ORGANIC LETTERS

2010
Vol. 12, No. 23
5486−5489

Synthesis of Oligoribonucleic Acid Conjugates Using a Cyclooctyne Phosphoramidite

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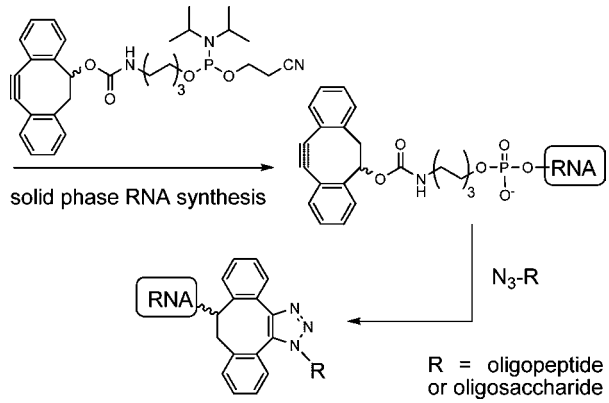
Received September 30, 2010

ABSTRACT



The conjugation of a ribonucleic acid 16-mer with the cationic amphiphilic peptide penetratin and an anionic hyaluronan tetrasaccharide by means of Cu-free "click" chemistry is reported. The alkyne-functionalized 16-mer was prepared by automated solid-phase synthesis, using a newly developed strained cyclooctyne phosphoramidite in the final coupling. Cycloaddition of the alkyne functionalized RNA to the azide containing biomolecules led to a clean conversion into the corresponding nucleic acid conjugates.

Oligonucleotides functionalized in such a manner that they can be reliably connected to other entities (biomolecules,[1,2] surfaces[3,4]) have attracted considerable interest in life sciences, nanotechnology, and adjacent fields of research. For instance, advances in the development of nucleic acid based diagnostics and therapeutics rely on the availability of well-defined oligonucleotides provided with reporter molecules such as fluorescent labels[5,6] or targeting devices. In this respect, research toward inhibition of gene expression via antisense, RNAi, or antigene approaches explores the use of oligonucleotides conjugated to peptides,[7,8] carbohydrates,[9] or small molecules[10] to acquire therapeutics with improved properties in terms of stability to enzymatic degradation, cellular uptake, and targeting.[11,12] Over the past decades, numerous conjugation methods, including amide, disulfide, thioether, imine, and oxime formation, have been

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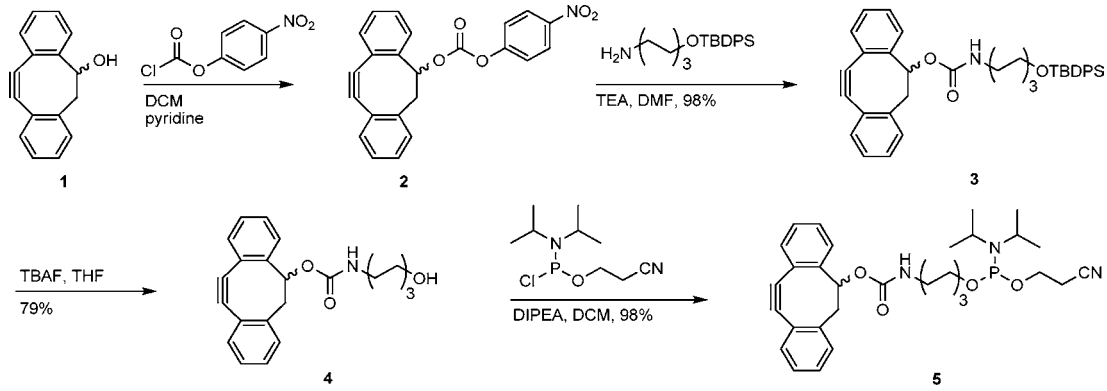
10.1021/ol102357u
Published on Web 11/04/2010

Scheme 1. Synthesis of the Strained Cyclooctyne Phosphoramidite



reported.[13−15] While most of these methods have their merits, they are not generally applicable due to limitations such as lack of selectivity and incomplete reactions.

