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Non-Nucleoside Building Blocks for Copper-Assisted and Copper-Free Click Chemistry for the Efficient Synthesis of RNA Conjugates

K. N. Jayaprakash,[†] Chang Geng Peng,[†] David Butler,[†] Jos P. Varghese,[‡] Martin A. Maier,[†] Kallanthottathil G. Rajeev,[†] and Muthiah Manoharan*,[†]

Drug Discovery, Alnylam Pharmaceuticals, Cambridge, Massachusetts 02142, United States, and Sanmar Speciality Chemicals Ltd., Chennai, Tamil Nadu, India

mmanoharan@alnylam.com

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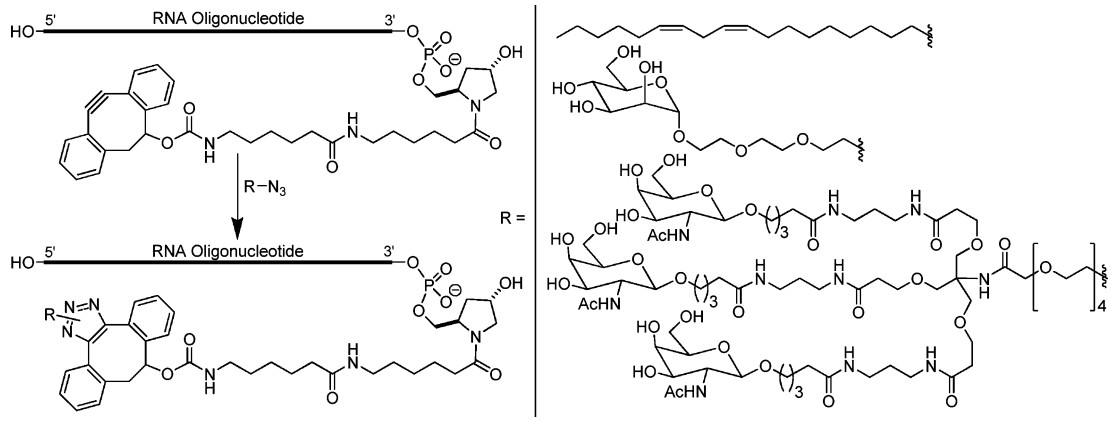
ABSTRACT



Novel non-nucleoside alkyne monomers compatible with oligonucleotide synthesis were designed, synthesized, and efficiently incorporated into RNA and RNA analogues during solid-phase synthesis. These modifications allowed site-specific conjugation of ligands to the RNA oligonucleotides through copper-assisted (CuAAC) and copper-free strain-promoted azide−alkyne cycloaddition (SPAAC) reactions. The SPAAC click reactions of cyclooctyne−oligonucleotides with various classes of azido-functionalized ligands in solution phase and on solid phase were efficient and quantitative and occurred under mild reaction conditions. The SPAAC reaction provides a method for the synthesis of oligonucleotide−ligand conjugates uncontaminated with copper ions.

Since the discovery of RNA interference (RNAi) as a means to silence expression of specific genes, small-interfering RNAs (siRNAs) have proven to be powerful tools for specifically eliciting degradation of targeted mRNAs of disease-causing genes. Although siRNAs have the potential to become a highly effective class of therapeutics,[1] efficient tissue distribution and intracellular delivery *in vivo* remain critical challenges.[2] Reported approaches to delivery of siRNA to particular cell types involve conjugation of lipids or other small molecules to one of the strands of the siRNA or formulations with lipid-, polymer-, or peptide-based delivery systems.[3]

[†] Alnylam Pharmaceuticals.
[‡] Sanmar Speciality Chemicals Ltd.

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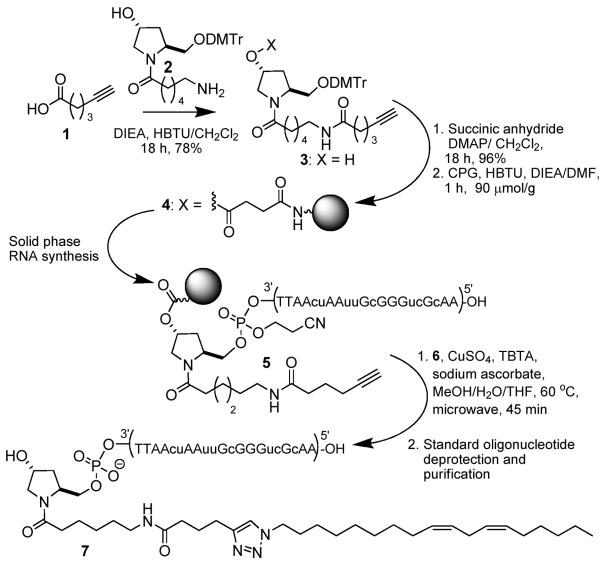
Cu(I)-catalyzed 1,3-dipolar [3 + 2] cycloadditions[4] between azides and alkynes (CuAAC), known generally as "click reactions", have been used extensively for conjugation of various ligands to DNA,[5] for DNA ligation,[6] and for fluorophore tagging of oligonucleotides[7] and modified nucleosides.[8] High chemical stability and selective reactivity of alkyne and azide precursors and excellent 1,4-regioselectivity[4] make CuAAC a promising approach for oligonucleotide−ligand conjugate synthesis.

