ChemBioChem

Research Article
doi.org/10.1002/cbic.202300701

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Orthogonal End Labelling of Oligonucleotides through Dual Incorporation of Click-Reactive NTP Analogues

Eva S. Schönegger[+],[a, b] Antony Crisp[+],[b] Marco Radukic,[c] Jonas Burmester,[b] Thomas Frischmuth,*[b] and Thomas Carell*[a]

Post-synthetic modification of nucleic acid structures with clickable functionality is a versatile tool that facilitates many emerging applications, including immune evasion, enhancements in stability, fluorescent labelling, chemical 5′-RNA-capping and the development of functional aptamers. While certain chemoenzymatic approaches for 3′-azido and alkynyl labelling are known, equivalent 5′-strategies are either inefficient, complex, or require harsh chemical conditions. Here, we present a modular and facile technology to consecutively modify DNA and RNA strands at both ends with click-modifiable functional groups. Our approach using γ-modified ATP analogues facilitates T4 PNK-catalysed 5′-modification of oligonucleotides, a process that is compatible with TdT-catalysed 3′-elongation using 3′-azido-2′,3′-ddGTP. Finally, we demonstrate that our approach is suitable for both oligo-oligo ligations, as well ssDNA circularization. We anticipate that such approaches will pave the way for the synthesis of highly functionalised oligonucleotides, improving the therapeutic and diagnostic applicability of oligonucleotides such as in the realm of next-generation sequencing.

Introduction

Over the past decades, site-specific bioconjugation of nucleic acids has allowed the burgeoning field of nucleic acid technology to occupy previously unimaginable niches, such as cellular tracking, encoding of small molecule libraries, and the synthesis of targeted oligonucleotide siRNA therapeutics.[1] These and other innovations rely entirely upon our ability to introduce specific chemical modifications into nucleic acid molecules, either during their synthesis, or post-synthetically. When it comes to post-synthetic modification of the 3′-ends of DNA and RNA molecules, an assortment of chemical and enzymatic options are available, with the most notable example being polymerase-mediated incorporation of a modified nucleoside triphosphate. 5′-end labelling, meanwhile, is typically limited by stochastic techniques such as nonspecific chemical crosslinking.[2] Motivated by the absence of suitable methods, we set out to develop a general click chemistry-based chemoenzymatic procedure to modify the 5′-end of single-stranded nucleic acids (Figure 1). A key requirement was for us that the new method is compatible with existing nucleotidylexotransferase mediated 3′-ligation reactions, such as those pioneered by the Kukhanova laboratory (Figure 1).[3–7] Such a method would allow enhanced detectability of oligonucleotides,[8] and could pave the way for dual 3′- and 5′-adapter ligations as required for next-generation sequencing library preparation.[9] In addition, such a method could enhance antibody and payload ligations to oligonucleotides,[10] post-synthetic 5′-capping, chemical gene synthesis,[11] and, as investigated here, nucleic acid circularisation.[9] The click-chemistry based method would also complement recent 5′-labelling approaches by the Rentmeister laboratory (Figure 1) that are based on promiscuous

[a] E. S. Schönegger,[+] Prof. Dr. T. Carell
Department Chemie
Ludwig-Maximilians-Universität München
Butenandtstr. 5–13, 81377 Munich (Germany)
E-mail: Thomas.carell@lmu.de

[b] E. S. Schönegger,[+] Dr. A. Crisp,[+] J. Burmester, Dr. T. Frischmuth
baseclick GmbH
Floriansbogen 2–4, 82061 Neuried (Germany)
E-mail: t.frischmuth@baseclick.eu

[c] Dr. M. Radukic
Technische Fakultät
Universität Bielefeld
Universitätsstraße 25, 33615 Bielefeld (Germany)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202300701