The advent of the Cu(I)-catalyzed[16,17] Huisgen [3 + 2] cycloaddition between an alkyne and an azide, commonly referred to as the "click" reaction, enables the selective formation of a specific triazole product in an aqueous and otherwise complex chemical environment. In recent years, DNA fragments have been conjugated to oligopeptides, oligosaccharides, and fluorescent dyes in this fashion. In a postsynthetic approach, oligonucleotides functionalized with either an alkyne or an azide are conjugated with complementary functionalized molecules after standard automated DNA synthesis and ensuing removal of the protecting groups.[18] In this approach, alkyne or azide functionalities are appended to nucleobases, the ribose, or the terminal phosphate of the synthetic DNA fragments.[19] Alkyne functions are more often incorporated in DNA fragments, as azides are usually incompatible with the phosphoramidite chemistry applied in the solid-phase synthesis of oligonucleotides. The necessity to use a Cu(I)-stabilizing ligand[20] and oxygen-free conditions to increase the cycloaddition rate and to minimize the degradation of DNA has shifted the attention to the development of Cu-free click reactions. This aim corresponds with the new metal-free bioorthogonal reactions developed in the field of chemical biology and involves the reaction of strained cycloalkynes with azides,[21−23] the reaction of strained alkenes with tetrazines,[24a,b] the reaction of oxabornadienes with alkynes,[25] and a strategy based on a photoreaction-mediated liberation of a strained alkyne for ensuing click ligation.[26] Recent methodologies to nucleic acid conjugates apply the nitrile oxide-norbornene[27] click chemistry and the Diels−Alder reaction.[28a,b] As part of a program[29] to design and evaluate nucleic acid conjugates, we became interested in the bioconjugation procedure of the group of Boons,[23] in which 4-dibenzocyclooctynols are applied for the visualization of metabolically labeled glycoconjugates. Dibenzocyclooctynols are easily synthesized and react rapidly with azides. We envisaged that derivatization of dibenzocyclooctynol and ensuing conversion into a phosphoroamidite would afford a phosphitylation agent applicable for automated solid-phase nucleic acid synthesis. The nucleic acid fragments, resulting from this approach, are provided with dibenzocyclooctynol function at the 5′ end, allowing cycloaddition with azides to give oligonucleotide conjugates having intact primary and secondary structure. Phosphoramidite **5** was designed as a suitable and easily accessible phosphitylating reagent.

Previously described cyclooctynol **1**[30] (Scheme 1) was treated with *p*-nitrophenyl chloroformate. The resulting carbonate **2** was used without further purification to react with 2 equiv of TBDPS-protected aminohexanol,[31] furnishing **3** in near quantitative yield. TBAF-mediated removal of the TBDPS group of **3** and subsequent phosphitylation of the

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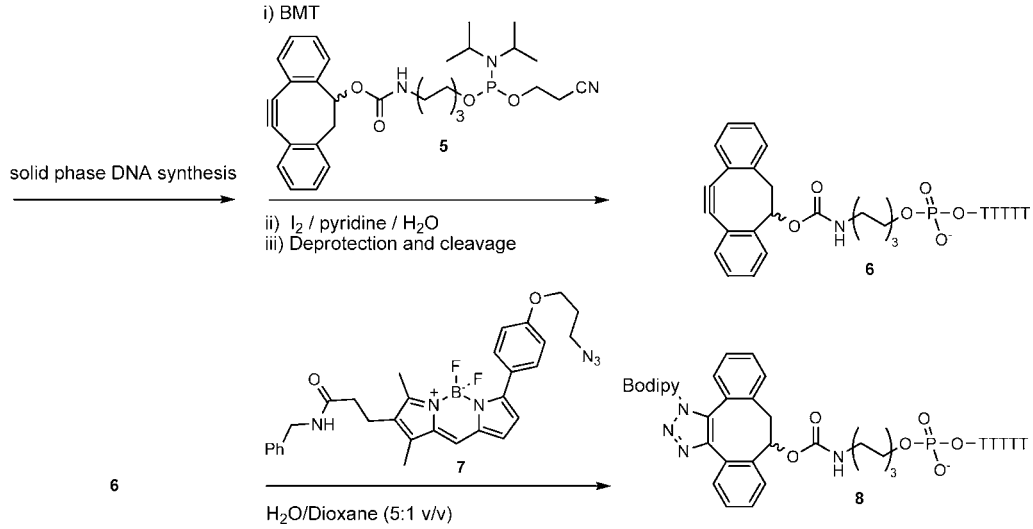
Scheme 2. Synthesis of the Alkyne-T_5 Sequence (**6**) and Subsequent Conjugation with the Fluorescent Label BODIPY



