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Tris(azidoethyl)amine Hydrochloride; a Versatile Reagent for Synthesis of Functionalized Dumbbell Oligodeoxynucleotides

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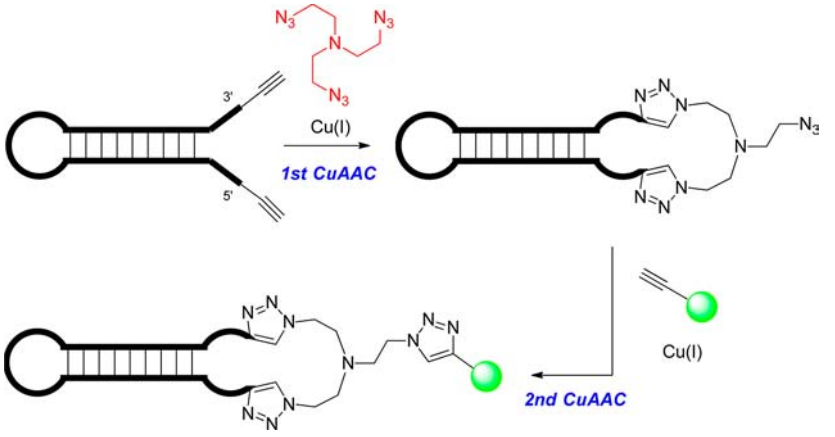
ABSTRACT



Triazole-cross-linked oligodeoxynucleotides were synthesized using the Cu(I) catalyzed alkyne−azide cycloaddition with tris(azidoethyl)amine hydrochloride and oligodeoxynucleotides possessing *N*-3-(propargyl)thymidine at both the 3′- and 5′-termini. Further installation of a functional molecule to the dumbbell oligodeoxynucleotides was achieved by utilizing the remaining azide group.

Our recent knowledge of novel aspects of DNA and RNA research has been motivational for the further development of nucleic acid based chemistry. Especially, relatively short stranded nucleic acids have potential as therapeutic agents including siRNAs, miRNAs, and aptamers, and much effort has been dedicated to functionalize DNA and RNA with appropriate properties in order to develop oligonucleotide therapeutics. In applying DNA or RNA as therapeutic agents, there are several shortcomings associated with the use of short stranded nucleic acids, making their use in biological systems unfeasible as yet. Generally, short double-stranded oligodeoxynucleic acids (ODNs) used in these studies possess less thermal stability under physiological conditions. Another drawback is the presence of extra- and intracellular nucleases, which cleave ODNs.[1,2] Cross-linking double-stranded ODNs are one of the solutions to achieve thermal stability and resistance to nucleases.[3−11] Dumbbell ODNs, which are circular nucleic

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acids consisting of double-stranded stem region and nucleotide loops at both of their termini, possess increased exonuclease resistance because they have no terminal nucleotide residues.[12−15] Chemical modification including cross-linking of ODNs by click chemistry is a growing field.[16] Recently we have reported that triazole-cross-linked ODNs were synthesized using the Cu(I) catalyzed alkyne−azide cycloaddition (CuAAC)[17,18] with ODNs possessing *N*-3-(azidoethyl)thymidine and *N*-3-(propargyl)-thymidine at the 3′- and 5′-termini (Figure 1a).[19] The newly synthesized ODNs possess thermal stability and showed excellent resistance against snake venom phosphodiesterase (3′-exonuclease), whose properties are necessary for decoy molecules to achieve biological responses leading to alteration of gene expression. However, the *N*-3-(iodoethyl)thymidine 3′-phosphoramidite was necessarily used as a precursor of the *N*-3-(azidoethyl)-thymidine residue because azido groups are known to be readily reduced by a trivalent phosphorus atom during the automated DNA synthesis.[20] Once introduced in solid phase DNA synthesis via the general phosphoramidite method, the iodo group is converted to the corresponding azide group via the on-column application of NaN_3. In order to overcome this shortcoming, we report herein the development of tris(azidoethyl)amine hydrochloride (**1**) as a novel cross-linking reagent of ODNs by the CuAAC (Figure 1b).

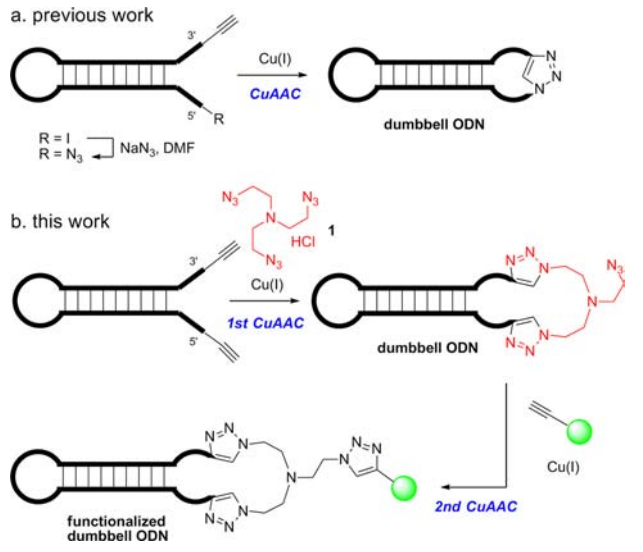
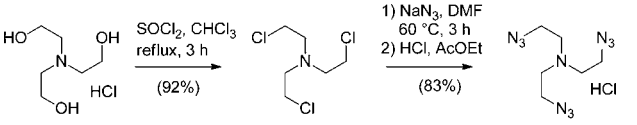


Figure 1. Formation of triazol cross-linked dumbbell ODNs by CuAAC.

Our improved strategy to access the dumbbell ODNs is to use the multivalent azide linker as an external cross-linking source and *N*-3-(propargyl)thymidine as a partner unit, which is incorporated into the ODN at both termini. This allowed us to avoid using any precursor amidite units that needed to be converted to the corresponding azide unit during the automated ODN synthesis. Furthermore, installation of a functional molecule to the dumbbell ODNs is now possible by utilizing the remaining azide group.

Scheme 1. Preparation of Tris(azidoethyl)amine Hydrochloride



Tris(azidoethyl)amine hydrochloride (**1**) was prepared as shown in Scheme 1. Tris(ethanol)amine hydrochloride (**2**) was treated with $SOCl_2$ in $CHCl_3$ at reflux for 3 h, and the resulting tris(2-chloroethyl)amine (**3**) was reacted with NaN_3 in DMF to give tris(azidoethyl)amine. After the aqueous workup, the free amine[21] was converted to its hydrochloride salt, which was crystallized from AcOEt. The salt has good solubility in water and is suitable for cross-linking biomolecules in aqueous media.

With the cross-linking reagent **1** in hand, the formation of the dumbbell ODN was examined. As in the case of our previous system, most of the strategies to cross-link ODNs are done in an intramolecular fashion. Different from these strategies, the first step of the reaction sequence in this study is an intermolecular CuAAC of **1** with either of the propargyl groups at the termini of double-stranded ODNs. The following CuAAC is in the intramolecular mode. The intermolecular CuAAC could be challenging because simultaneous CuAAC at both propargyl groups at one terminal could proceed, resulting in termination of the cross-linking or oligomerization. In order to see the ability of **1** to cross-link ODN, a single stranded hairpin ODN (**hpODN**), where two *N*-3-(propargyl)thymidines were attached at the 5′- and 3′-ends, was first investigated for cross-linking at the single terminus (Scheme 2). Thus, after the annealing of **hpODN**, the CuAAC was carried out by

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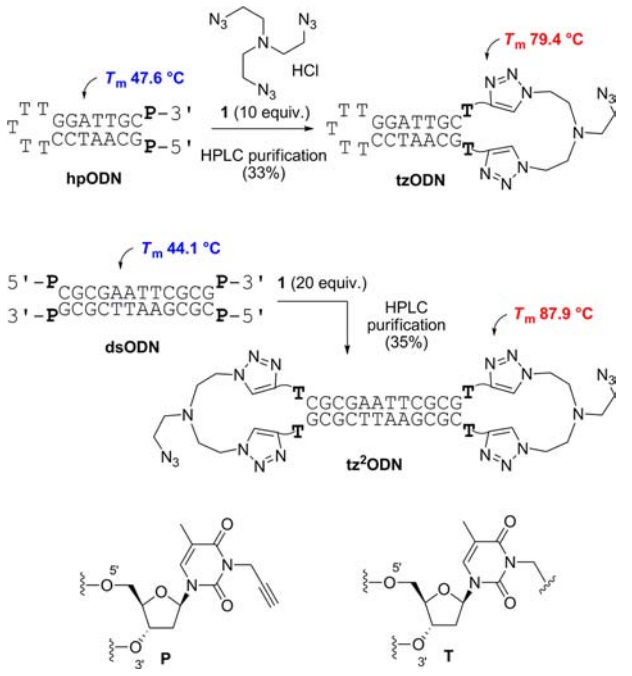
Scheme 2. Formation of Dumbbell ODN by CuAAC[a]