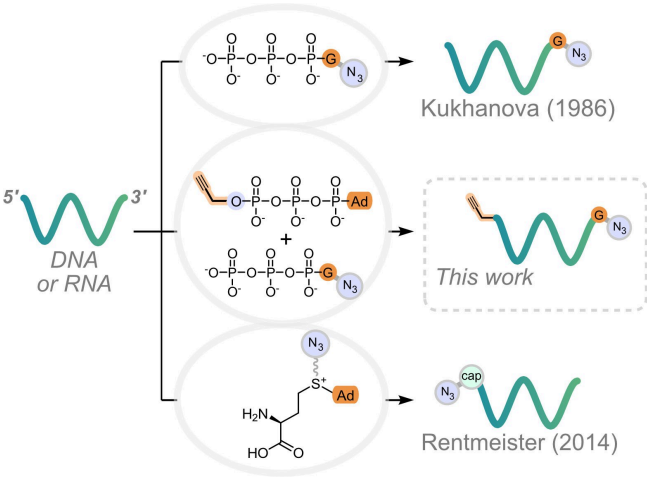


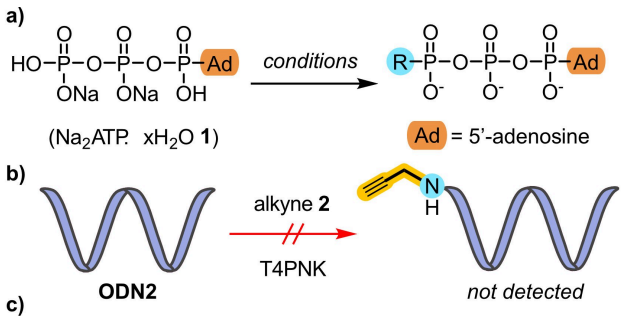
Figure 1. Schematic representation of chemoenzymatic oligonucleotide end-labelling methods that are currently in use.

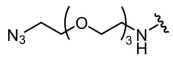
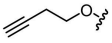
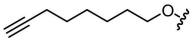
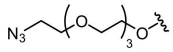
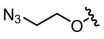
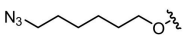
methyltransferase (Ecm1).[8] Furthermore, our approach has the advantage that it involves only one synthetic step per label, and minimal chemical alteration to the DNA or RNA structure (Figure 1). We anticipated that this could be accomplished via the T4 PNK-catalysed transfer of an alkyne- or azido-modified γ-phosphate group from adenosine triphosphate (ATP)[12–14] to 5′-hydroxy groups of nucleotides and deoxyribonucleotides – a process that is inherently compatible with TdT-catalysed 3′-elongation reactions (Figure 1).

Results and Discussion

Motivated by the great potential of γ-phosphate modified ATP analogues for protein kinase catalysed reactions advertised by Lee and co-workers,[15] we prepared a small collection of suitably γ-labelled adenosine 5′-triphosphates, with embedded click- functionality, according to a method previously published by our laboratory. In that study, fluorophores were coupled to γ-modified ATP-analogues by means of Cu^{I}-catalysed alkyne-azide cycloaddition (CuAAC) reactions.[16] For the synthesis, treatment of adenosine 5′-triphosphate (ATP) disodium salt hydrate **1** (Figure 2) with propargylamine in the presence of EDC-HCl afforded compound **2** in a highly satisfactory yield (97%), as determined by analytical RP-HPLC. In order to prepare **2** for use with the enzyme T4-PNK, an ion-exchange method was applied to achieve efficient desalting (Chelex® 100 sodium form), followed by a dialysis for 12 h. We next prepared compound **3** using the same method, which provided **3** in a more modest, albeit practicable yield (48%). Originally, phosphoramidates were synthesised, based on their potential application as T4 PNK substrate published by Anthony and co-workers.[17] However, in anticipation that phosphoramidates such as **2** or **3** might be prone to acid-mediated solvolysis,[18,19] we further prepared a subset of phosphate monoester-linked alkynes (compounds **3–6**) and azide (compound **7**), which was obtained in up to 68% yield by nucleophilic substitution, involving either the corresponding alkyl halide or *p*-toluenesulfonate (Supporting Information, Section 3.2). In addition, we prepared the organothiophosphate **8** using similar conditions (Supporting Information). Finally, compounds **9** and **10** were purchased for the purpose of further reactivity screening.