obtained alcohol **4** with commercially available 2-cyanoethoxy-*N*,*N*-diisopropylamino-chloro-phosphine and DIPEA in DCM led to the isolation of new phosphitylation reagent **5** in 75% overall yield.

The adequacy of reagent **1** for application in automated DNA synthesis was explored by the construction of alkyne-functionalized thymine pentamer **6** (Scheme 2). The pentanucleotide T_5 was assembled by the use of a standard four-step elongation cycle, comprising coupling with 5 equiv of commercially available thymidine phosphoramidite under influence of the activating agent 5-(benzylmercapto)-1*H*-tetrazole (BMT), oxidation with I_2/pyridine/H_2O, capping with acetic anhydride, and finally removal of the dimethoxytrityl group by dichloroacetic acid. In the ultimate and crucial steps of the solid-phase protocol, the immobilized pentamer was functionalized by coupling with 6 equiv of amidite **5** and ensuing oxidation[32] of the intermediate phosphite using the standard conditions. Deprotection and cleavage from the CPG solid support using aqueous ammonia was followed by purification with reversed-phase HPLC to afford the target alkyne-functionalized pentamer **6** (Scheme 2). Notably, the presence of the hydrophobic dibenzocyclooctyne moiety increased the retention time of our target compound in comparison with the unfunctionalized oligomer significantly, thereby simplifying purification by RP-HPLC.

For the first conjugation event, we selected the azide-functionalized Azido-BODIPY derivative (**7**), obtained as recently reported by us,[33] as a reaction partner for the cycloaddition with the alkyne-T_5 oligonucleotide (**6**). The progress of the conjugation of stoichiometric amounts of azide label **7** and alkyne-funtionalized pentamer **6** was monitored by LC−MS analysis. After stirring the reaction overnight, we found the reaction to be complete as indicated by a single peak in the LC trace, which was confirmed to be the product **8** by mass spectrometry data.

Following our success in synthesizing a fluorescently labeled DNA fragment, we shifted our attention to the construction of conjugates of the less stable and therefore more challenging RNA oligomers. We selected a 16-mer RNA fragment, in which all common nucleosides are incorporated, for functionalization with the dibenzocyclooctynol moiety. The alkyne-functionalized RNA fragment **9** (Scheme 3) was constructed by a standard RNA automated solid-phase synthesis protocol, again employing the powerful BMT activating agent. At the end of the synthesis, deprotection of the phosphotriesters and the exocyclic amine groups of the nucleobases with methylamine in methanol was accompanied by cleavage from the solid support to yield the 2′-OTBS-protected oligomer. Removal of the silyl groups with TEA·HF and subsequent purification by anion exchange chromatography yielded target RNA fragment **9**.

For conjugation with oligonucleotide **9**, the cationic amphiphilic peptide penetratin **11** (Scheme 3) and a tetrameric fragment of hyaluronan **10** were selected. Penetratin, a 16-amino acid peptide having the sequence Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2 is known for its ability to internalize covalently attached

