the construction of various ON-based functional tools.[7,8]

Obika and Hari reported the thermal stability of ONs possessing 1-substituted 1,2,3-triazole deoxyribonucleosides prepared from DNA oligomers bearing 1,2-dideoxy-1-ethynyl-β-D-ribofuranose, a 2-deoxy analog of $R^E$, via CuAAC with several simple aliphatic and aromatic azides.[9] These DNA oligomers carrying 1,2,3-triazoles stabilize the duplex better than the corresponding DNA oligomers before CuAAC. Against this background, we became interested in the thermal stability of ONs bearing nucleobase-substituted 1,2,3-triazole nucleosides derived from $R^E$. Here, we describe the synthesis of RNA oligomers bearing $R^E$ and the thermal stability of the ONs before and after CuAAC with azide compounds, including nucleobases with azide moieties (Scheme 1). In this study, 9-azidomethyladenine (**2**) and 1-azidomethylthymine (**3**) were used as purine- and, pyrimidine-type azides, respectively.

The phosphoramidite derivative of $R^E$ (**1**) was prepared from the corresponding unprotected $R^E$ through a general synthetic protocol by the following series of reactions[7]: 4,4′-dimethoxytrityl (DMTr) protection of 5-OH, *tert*-butyldimethylsilyl (TBDMS) protection of 2-OH, and phosphitylation of 3-OH. However, the protocol afforded the 2- and 3-protected isomers, which are frequently difficult to separate, in the second silylation step. Even in the case of $R^E$, it is

* Corresponding author at: Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.
*E-mail address:* ykkitade@gifu-u.ac.jp (Y. Kitade).

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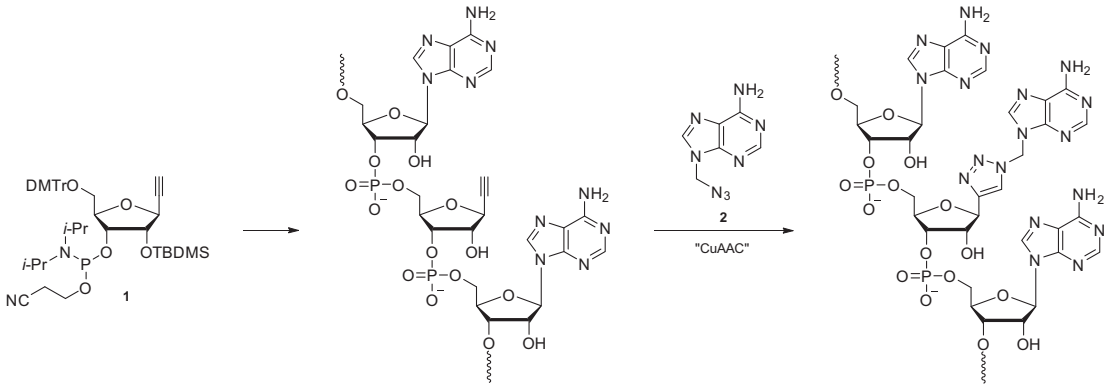


**Scheme 1.** Synthesis of oligonucleotides bearing nucleobase-substituted 1,2,3-triazole nucleosides by CuAAC post-modification method.

not easy to isolate the desired 2-protected isomer by column chromatography on silica gel. To synthesize several ONs containing $R^E$, we have developed a scalable synthetic method for **1** without selective protection of 2-OH in the presence of both 2-OH and 3-OH.[10] In this protocol, the key intermediate **7** can be obtained from **6** with complete stereoselectively. Nucleobases bearing azide moieties **2** and **3** were prepared according to previous reports (Scheme S1 in Supporting Information).[11–13]

Three RNA oligomers bearing $R^E$ (ON **14**–**16**) were synthesized using an automated nucleic acid synthesizer with phosphoramidite derivative **1**. Control and/or complementary strands (ON **10**–**13**) without $R^E$ were also prepared (Table 1). Subsequently, the click reaction[14] between ONs **14**–**16** and a nucleobase bearing an azide moiety (**2** or **3**) was examined under our previously optimized conditions (Scheme 1).[4] The sequences of the corresponding RNA oligomers containing nucleobase-substituted 1,2,3-triazole nucleosides (ONs **17**–**22**) are depicted in Table 1. The structures of these ONs were confirmed by MALDI-TOF/MS analysis. ON **16**, ON **21** and ON **22** was used to investigate the effect of an extra mismatch nucleoside adjacent to $R^E$ or nucleobase-substituted 1,2,3-triazole nucleosides on duplex stability.