To achieve CuAAC-mediated RNA conjugation to different ligands, we initially explored a hydroxyprolinol-derived alkyne derivative. The key alkyne intermediate **3** for the synthesis of the long-chain aminoalkyl controlled pore glass (lcaa CPG) support **4** (Scheme 1) and the corresponding

Scheme 1. CuAAC Reaction with Solid-Supported RNA[a]



[a] The lowercase letters indicate 2′-*O*-methyl-ribonucleotides.

phosporamidite (Scheme S1, Supporting Information) for incorporation of the alkyne moiety to internal and/or 5′-end of the oligonucloetide were obtained from *trans*-4-hydroxyprolinol derivative **2**[9] and 5-hexynoic acid (**1**). The secondary hydroxyl group of **3** was reacted with succinic anhydride in the presence of DMAP to afford the hemisuccinate, which was then loaded onto lcaa CPG support under peptide coupling conditions to obtain the desired CPG-alkyne support **4** with loading of 90 μmol/g.

The alkyne-functionalized hydroxyprolinol moiety was incorporated at the 3′ end of the oligonucleotide using standard solid-phase synthesis to afford the support-bound alkyne-RNA **5** (Supporting Information). We evaluated click reactions of the solid support-bound oligonucleotide **5** with linoleyl azide[10] (**6**, Scheme 1) under conditions reported for the synthesis of various solid-supported carbohydrate−DNA conjugates.[11] The goal of linoleyl conjugation was to improve the tissue distribution and cellular permeation of siRNAs, similar to what we achieved with cholesterol conjugation earlier.[12] The reaction went to completion upon microwave irradiation (60 °C, 45 min) of the reaction mixture of alkyne/azide/$CuSO_4$/tris(benzyltriazolyl-methyl) amine[13] (TBTA)/sodium ascorbate with 1:5.8:0.8:5.6:6.4 molar ratio in methanol/water/THF (2:2:1 by volume).

The main drawback of the CuAAC reaction is contamination of the product with copper ions; this is especially a problem when the reaction is performed in the solution phase using an unprotected alkyne or azido functionalized oligonucleotide (unpublished results). Contamination with Cu^+/Cu^{2+} could be problematic due to the potential for increased nucleic acid hydrolysis[14] and potential cytotoxicity due to heavy metal ion contamination. The development of the strain-promoted azide−alkyne cycloaddition (SPAAC) reaction, also known as Cu-free click chemistry, has provided a solution to this issue.[15] In addition to this approach, strained alkene−nitrile oxide[16] and alkyne−nitrile oxide[17] click cycloaddition reactions have also been reported for the modification of DNA in the absence of metal catalysts.

Two research groups have reported the synthesis of activated cycloalkynes for click chemistry that do not require Cu(I) catalysis. Bertozzi's laboratory reported strained cyclooctynes **8**,[15a] **9**,[15b,c] **10**,[15d] and **11**,[15e] and Boon's laboratory reported the cyclooctyne **12**[15g] (Figure 1). These compounds can undergo azide−alkyne cycloaddition in the absence of copper and enabled the SPAAC[15a] process. For

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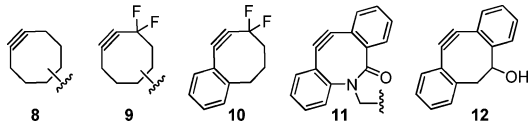
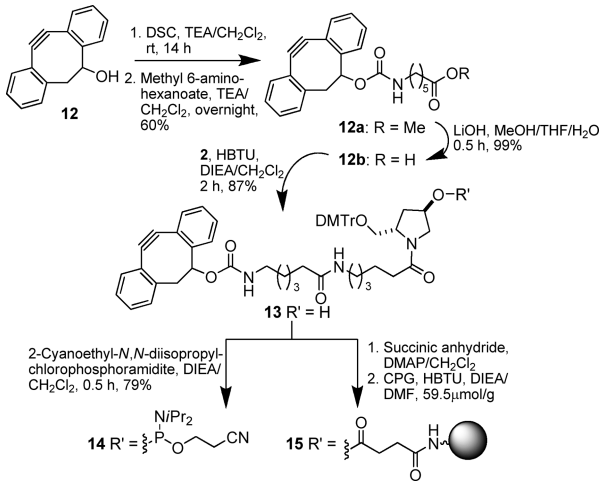


Figure 1. Strained cyclooctynes enable click reaction with azides in the absence of copper.[15]

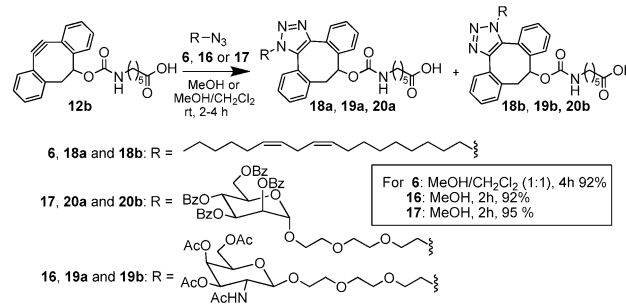
compound **8**, activation of cyclooctyne is due to ring strain, and the *gem*-difluoro substitution of compound **9** adjacent to the triple bond further enhances reactivity. In compounds **11** and **12**, activation is accomplished by the orthodibenzo substitution. Compound **10** combines features of aryl and *gem*-difluoro substitutions. We herein describe the syntheses of conjugates between RNA oligonucleotides and various ligands that are capable of improving cellular delivery of siRNAs. Our synthetic adaptations will allow the syntheses of a variety of RNA conjugates using a copper-free click protocol.

Scheme 2. Synthesis of Non-Nucleosidic SPAAC Alkyne Monomers for RNA Synthesis



As outlined in Scheme 2, the cyclooctyne derivative **12**[15g] was activated with disuccinimidyl carbonate and further reacted with methyl 6-aminohexanoate to afford compound **12a** in 60% yield. The ester **12a** was hydrolyzed to afford the carboxylic acid-functionalized compound **12b** in quantitative yield. The carboxylic acid **12b** was coupled to compound **2**[9] to afford compound **13** in 87% isolated yield. Phosphitylation of compound **13** afforded the phosphoramidite **14** (79% yield), which was used to introduce the cyclic alkyne moiety at the 5′-end and at internal positions of oligonucleotides. Succinylation of compound **13** enabled loading onto lcaa CPG under peptide coupling conditions to obtain the support **15** (loading 59.5 μmol/g). The support **15** was then used to introduce the alkyne at the 3′-end of oligonucleotides by standard oligonucleotide synthesis protocols (Supporting Information). Both strained alkyne building blocks **14** and **15** were stable under solid-phase oligonucleotide synthesis and deprotection conditions.

Scheme 3. Model Cu-Free Click Reactions with Azido Derivatives



As shown in Scheme 3, model cycloaddition reactions with different azido derivatives were evaluated to ensure that the reactivity of cyclic alkyne **12b** was not compromised after derivatization with a carboxylic acid and to evaluate the regiochemistry of the products. Compound **12b** was reacted with 1 mol equiv of linoleyl azide **6** or azides derived from monosaccharides *N*-acetylgalactosamine (GalNAc) **16**[18] or mannose **17**.[19] These sugars, respectively, target the drugs to either the asialoglycoprotein receptor, ASGPR, and mediate delivery to hepatocytes of liver[20] or to mannose binding proteins and enable delivery to dendritic cells and macrophages.[21] The click reaction generated *1,4-*/*1,5*-triazole derivatives **18a/18b** (linoleyl 92%), **19a/19b** ([GalNAc]^{Ac} 92%), and **20a/20b** ([Mannose]^{Bz} 95%) in high isolated yields as inseparable mixtures of regioisomers. The rate of reaction depended upon the nature of the substrate. Lipid derivative **6** took longer to react than the sugar derivatives **16** or **17**, a difference we attribute to the poor solubility of **6** in MeOH compared to that of the other azides.

Next we examined the Cu-free click reaction in solution between deprotected 5′-alkyne-RNA **21** and [GalNAc]^{Ac}-azide **16** (Scheme 4). The oligoribonucleotide **21** with the activated alkyne moiety at the 5′-end underwent cycloaddition reaction with the azide **16** at room temperature in MeOH/THF/water (1:1:1.5, v/v). The progress of the reaction was monitored by HPLC. The cycloaddition reaction was very efficient; within 45 min all the starting materials were consumed to afford the product **27**. The identity and integrity of the conjugate **27** were confirmed by LC-MS and HPLC analysis. We then studied the reaction between **21** and the