Having this library of compounds in hand, we next investigated their applicability for 5′-labelling followed by a CuAAC click reaction.[20] Initially, we synthetically prepared the longer 43-mer oligonucleotide **ODN 1** and the shorter 17-mer nucleotide **ODN 2** (Table S1 in the Supporting Information). For the first experiment, **ODN 2** was incubated with alkyne **2** and T4 PNK (37 °C, 50 U). Upon the anticipated completion of the enzymatic reaction after 3 h, the resulting 5′-modified oligodeoxynucleotide was subsequently treated with either CalFluor-647-azide *or* AzDye™-350-azide in the presence of Cu^{I} to initiate the click reaction. Contrary to our expectations, however, neither experiment yielded any triazole-containing cycloadduct, as inferred from denaturing polyacrylamide gel electrophoresis (dPAGE) images. Instead, mass-spectrometric (MALDI-TOF) data hinted at γ-phosphoramidyl-hydrolysis, which provided the *m/z*



Entry	R	Conditions	Conversion*
2		(ii)	97%
3		(ii)	48%
4		(i)	64%
5		(ii)	15%
6		(i)	68%
7		(i)	46%
8		(iii)	96%
9		-	-
10		-	-

*conversion monitored by analytical RP-HPLC.

Figure 2. a) Synthesis of azide and alkyne γ-labelled nucleoside 5′-triphosphates. b) Unsuccessful first labelling experiment with alkyne **2** on **ODN 2**. c) Compound library, reagents and conditions: (i) Step 1: **1**, CHELEX-100, $[Bu_4N]Br$, Step 2: alkylating tosylate/bromide reagent (Supporting Information), anhydrous DMF, overnight, Ar, RT; (ii) **1**, EDC·HCl, alkylating amine, H_2O, (DMF), 18 h, 25 °C; (iii) ATPγS, DMF/D_2O, $NaHCO_3$, propargyl bromide, RT, overnight.

values for the 5′-phosphorylated oligodeoxynucleotide **ODN 2 (5′-PHO-ODN 2)** as the sole reaction product. This result is consistent with previous observations of a rapid hydrolysis of phosphoramidates under mildly acidic conditions.[18,19] Similar outcomes were also observed when the azide **3** was employed as the reactive triphosphate (Table S2).

We therefore began to experiment with γ-O-ATP esters such as compounds **9** and **4**, anticipating that these might not suffer the same drawbacks as their γ-N-ATP analogues, and given their reported comparative stabilities at pH values as low as 2.0.[19] Gratifyingly, when the same process was conducted using the 43-mer **ODN 1** with azide **9** as the substrate, the product **ODN 1-a** was indeed obtained in up to 37% yield (Figure 3, Table S3).

When treated with alkyne **4**, the yield of the product **ODN 1-b** (**ODN 1_propargyl-PHO**) was higher (57%, Table S3). In order