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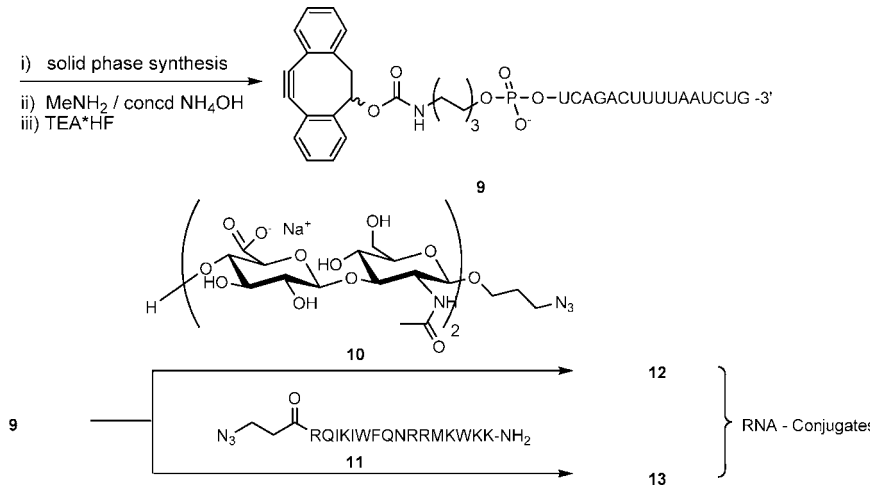
(32) Solution-phase experiments revealed that the alkyne is also stable towards *t*-BuOOH (100 equiv, 90 min).

(33) The final synthetic step and full compound characterization can be found in the Supporting Information. For further synthetic information and intermediates, see: Verdoes, M.; Florea, B. I.; Hillaert, U.; Willems, L. I.; van der Linden, W. A.; Sae-Heng, M.; Filippov, D. V.; Kisselev, A. F.; van der Marel, G. A.; Overkleeft, H. S. *ChemBioChem* **2008**, *9*, 1735–1738.

Scheme 3. Synthesis of 5′-Alkyne Functionalized Ribonucleic Acid 16-mer and Its Conjugation to Hyaluronic Acid Tetramer and Penetratin



peptides and oligonucleotides into many cell types.[7] Hyaluronic acid (HA), a linear polysaccharide of the glycosaminoglycan family, is involved in a wide variety of biological processes, such as cell migration and proliferation. HA and fragments thereof are known to be recognized by the CD44 protein, and studies have revealed that conjugates of this carbohydrate showed an increased uptake in cancer cells due to overexpression of the CD44 protein.[34] The preparation of azido-functionalized penetratin **11** started with automated solid-phase peptide synthesis (SPPS), using RAM Tentagel and Fmoc chemistry, followed by manual coupling with azidopropionic acid succinimidyl ester.[35] Final deprotection and cleavage from the solid support followed by HPLC purification yielded the peptide penetratin equipped with an azide moiety **11**. Conjugation with the RNA oligomer (**9**) was performed in a phosphate buffer saline pH 7.3. Although the solubility of the reaction partners was initially incomplete, the reaction proceeded toward a clear solution and after 5 h. LC−MS analysis showed the formation of a single product.

Next the conjugation of the RNA fragment with a hyaluronan tetramer was undertaken. The tetrasaccharide **10** containing an azide spacer on the reducing end was obtained as previously reported by our group.[36] Similar cycloaddition of tetrasaccharide **10** with the RNA oligomer **9** in water as the solvent and shaking for 4 h resulted in complete disappearance of starting materials and formation of conjugate **12**, as observed by LC−MS. HPLC purification afforded the target conjugates **12** and **13**. Both the DNA and the RNA conjugates were purified for analytical purposes. Purification on a semipreparative extended polar selectivity column allowed only partial separation of the two putative regioisomers. Hence, the conjugates were isolated as mixtures.

The facile preparation of dibenzocyclooctynol containing phosphoramidite **5**, the seamless implementation of this new reagent in standard automated nucleic acid synthesis, as well as the efficiency of the ensuing cycloaddition reaction of equimolar quantities of alkyne-functionalized RNA (DNA) oligomers with azide-containing biomolecules make this method a valuable alternative for existing procedures toward RNA (DNA) conjugates. The existence of a variety of azide-functionalized entities such as nanoparticles and surfaces allows the application of the here presented cyclooctyne-functionalized oligonucleotides to other fields of research. Future work from our side includes the conjugation of oligonucleotides to proteins and the application of other nucleic acid derivatives such as thiophosphoryl DNA[29a] and 2′-*O*Me RNA.

Acknowledgment. This work was funded by the Dutch Organization for Scientific Research (NWO).

Supporting Information Available: Spectroscopic data and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

OL102357U

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