[a] **P** = *N*-3-(propargyl)thymidine residue; **T** = *N*-3-(substituted) thymidine residue. Reaction was conducted under the conditions with **1** (20 equiv), $CuSO_4$ (10 equiv), TG-TBTA(10 equiv), Na ascorbate (20 mM), NaCl (50 mM) in MOPS buffer (pH 7.0, 2 mM) at room temperature for 6 h.

adding a solid-supported copper(I) catalyst immobilized with tris(benzyltriazolyl)amine onto a Tentagel resin[22] (TG-TBTA, 10 equiv) to a solution of **hpODN** (0.1 μM) and **1** (10 equiv) in 2 mM MOPS buffer (pH 7.0) containing 20 mM sodium ascorbate and 50 mM NaCl at room temperature. The progress of the reaction was monitored by HPLC (Figure 2a,c). A new peak was observed at a retention time of 9.5 min (10 min for **hpODN**), and the reaction was complete within 6 h. The structure of the newly formed ODN (**tzODN**) was determined by analyzing MALDI-TOF MS and a composition of nucleosides as follows. Thus, the resulting **tzODN** was purified by reversed-phase HPLC (33% isolated yield),[23] and enzymatic digestion of the ODN showed a composition of nucleosides. As a result, no peak corresponding to *N*-3-(propargyl)thymidine (**P**) was detected by HPLC analysis, and the bistriazole-bridged thymidine **4** was formed instead (Figure 2b,d).[24] The resulting nucleoside composition was in good accordance with the desired one, and mono- or tris-triazole derivative was not observed in the reaction mixture. Of note is a rather preferred intramolecular CuAAC with ring closure for **hpODN**. The use

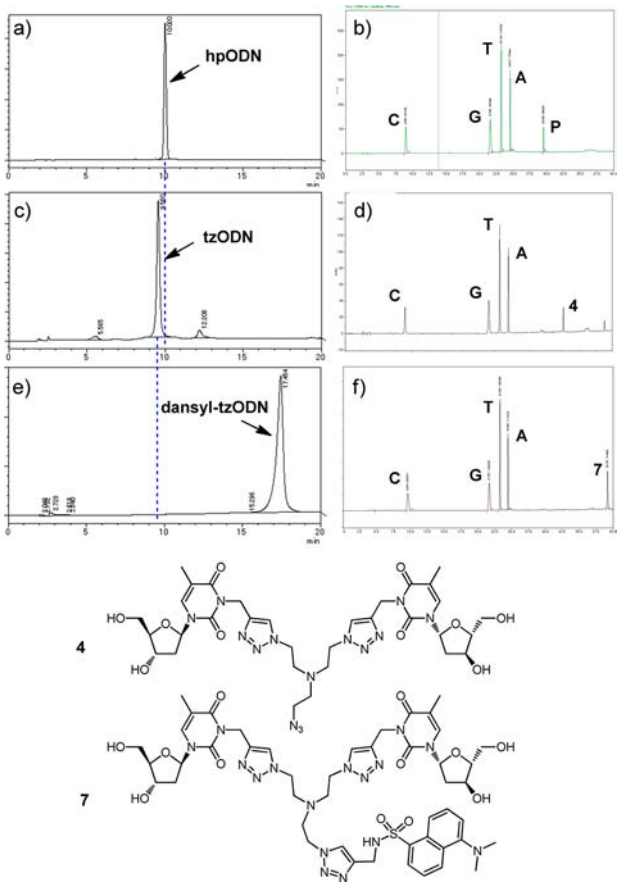


Figure 2. Reversed-phase HPLC chromatograms (UV absorbance vs time) in the CuAAC (a, c, and e) and their nucleoside composition analysis (b, d, and f). (a) **hpODN** in the absence of Cu[I] catalysis; (b) enzymatic digestion of **hpODN**; (c) after the CuAAC of **hpODN**; (d) enzymatic digestion of **tzODN**; (e) after the CuAAC of **tzODN** with **5**; (f) enzymatic digestion of **dansyl-tzODN**.

of an excess amount of tris(azidoethyl)amine (up to 1000 equiv) still gave the **tzODN** as a main product. It would be because the increased number of the reactive sites with two azide groups, which can be cross-coupled with the alkyne in the opposite strand, and these reactive groups are in close proximity in space at the terminus of the strand for the second CuAAC. Next, cross-linking by **1** was investigated with a double-stranded ODN (**dsODN**), which has the cross-linking site at both termini. When the same conditions to prepare **tzODN** were applied to **dsODN**, the desired **tz²ODN** was afforded supported by a composition analysis of nucleosides (Figures S2, S5). This would imply that the CuAAC sequence was successfully achieved and the triazole cross-linking indeed occurred at both termini providing a dumbbell ODN. UV melting experiments were then conducted to characterize the thermally induced denaturation of ODNs. Comparing the T_m values for cross-linked **tzODN** (79.4 °C) or **tz²ODN** (87.9 °C) with that of the control **hpODN** (47.6 °C) or **dsODN** (44.1 °C) revealed a dramatic increase (> 30 °C) in thermal

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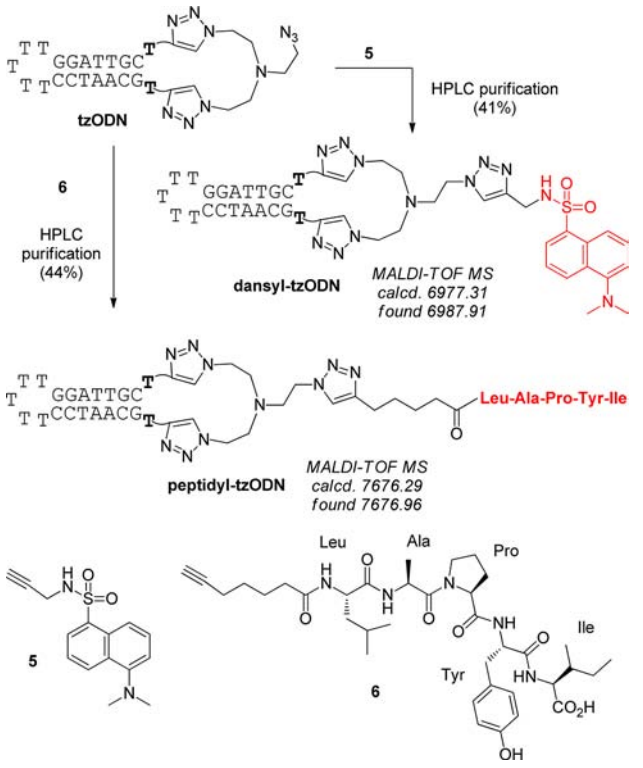
(23) The isolated yields of dumbbell ODNs after CuAAC were moderate (33−41% yield after HPLC purification) although the HPLC analysis of the reaction mixture showed relatively clean conversion (see Figure 2c). This is true for any CuAAC reactions in this study although the reason is unclear.
(24) Authentic triazole derivatives were also prepared as shown in Scheme S1.

Scheme 3. Functionalization of Dumbbell ODN by CuAAC[a]



[a] Reaction was conducted under the conditions with alkyne derivative (100 equiv), $CuSO_4$ (10 equiv), TG-TBTA(10 equiv), Na ascorbate (20 mM), NaCl (50 mM) in MOPS buffer (pH 7.0, 2 mM) for 6 h.

stability with the triazole cross-link. The control ODNs (**hpODN** and **dsODN**) displayed a characteristic B-DNA spectrum,[25] and **tzODN** and **tz^{2}ODN** possessing the triazole cross-linked thymidines at the end(s) of the helix displayed similar CD spectra (Figure S7). These results suggest that the terminal nucleobase modifications do not significantly distort the helical geometry of the duplex.

Having established the cross-linking of **hpODN** by the CuAAC, the next CuAAC was examined on **tzODN**. As representative examples, a fluorophore and a peptide were chosen as the functional molecules. The CuAAC of **tzODN** and *N*-propargyldansylamide[26] (**5**, 100 equiv) was carried out (Scheme 3). As a result, complete consumption of **tzODN** occurred, and a new peak possessing absorption at 340 nm was observed in the HPLC analysis (Scheme S2b). MALDI-TOF MS and a composition analysis of the newly formed peak unambiguously determined the formation of the desired **dansyl-tzODN** (41% isolated yield, Figure 2e,f),[23] a functionalized dumbbell ODN. Reaction of **tzODN** with peptide **6** also gave **peptidyl-tzODN** (44% isolated yield, Figure S3).

In conclusion, tris(azidoethyl)amine hydrochloride (**1**) was developed as a novel cross-linking and functionalizing reagent of ODNs by the CuAAC.

Triazole-cross-linked functionalized dumbbell ODNs were successfully synthesized by two sequential CuAACs with ODNs possessing *N*-3-(propargyl)thymidine at the 3′- and 5′-termini. This strategy is effective for the preparation of cross-linked ODNs because the precursor ODNs are stable and easy to prepare. This study should now enable us to functionalize DNA and RNA with appropriate properties in order to develop oligonucleotide therapeutics.

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Supporting Information Available. Experimental procedures, NMR spectra for new compounds, HPLC chromatograms, CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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