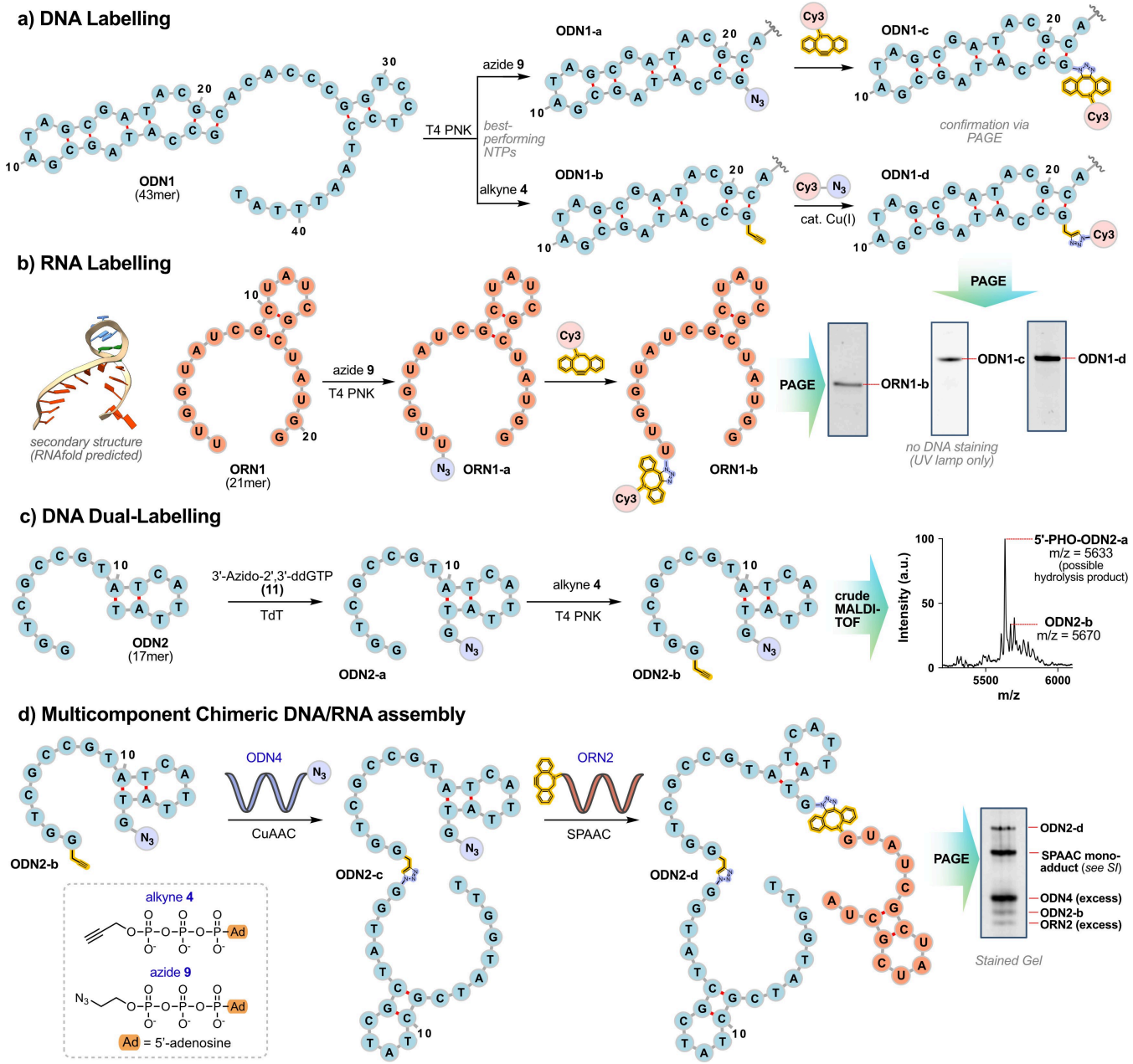


Figure 3. Schematic illustration of several different applications of sequential 3′- and 5′- labelling. a) A DNA 43-mer (**ODN 1**) is labelled at the 5′-end with either an alkyne or an azide, and subsequently click-ligated with a fluorophore (Cy3) for dPAGE visualisation (Figures S1 and S2). b) An RNA 21-mer (**ORN1**) is labelled at the 5′-end with an azide, and subsequently ligated with Cy3 (Figure S3). c) A DNA 17-mer (**ODN 2**) is labelled with an azide (**11**) at the 3′-end, and then an alkyne (**4**) at the 5′-end (Table S5). d) The product from the previous reaction is subsequently treated with two new alkyne- and azido-labelled oligonucleotides, thus affording a new DNA–RNA hybrid product assembled from three oligonucleotide components (for full gel image see Figure S4). For illustrative purposes, the depicted secondary structures were calculated using either RNAComposer[21,22] or UNAFold[23] using the default parameters, and then visualised using forna[24] and ChimeraX[25]. • = DNA, • = RNA.

to gain further proof for the successful enzymatic modification and subsequent click reaction, we next carried out click reactions using the fluorophore Cy3 which allows visualisation of the reaction outcome by dPAGE. For the experiment we treated **ODN 1-a** with dibenzocyclooctyne-Cy3 (DBCO–Cy3) and indeed cleanly afforded **ODN 1-c**, as confirmed by dPAGE, both with and without GelRed stain (Figures 3a and S2, confirmed by RP-HPLC, Table S4). Encouraged by this result, we subsequently performed an analogous experiment using **ODN 1-b** in combination with Cy3-azide, which once again, yielded the dye-adduct **ODN 1-d** in a clean reaction (Figures 3a and S1).

Given that single-stranded ribonucleic acids are known to be natural substrates of T4 PNK, we were curious as to whether a suitable oligoribonucleotide (ORN) could also be labelled. Thus, we next conducted an experiment where we treated the RNA 21-mer **ORN 1** (Table S1) with azide **9** in the presence of T4 PNK. To our delight, this provided the labelled product, which was visualised via a SPAAC reaction,[26] involving either

DBCO–Cy3, or alternatively a 3′-DBCO labelled oligodeoxynucleotide (**ODN 3**, Table S1), which produced the expected 21 nucleotide shift upon dPAGE analysis (Figure S3). The yield of the 5′-end labelling and thus also the yield of the subsequent SPAAC is lower compared to the performed experiments with **ODN 1** and **ODN 2** (Figure S2). This can be explained by well-known observations, that the labelling efficiency by T4 PNK is sequence specific. Thus, factors, such as the identity of the 5′-terminal nucleotide or the ORN folding state can have an impact on the 5′-labelling efficiency.[27] A natural extension of this strategy was to assemble a longer hybrid-DNA/RNA oligonucleotide by incrementally combining two DNA and one RNA oligonucleotide. We envisaged that this could be accomplished by site-selectively labelling both the 5′- and 3′-ends of a template oligo with distinct azido and alkynyl functionality followed by two sequential click reactions. To this end, the 17-mer **ODN 2** was first treated with 3′-azido-2′,3′-ddGTP (**11**) in the presence of catalytic TdT, which gave upon spin column purification, the anticipated 3′-azido labelled oligodeoxynucleotide **ODN2-a**, as confirmed by MALDI-TOF-MS (Table S5). **ODN 2-a** was then treated with alkyne **4** in the presence of T4 PNK, to afford **ODN 2-b**. In this case, the crude MALDI-TOF showed formation of both the expected products, as well as another peak at *m/z* 5633 (**5′-PHO-ODN 2-a**), possibly indicating hydrolysis of the formed alkyne. Anticipating that this might be caused by the ionisation step during MALDI-TOF analysis, we directly continued with the click reaction. Indeed, the dual click reactions of **ODN 2-b** together with first the 3′-azido oligodeoxynucleotide **ODN 4**, (10× molar excess, Table S1) and second with the 5′-DBCO modified oligoribonucleotide **ORN 2** (2× molar excess, Table S1) provided the chimeric DNA/RNA product **ODN 2-d**, with only traces of 5′-unligated starting material (visible by dPAGE, Figure S4). This strengthened our hypothesis that the dominant signal in the crude MALDI-TOF corresponded to the ionisation-induced 5′-hydrolysis product. The double chemoenzymatic addition of azide and alkyne functionalities and even the double click reaction had worked as inferred from the fact that the correct oligonucleotide was among the dominant species detected by dPAGE, taken into account, that **ODN 4** and **ORN 2** were added in excess (Figures 3d and S4).

In a further experiment meant to examine the synthetic utility of our method, we set the goal to achieve circularisation of a medium length (100 nt) oligodeoxynucleotide, **ODN 5** (Table S1), which was specifically selected for generating a self-annealing stem loop at the ends. Beyond their many biological roles,[28] circular nucleic acids have recently attracted enormous interest due to their asserted exonuclease resistance,[29] which makes them highly attractive candidates as novel RNA therapeutics.[30] In order to accomplish this task, **ODN 5** was initially both 5′- and 3′-azido labelled, according to the procedure described above to afford **ODN 5-a** (Figure 4). The purified and labelled oligodeoxynucleotide was then reannealed, and treated with the bifunctional crosslinker **12**, DBCO-PEG4-DBCO, to give **ODN 5-b**. Upon completion of the reaction, we proved the circularization by treating the reaction mixture with MspI, a restriction enzyme that recognises the sequence CCGG. Remarkably, 10% dPAGE of digested and undigested

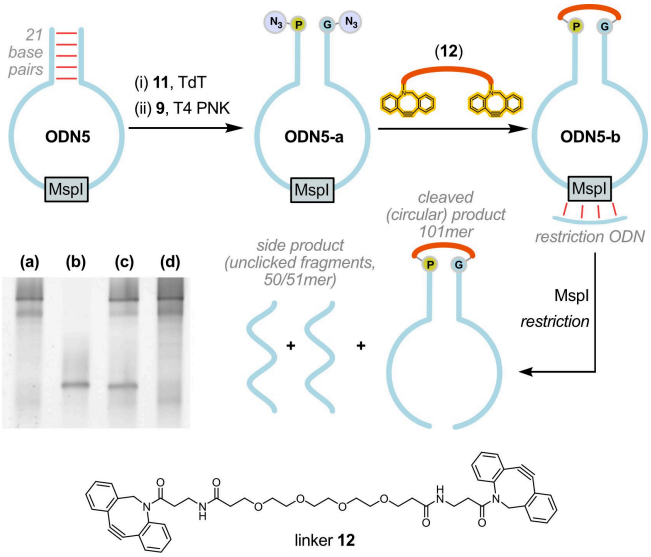
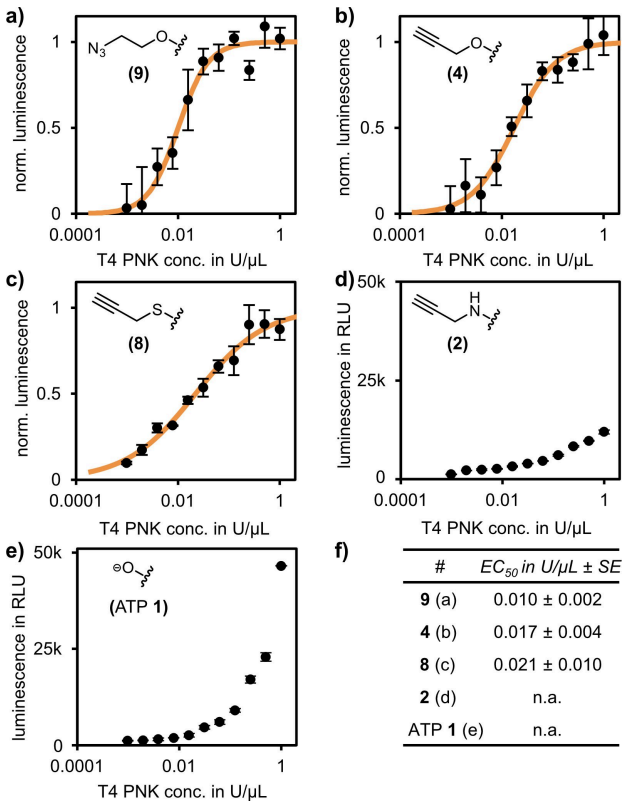


Figure 4. Schematic illustration of **ODN 5** circularisation and subsequent proof of concept with MspI. 10% dPAGE: a) **ODN 5**: 100-nt oligonucleotide (Supporting Information), b) **ODN 5** treated with MspI to give 50-nt oligonucleotide digestion products, c) Crude reaction product containing **ODN 5**, **ODN 5-a**, and **ODN 5-b** treated with MspI give mixture of circular 101-nt and 50-/51-nt linear digestion products, d) Crude mixture containing **ODN 5**, **ODN 5-a**, and **ODN 5-b**, without MspI digestion. P═PHO (monophosphate), G–N_3 = 3′-azido-2′,3′-ddGMP.

ODN 5 showed an almost complete disappearance of the 100 nt upper band, whereas the clicked product **ODN 5-b** did not, thus indicating a successful circularization process (for full gel image, see Figure S5).

Beyond the γ-modified ATP analogues alkyne **4** and azide **9**, we also screened the other synthesised candidates for their ability to perform 5′-labelling of the 43-mer **ODN 1**. The screening revealed that shorter γ-O-linked phosphate esters such as **4** and **9** perform far better as substrates for the T4 PNK catalysed kinase reaction than those with slightly longer linkers. **ODN 1** for example was labelled in only 19% and 7% conversions with alkyne **5** and azide **10**, respectively (Table S3). The even longer linkers produced no detectable product. Based on these results, we postulated that the active pocket of the T4 PNK might be sterically constrained, such that larger or more hydrophobic linkers are not suitable to 5′-labelling. To further investigate our hypothesis, we determined binding characteristics of the compounds to T4 PNK.

One indirect but relatively straightforward way to achieve this goal is to measure T4 PNK-mediated hydrolysis of the compounds, enabled by an ADP-dependent luciferase assay (ADP-Glo assay, Figure 5). In this assay, we observed that the azido and alkynyl esters **9** and **4** indeed showed the expected sigmoidal curve of the luminescence signal depended on T4 PNK concentration. The obtained EC_{50} values as proxy metrics for their affinities towards T4 PNK were also similar with $0.010 \pm 0.002\ U\,\mu L^{-1}$ for azide **5** and $0.017 \pm 0.004\ U\,\mu L^{-1}$ for alkyne **4**. The N-linked alkyne **2** also showed expectedly no enzyme dependency. Surprisingly, the S-linked alkyne **8** indicated an unexpected luminescence signal intensity and curve shape (Figure 5c), although no ODN labelling was



#	EC_{50} in U/µL ± SE
9 (a)	0.010 ± 0.002
4 (b)	0.017 ± 0.004
8 (c)	0.021 ± 0.010
2 (d)	n.a.
ATP **1** (e)	n.a.

Figure 5. T4 PNK-mediated hydrolysis of ATP analogues under optimised reaction conditions with 1 µM **ODN 1**, at various enzyme concentrations, determined by an ADP-dependent luciferase assay. The standard T4 PNK concentration is 1 U µL^{-1} (the respective rightmost data points). a–c Results are shown as luminescence normalised to the upper and lower asymptote of each fit (orange line, logistic, four-parameter) versus T4 PNK concentration. d, e Results are shown as absolute luminescence signal from the plate reader since no fit was possible. a γ-Azido analogue, entry 9, b γ-propargyl analogue, entry 4, c γ-propargyl-thio analogue, entry 8, d γ-propargyl-imido analogue, entry 2, e ATP (**1**). Note the logarithmic scale of the *x*-axes; error bars represent the standard deviation from two technical replicates; RLU: relative fluorescence units. f Table of EC_{50} values from the fits; SE: standard error.

detected. Instead, it showed a sigmoidal response curve similar to compounds **9** and **4**, with a highly comparable EC_{50} value of 0.021 ± 0.010 U µL^{-1}. We hypothesise that S-linked alkyne **8** is hydrolysed by T4 PNK independently from the ODN, explaining the large and increasing signal, a finding in support of previous observations.[17] Notably, the molecular mechanism of this background hydrolysis is not obvious as it is thought that the γ-phosphate undergoes initial nucleophilic attack, where it is directly transferred to the ODN 5′-OH without first forming a phosphoryl-enzyme intermediate.[14] Finally, in order to rationalise the data, we performed rigid receptor docking studies using the ATP analogues described above (Supporting Information). This analysis revealed a high positional variability for the adenosine moieties of all compounds, likely reflecting the known acceptance of T4 PNK towards different NTP substrates.[12] Overall, the model provided predictive value, in that relative binding energies appeared to be good predictors of the general possibility of labelling.

Conclusions

In summary, we have demonstrated the application of a novel chemoenzymatic method for the site-specific 5′-labelling of DNA and RNA molecules with either alkynes or azides. Notably, our method is compatible with a TdT-catalysed 3′-labelling reaction, which opens up the possibility to generate multi-component DNA assemblies. Importantly, our new method allows efficient chemically induced post-synthetic circularisation of DNA as demonstrated here using a ssDNA 100-mer. This is currently not feasible with existing methods, except in highly sequence-specific contexts. Our method offers superior orthogonality to related commercial technologies, such as the 5′ EndTag Kit from Vector Laboratories. This is due to the nature of click chemistry, which largely avoids side reactions, such those that can occur when thiophosphates are employed. These findings thus open up new avenues for the synthesis of novel complex oligonucleotide structures and procedures, including those needed for next-generation sequencing or for the development of new nucleic acid therapeutics.

Acknowledgements

This work has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 861381 (NATURE-ETN). We would like to express our gratitude to Dr. Markus Müller and Dr. Fabio Spada for fruitful scientific discussions. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

The authors declare no conflict of interests.

Data Availability Statement

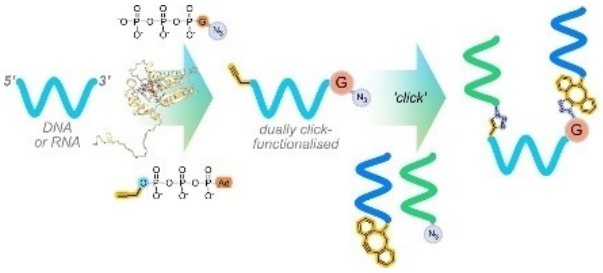
The data that support the findings of this study are available in the supplementary material of this article.

Keywords: chemoenzymatic · click chemistry · click-functionalised nucleotides · labelling · nucleic acids

[1] N. Z. Fantoni, A. H. El-Sagheer, T. Brown, *Chem. Rev.* **2021**, *121*, 7122–7154.
[2] N. R. Zearfoss, S. P. Ryder, *Methods Mol. Biol.* **2012**, *941*, 181–193.
[3] S. Croce, S. Serdjukow, T. Carell, T. Frischmuth, *ChemBioChem* **2020**, *21*, 1641–1646.
[4] E. S. Schönegger, A. Crisp, M. Müller, J. Fertl, S. Serdjukow, S. Croce, M. Kollaschinski, T. Carell, T. Frischmuth, *Bioconjugate Chem.* **2022**, *33*, 1789–1795.
[5] I. Manuguerra, S. Croce, A. H. El-Sagheer, A. Krissanaprasit, T. Brown, K. V. Gothelf, A. Manetto, *Chem. Commun.* **2018**, *54*, 4529–4532.
[6] Z. G. Chidgeavadze, R. S. Beabealashvilli, A. A. Krayevsky, M. K. Kukhanova, *Biochim. Biophys. Acta* **1986**, *868*, 145–152.
[7] R. S. Beabealashvilli, A. V. Scamrov, T. V. Kutateladze, A. M. Mazo, A. A. Krayevsky, M. K. Kukhanova, *Biochim. Biophys. Acta* **1986**, *868*, 136–144.

[8] J. M. Holstein, D. Stummer, A. Rentmeister, *Chem. Sci.* **2015**, *6*, 1362–1369.
[9] S. Barberán-Soler, J. M. Vo, R. E. Hogans, A. Dallas, B. H. Johnston, S. A. Kazakov, *Genome Biol.* **2018**, *19*, 105.
[10] I. Dovgan, A. Ehkirch, V. Lehot, I. Kuhn, O. Koniev, S. Kolodych, A. Hentz, M. Ripoll, S. Ursuegui, M. Nothisen, S. Cianférani, A. Wagner, *Sci. Rep.* **2020**, *10*, 7691.
[11] M. Kukwikila, N. Gale, A. H. El-Sagheer, T. Brown, A. Tavassoli, *Nat. Chem.* **2017**, *9*, 1089–1098.
[12] C. C. Richardson in *Enzymes*, Academic Press, **1981**, pp. 299–314.
[13] J. H. Eastberg, J. Pelletier, B. L. Stoddard, *Nucleic Acids Res.* **2004**, *32*, 653–660.
[14] R. L. Jarvest, G. Lowe, *Biochem. J.* **1981**, *199*, 273–276.
[15] S. E. Lee, L. M. Elphick, A. A. Anderson, L. Bonnac, E. S. Child, D. J. Mann, V. Gouverneur, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3804–3807.
[16] S. Serdjukow, F. Kink, B. Steigenberger, M. Tomás-Gamasa, T. Carell, *Chem. Commun.* **2014**, *50*, 1861–1863.
[17] T. M. Anthony, M. K. H. Pflum, *Bioorg. Med. Chem.* **2018**, *26*, 2331–2336.
[18] W. S. Wadsworth, *J. Org. Chem.* **1987**, *52*, 1748–1753.
[19] S. M. Hacker, M. Mex, A. Marx, *J. Org. Chem.* **2012**, *77*, 10450–10454.
[20] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021.
[21] M. Popenda, M. Szachniuk, M. Antczak, K. J. Purzycka, P. Lukasiak, N. Bartol, J. Blazewicz, R. W. Adamiak, *Nucleic Acids Res.* **2012**, *40*, e112.
[22] M. Antczak, M. Popenda, T. Zok, J. Sarzynska, T. Ratajczak, K. Tomczyk, R. W. Adamiak, M. Szachniuk, *Acta Biochim. Pol.* **2016**, *63*, 737–744.
[23] N. R. Markham, M. Zuker, *Methods Mol. Biol.* **2008**, *453*, 3–31.
[24] P. Kerpedjiev, S. Hammer, I. L. Hofacker, *Bioinformatics* **2015**, *31*, 3377–3379.
[25] E. F. Pettersen, T. D. