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COMMUNICATION

2'-Modified thymidines with bioorthogonal cyclopropene or sydnone as building blocks for copper-free post-synthetic functionalization of chemically-synthesized oligonucleotides

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Novel 2'-functionalized nucleoside phosphoramidite monomers comprising bioorthogonal methylcyclopropene or sydnone moieties were developed and applied for the first time to oligonucleotide solid-phase synthesis. Traceless oligonucleotide post-synthetic modifications with reactive complementary probes were successfully achieved through either inverse electron-demand Diels-Alder (IEDDA) reaction or strain-promoted sydnone-alkyne cycloaddition (SPSAC).

Methods for conjugating different agents (fluorophores, carbohydrates, peptides, lipids...) onto oligonucleotides are important for various chemical biology studies and have diverse applications in addressing oligonucleotide delivery in the field of medicinal chemistry.^{1,2} Two strategies could be envisioned to introduce, internally, these species onto oligonucleotides (*i*) at an early stage, before oligomerization through the incorporation of a phosphoramidite building block with the final desired functionality; (*ii*) after oligonucleotide synthesis through post-synthetic modification of a nucleoside containing a small reactive group. In the first approach, the highly modified monomer has to withstand either the harsh chemical conditions in the case of solid-phase synthesis (SPS) or the polymerase enzymatic conditions. The second approach offers a more modular and efficient way to synthesize diverse labelled oligonucleotides from a single common precursor. Incorporating a modified monomer with a small chemical tag does not significantly affect the overall yield of the synthesis. In a subsequent step, the resulting oligonucleotide is functionalized with the desired reactive moiety in better yield. Among the multitude of chemical strategies available for post-

synthetic conjugation, bioorthogonal chemistry has emerged as a particularly robust and versatile approach, enabling highly efficient and selective chemical modifications of oligonucleotides in vitro, in cells, or even in living organisms. Thus, various DNA or RNA phosphoramidites and/or triphosphate building blocks containing most of the classical bioorthogonal groups (azide, alkyne, tetrazine, cyclooctyne, methylcyclopropene) have been developed.^{3,4}

The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) remains one of the most popular bioorthogonal reaction because of its fast kinetics and high efficiency. It has proven to be a valuable reaction for DNA and RNA modifications with different dyes and labels.^{5,6,7} Several nucleosides bearing an alkyne reporter group either on the nucleobase (C-5 position of pyrimidines or C-7 position on 7-deazapurines) or on the 2'-OH position of ribose, were prepared as phosphoramidite or as triphosphate derivatives and subsequently used in chemical or polymerase oligonucleotide synthesis.^{8,9,10,11,12} The backbone post-synthetic functionalization by CuAAC was also developed using an alkynyl phosphinoamidite, which is compatible with conventional solid-phase oligonucleotide synthesis.¹³ The azide group, however, is not compatible with phosphoramidite synthesis on solid support due to its reactivity with phosphorous (III). Alternatively, the incorporation of a building block with a P(V) and an azidomethyl substituent on the 2'-hydroxy group could be performed using the phosphotriester method.¹⁴ Additionally, azido moieties can be introduced onto oligonucleotides through post-functionalization using, for instance, 6-azidohexanoic acid and 2'-aminoethoxy-modified ribose.¹⁵ Azide-modified ribo- and deoxyribonucleotide triphosphates can also be incorporated into nucleic acids via polymerase-based methods for efficient CuAAC labelling of modified RNA or DNA.^{16,17}

More recently, copper-free cycloadditions have been applied to the post-synthetic modification of oligonucleotides. Among the reported strategies, strain-promoted alkyne-azide cycloaddition (SPAAC) and inverse electron demand Diels-Alder (IEDDA) have proved useful for internal labelling. The

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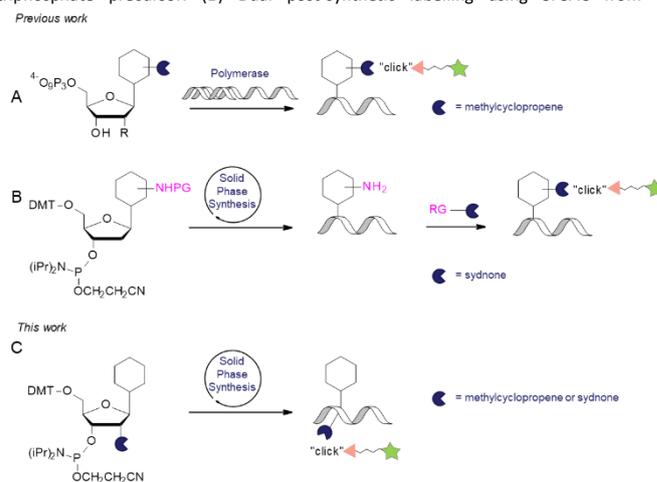
† Electronic Supplementary Information (ESI) available: Experimental procedures, characterization and supplementary data. See DOI: 10.1039/x0xx00000x

first approach involved nucleoside-based building blocks (phosphoramidite and/or triphosphate) in which cyclooctynes (DIFO¹⁸, DIBO^{15,19}, BCN^{15,19}, COMBO²⁰) were grafted to either nucleobase or ribose. In the second approach, several nucleosides containing dienes or dienophiles were also reported. As dienes, 1,2,4-triazines-modified nucleotides have already been incorporated enzymatically in oligodeoxynucleotides (ODN).^{21,22,23} Despite their established utility in bioorthogonal chemistry, 1,2,4,5-tetrazine moieties have seen limited use in the context of oligonucleotide synthesis. However, both dienes, phosphoramidite²⁴ and triphosphate,²³ were incorporated in ODNs. *Trans*-cyclooctene (TCO) and norbornene dienophiles were only introduced on nucleobases and the resulting building blocks were used to synthesize ODNs through enzymatic^{25,26,27,28,29} or chemical approaches.³⁰ Notably, TCO was found to be sensitive to standard aqueous iodide oxidation and requires alternative conditions.³¹

Expanding the range of nucleosides functionalized with bioorthogonal reactive groups has made it possible to develop highly selective one-pot or sequential labelling of nucleic acids with mutually exclusive pairs of bioorthogonal labelling reagents.^{32,33,31} In this context, we were interested in expanding the toolbox of phosphoramidite building blocks containing small bioorthogonal functions that can be labelled in copper-free conditions, thus allowing their use in living cells. We therefore focused our attention on the methylcyclopropene and the sydnone reactive groups, allowing iEDDA and strain-promoted sydnone-alkyne cycloaddition (SPSAC)^{34,35}, respectively. Importantly, these reactions exhibit faster kinetics than SPAAC.³⁶ To our knowledge, the introduction of these two minimal bioorthogonal phosphoramidite monomers directly into a chemical solid-support synthesis cycle has not yet been reported. Recently, methylcyclopropene and sydnone moieties have been incorporated into oligonucleotides utilizing two different pathways. For instance, methylcyclopropene-modified triphosphate nucleotides have been introduced enzymatically into oligoribonucleotides (ORN) by T7 *in vitro* transcription³⁷ or in ODN by standard DNA polymerases by Kath-Schor and Wagenknecht teams, respectively (Fig. 1A).^{38,23,32} Furthermore, they validated the site-specific labelling using post-synthetic reactions with tetrazine-based probes. The sydnone moiety was introduced into an ODN by Wagenknecht's team using a C-5-amino-modified thymidine phosphoramidite post-synthetic approach and an amide coupling reaction.³⁹ Finally, cyclooctyne probes were introduced, allowing the labelling of the sydnone-modified ODN through a dual post-synthetic strategy. This sequence demonstrated the compatibility of sydnone as bioorthogonal function to further label oligonucleotides (Fig. 1B).³⁹

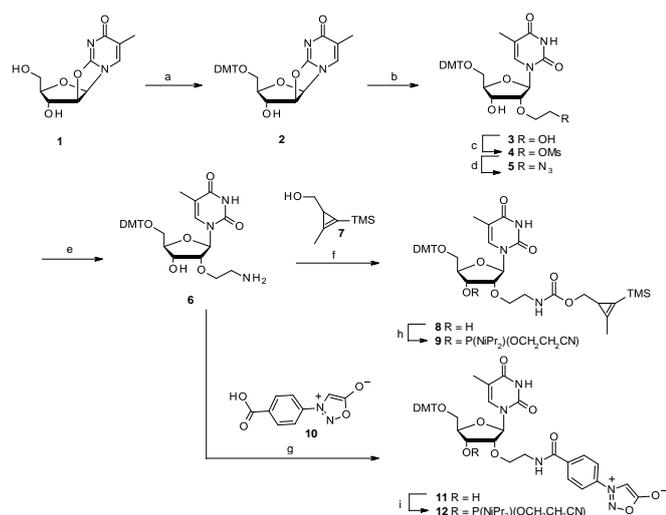
Here, we report *i)* the synthesis of two new phosphoramidite building blocks bearing either methylcyclopropene or sydnone bioorthogonal moieties, *ii)* their use and compatibility in oligonucleotide solid phase synthesis, and *iii)* their post-synthetic labelling with tetrazine and cyclooctyne probes, respectively (Fig. 1C).

Fig. 1. Post-synthetic labelling of ODN/ORN. (A) Labelling using iEDDA starting from a triphosphate precursor. (B) Dual post-synthetic labelling using SPSAC from a



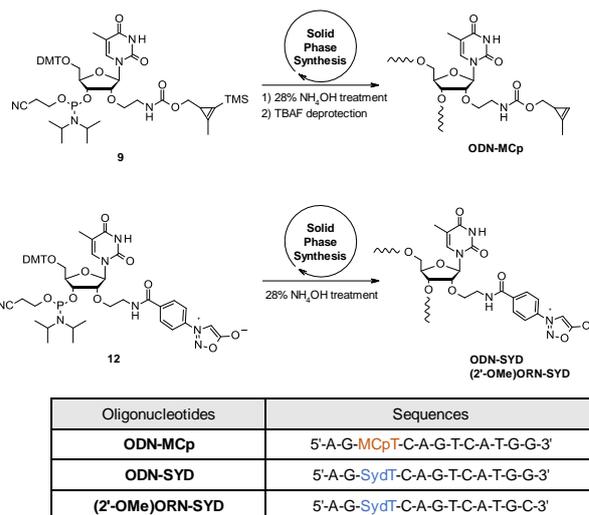
phosphoramidite precursor. (C) Our work: 2'-functionalization from a phosphoramidite precursor demonstrating the compatibility of methylcyclopropene and sydnone bioorthogonal groups in solid-phase oligonucleotide synthesis.

To this aim, we designed two new phosphoramidite monomers containing either a methylcyclopropene or an arylsydnone attached *via* an aminoethoxy linker to the 2'-O-position of the ribothymidine. The attachment of these bioorthogonal groups to the 2'-position may enhance duplex stability, and nuclease resistance.⁴⁰ These building blocks have the potential to be valuable monomers for post-functionalization of oligonucleotide, particularly in antisense applications. Both 2'-modified phosphoramidite building blocks **9** and **12** were obtained from the similar intermediate 2'-aminoethoxy ribothymidine **6** (Scheme 1). The amine intermediate **6** was synthesized starting from the commercially available 2,2'-anhydrothymidine **1** using the procedure reported by Brown *et al.*⁴¹ In brief, after tritylation, the DMT protected 2,2'-anhydrothymidine **2** was subjected to nucleophilic ring opening by ethylene glycol in the presence of titanium(IV) isopropoxide. The resulting primary alcohol **3** was subsequently treated with MsCl at a low temperature, followed by displacement of the mesyl group by sodium azide and final Staudinger reduction of the azide moiety yielded the desired 2'-aminoethoxy ribothymidine **6** with an overall yield of 12%. A carbamate linker was chosen to introduce the methylcyclopropene moiety since tetrazine-cyclopropene ligation had been shown to proceed significantly faster with the more electron-rich carbamate compared to the amide linker.⁴² The precursor **6** was converted to the desired carbamate **8** *via* a carbonyldiimidazole (CDI) coupling with TMS-methylcyclopropene alcohol **7** in a moderate 46% yield. The functionalized amide phenylsydnone **11** was prepared in 72% yield by coupling the phenylsydnone acid **10** with the precursor **6** according to standard coupling conditions. Finally, phosphorylation of **8** and **11** afforded phosphoramidite monomers **9** and **12** in 62% and 74% yields, respectively.