Hybridization experiments were performed using double-stranded RNA (dsRNA) (ON **10**/ON **11**) and RNA/DNA duplex (ON **11**/ON **12**) as reference molecules. The $T_m$ values of dsRNAs are summarized in Table 1. The dsRNA oligomers with $R^E$ at the centre of the sequence (ON **11**/ON **14** and ON **10**/ON **15**) had almost equal stability. The $T_m$ value of the dsRNA between RNA oligomers including cytidine, which can be flipped out, adjacent to $R^E$ (ON **16**) and ON **10** resembled that of dsRNA with $R^E$ at the centre (ON **10**/ON **15**). ONs tethered to AT base-pairing nucleobases via CuAAC greatly stabilized the duplex in all sequences. The $T_m$ values of ON **11**/ON **18** ($T_m$ = 27.2 °C), ON **10**/ON **19** ($T_m$ = 28.7 °C) and ON **10**/ON **21** ($T_m$ = 29.8 °C) were comparable with the full complementary dsRNA (ON **10**/ON **11**, $T_m$ = 28.6 °C). However, duplexes of ONs bearing mismatched nucleobases were less stable than those of ONs containing $R^E$. Furthermore, stability was significantly reduced in dsRNAs bearing a flipped nucleoside next to mismatched nucleobases (ON **10**/ON **22**). According to energy-minimized molecular models using Gaussian, a nucleobase linked to 1,2,3-triazole can form a base pair with a complementary nucleobase of the opposite strand (Fig. S1 in Supporting Information). Examination of the RNA/DNA duplexes showed that base-pairing nucleobases introduced by CuAAC enhanced the stability of the duplexes. The thermal stability of the RNA/DNA duplexes containing base-pairing nucleobase-substituted 1,2,3-triazole nucleosides was lower than that of corresponding complementary RNA/DNA molecules (ON **11**/ON **12** vs ON **12**/ON **19**). Interestingly, the degree of stability of ON **12**/ON **21** ($T_m$ = 21.9 °C) was superior to

**Table 1**
$T_m$ values of double-stranded ONs containing $R^E$ or 1,2,3-triazole nucleosides.

| No. of ON | Sequence | $T_m$ (°C) | No. of ON | Sequence | $T_m$ (°C) |
|---|---|---|---|---|---|
| ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 28.6 | ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 29.8 |
| ON **11** | 5′-aaaaaaaaaaaaaaa-3′ | | ON **21** | 5′-aaaaaaaR$^A$caaaaaaa-3′ | |
| ON **14** | 5′-uuuuuuuR$^E$uuuuuuu-3′ | 21.3 | ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 11.9 |
| ON **11** | 5′-aaaaaaaaaaaaaaa-3′ | | ON **22** | 5′-aaaaaaaR$^I$caaaaaaa-3′ | |
| ON **17** | 5′-uuuuuuuR$^A$uuuuuuu-3′ | 14.0 | ON **12** | 5′-TTTTTTTTTTTTTTT-3′ | 35.0 |
| ON **11** | 5′-aaaaaaaaaaaaaaa-3′ | | ON **11** | 5′-aaaaaaaaaaaaaaa-3′ | |
| ON **18** | 5′-uuuuuuuR$^T$uuuuuuu-3′ | 27.2 | ON **12** | 5′-TTTTTTTTTTTTTTT-3′ | 20.7 |
| ON **11** | 5′-aaaaaaaaaaaaaaa-3′ | | ON **15** | 5′-aaaaaaaR$^E$aaaaaaa-3′ | |
| ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 20.2 | ON **12** | 5′-TTTTTTTTTTTTTTT-3′ | 23.6 |
| ON **15** | 5′-aaaaaaaR$^E$aaaaaaa-3′ | | ON **19** | 5′-aaaaaaaR$^A$aaaaaaa-3′ | |
| ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 28.7 | ON **12** | 5′-TTTTTTTTTTTTTTT-3′ | 15.0 |
| ON **19** | 5′-aaaaaaaR$^A$aaaaaaa-3′ | | ON **16** | 5′-aaaaaaaR$^E$caaaaaaa-3′ | |
| ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 18.7 | ON **12** | 5′-TTTTTTTTTTTTTTT-3′ | 21.9 |
| ON **20** | 5′-aaaaaaaR$^T$aaaaaaa-3′ | | ON **21** | 5′-aaaaaaaR$^A$caaaaaaa-3′ | |
| ON **10** | 5′-uuuuuuuuuuuuuuuu-3′ | 20.1 | ON **13** | 5′-TTTTTTTGTTTTTTT-3′ | 20.7 |
| ON **16** | 5′-aaaaaaaR$^E$caaaaaaa-3′ | | ON **16** | 5′-aaaaaaaR$^E$caaaaaaa-3′ | |

[a]Small letters indicate ribonucleosides and capital letters show 2′-deoxyribonucleosides.
[b]$R^E$, $R^A$ and $R^T$ denote 1-deoxy-1-ethynyl-β-D-ribofuranose, 1-[1-(adenine-9-yl)methyl-1*H*-1,2,3-triazol-4-yl]-1-deoxy-β-D-ribofuranose (**23**), and 1-[1-(thymine-1-yl)methyl-1*H*-1,2,3-triazol-4-yl]-1-deoxy-β-D-ribofuranose (**24**), respectively.
[c]Measurements were carried out in 10 mM $Na_2HPO_4/NaH_2PO_4$ (pH 7.0), and 100 mM NaCl, with 3.0 μM of each oligonucleotide.