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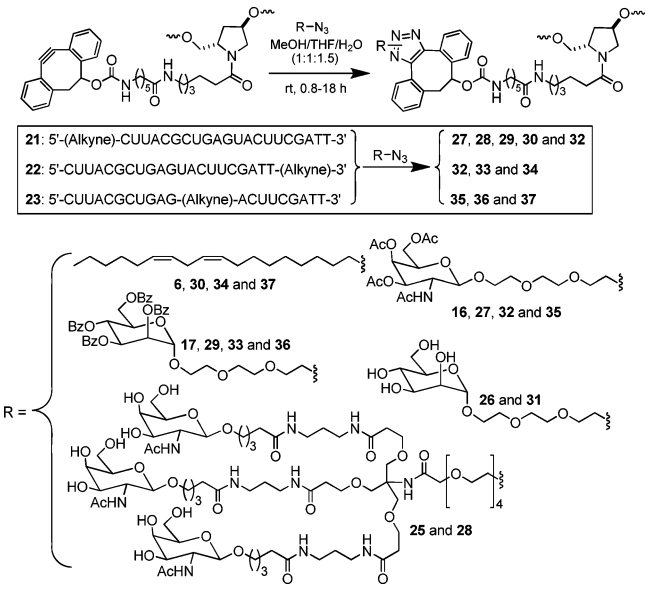
Scheme 4. RNA Conjugation by Cu(I)-Free Click Reactions



trivalent $GalNAc_3$-azide **25** to test the efficiency of the coupling with a large hydrophilic azide. Figure 2 depicts the

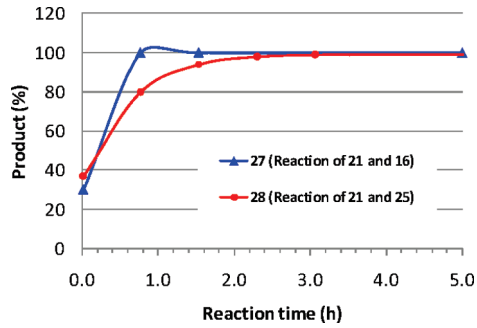


Figure 2. Progress of the click reaction of deprotected 5′-alkyne-RNA **21** with $[GalNAc]^{Ac}$-azide **16** and $GalNAc_3$ azide **25** monitored by HPLC.

time course of the SPAAC reaction of the sugar azides **16** and **25** with alkyne-RNA **21** in solution as monitored by HPLC. Both reactions were complete within 3 h at ambient temperature.

Reactions of alkyne-oligoribonucleotide **21** with other azido derivatives (**6**, **17**, and **26**[22]) were also quantitative (Table S3, Supporting Information). For the hydrophobic azide **17**, additional THF was required to improve solubility. Reaction of the hydrophobic linoleyl azide **6** with the alkyne **21** was slow in comparison to the sugar azides; HPLC analysis showed only 38% conversion in the first 3 h with completion of reaction in 18 h (Figure S1d, Supporting Information). The two product peaks shown in the HPLC profile correspond to compounds with the same molecular weight as shown by LC-MS analysis. We presume that these are the two regioisomers expected to form from the click reaction. The regioisomers obtained from the azides **16**, **17**, **25**, and **26** were inseparable under the HPLC conditions evaluated. All click products had the expected molecular weights upon LC/MS analysis.

We also examined the click reactions of alkynes with oligonucleotides derivatized at the 3′ terminus (**22**) and at an internal position (**23**). All the reactions were clean and quantitative. The click reactions between solid-support-bound **21** and azides proceeded to completion after stirring overnight at room temperature (Supporting Information). The heterogeneity of the reaction presumably contributed to the slow reactions between support-bound alkyne−oligoribonucleotide and the azides tested. Several fluorophore-tagged azides were also incorporated into the RNA in a similar fashion (data not shown).

Thus, using this strategy one can choose *where* to place a ligand and also at *what stage* of the RNA synthesis to incorporate it. For example, when screening a particular biological target using a library of ligands, it may be preferable to obtain purified RNA precursors to be utilized for multiple, parallel click reactions in solution, whereas for large-scale synthesis, conjugation of the ligand to the oligonucleotide on solid support may confer a considerable economic advantage as purification steps can be avoided.

To summarize, we have demonstrated that a Cu-free strained alkyne−azide click reaction is applicable to the general synthesis of oligonucleotide−ligand conjugates. All common steps of phosphoramidite chemistry, including the oxidation with iodine, are compatible with the activated, strained cyclooctyne derivative. Copper-free click reactions of RNA oligonucleotides functionalized at 5′ or 3′ termini or at an internal position were efficient in the solution phase as well as on solid support. The various azides evaluated reacted quantitatively and efficiently as determined by HPLC analysis. These procedures will serve as the foundation for the development of methods for the introduction of all kinds of functional molecules into RNA, DNA, and chimeric oligonucleotides without the generation of toxic byproducts or reactive intermediates. As pointed out, the only remaining limitation with this approach appears to be the mixed regiochemistry of the products. We are in the process of identifying methods to separate the isomers and evaluating their uptake, delivery, and gene silencing improvements of siRNAs derived from these RNA conjugates and will report the results in due course.

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Supporting Information Available: Experimental procedures and compound characterization; HPLC profiles; and MS characterizations of oligonucleotides and conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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