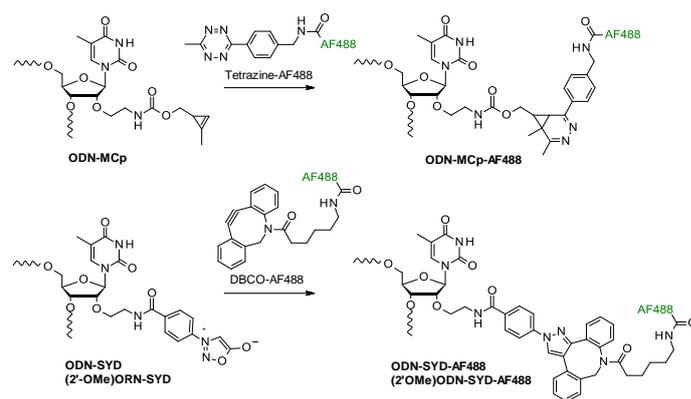
Scheme 1. Synthesis of methylcyclopropene and syndnone-based phosphoramidite building blocks **9** and **12**. Reagents and conditions: a) DMT-Cl, pyridine, rt, 2.5 h, 75%; b) ethylene glycol, Ti(OiPr)₄, DMF, 150 °C, 24 h, 62%; c) MsCl, Et₃N, CH₂Cl₂, -70 °C, 4 h, 57%; d) NaNa₃, 18-crown-6, DMF, 60 °C, 18 h, 90%; e) PPh₃, THF, H₂O, 45 °C, 18 h, 84%; f) **7**, CDI, THF, rt, 3 d, 46%; g) **10**, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), DIPEA, DMF, rt, 1 h, 66%; h) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, DIPEA, CH₂Cl₂, rt, 6.5 h, 62%; i) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH₂Cl₂, rt, 3 h, 74%.

Next, we verified the stability of **8** and **11** toward the reagents used during oligonucleotide solid-phase chemistry (acidic detritylation, capping, oxidation and basic cleavage). Preliminary results of I₂-mediated oxidation of **8** to convert the P(III) into P(V) showed a mixture of by-products. (Camphorsulfonyl)oxaziridine (CSO) replaced iodine as an oxidative reagent; interestingly, under these optimized conditions, no by-products were observed (Table S1, ESI[†]). In addition, several basic conditions used for resin-mediated cleavage were tested (Table S1, ESI[†]). Aqueous ammonia treatment of **8** should be performed at room temperature to avoid partial degradation. Next, phosphoramidites **9** or **12** were successfully involved in synthesizing two dinucleotides with 2'-*O*-methoxy-3'-acetate uridine under standard coupling conditions in solution (Scheme S1, ESI[†]). These dinucleotides were then subjected to iEDDA and SPSAC reactions, in aqueous conditions, with a tetrazine and a cyclooctyne, respectively to afford the expected products in good yields (Schemes S3-S4, ESI[†]). To validate the incorporating of these new synthesized bioorthogonal building blocks via solid-phase synthesis, the preparation of 12-mer oligonucleotides in ODN and (2'-OMe)ORN series including **9** or **12** at internal positions was undertaken. Oligonucleotides **ODN-SYD**, (2'-OMe)ORN-SYD containing syndnone **12** and **ODN-MCp** containing cyclopropene **9** were successfully synthesized in an oligonucleotide synthesizer at a 1 μmol scale (Scheme 2, Fig. S1 ESI[†]). The synthesis of the **ODN-MCp** was, however, less efficient than that of the **ODN-SYD** due to some degradation during the desilylation and basic cleavage steps. Moreover, we were unable to synthesize the MCp-modified ORN strand.



Scheme 2. Automated solid phase synthesis of syndnone or methylcyclopropene-modified 12-mers oligonucleotides.

Once the modified 12-mers were obtained, we investigated both click reactions according to the procedure reported by Wagenknecht (i.e. low concentration, aqueous conditions).³⁹ Oligonucleotides were treated with AlexaFluor 488-functionalized tetrazine or dibenzocyclooctyne (DBCO) as complementary reagents for iEDDA and SPSAC, respectively (Scheme 3, Scheme S5, ESI[†]).



Scheme 3. iEDDA and SPSAC reactions on 12mers (2.5 μM) treated with 10 equiv. of AlexaFluor 488-functionalized tetrazine or DBCO in phosphate buffer, pH 7.0 at 37 °C.

After a 60 minutes reaction time, the LC/MS traces indicated good conversions with 68%, 71%, and 53% for **ODN-SYD**, (2'-OMe)ORN-SYD and **ODN-MCp**, respectively (Fig. 2, Fig. S4-S6 ESI[†]). The reactions were completed in approximately 180 minutes. Interestingly, the kinetics of SPSAC showed no significant difference between the (2'-OMe)ORN strand and the ODN strand. On the other hand, iEDDA was shown to be slower than SPSAC.

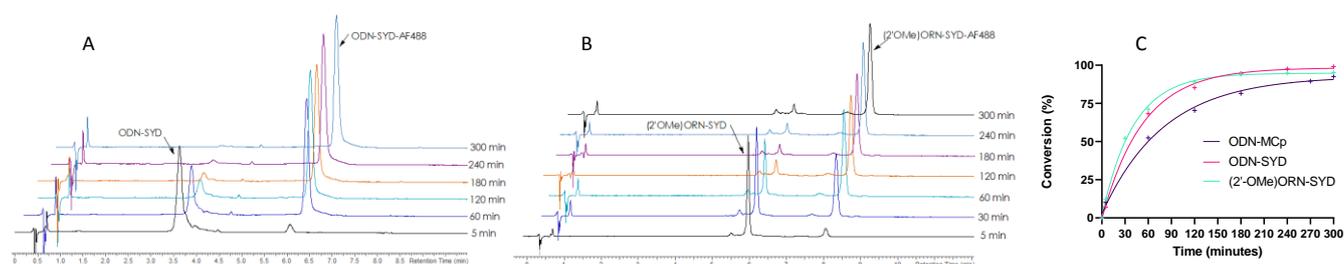


Fig 2. LC monitoring of click reactions at $\lambda = 260$ nm. (A) SPSAC on **ODN-SYD**, (B) SPSAC on **(2'-OMe)-ORN-SYD**, (C) Time-dependent evolution of labelling reactions.

In conclusion, this study increases the diversity of phosphoramidite building blocks including copper-free bioorthogonal moieties. We describe herein for the first time the compatibility of sydnone and cyclopropene moieties with oligonucleotide solid phase synthesis conditions. Furthermore, we verified the stability of synthesized oligonucleotides and showed that sydnone-modified ones are stable. Conversely, some degradation of carbamate-linked **ODN-MCp** was observed. Such instability has already been reported after an extended period of storage and several freeze-thaw cycles.⁴³ Conjugation with other linkers will be explored in due course. We successfully synthesized a sydnone-modified ORN, demonstrating the potential utility of the corresponding new phosphoramidite for the synthesis and functionalization of oligonucleotide therapeutics such as ASOs. Interestingly, the copper-free post-synthetic functionalization conditions showed reaction rates compatible with further studies in cells. These new bioorthogonal building blocks are also interesting for future use in dual labelling as they are potentially orthogonal to each other.

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Conflicts of interest

There are no conflicts to declare.

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In this study, we introduced for the first time phosphoramidite building blocks bearing sydnone or methylcyclopropene bioorthogonal moieties in oligonucleotides through solid-phase synthesis. Oligonucleotide post-synthetic modifications with complementary reactive probes were successfully achieved.

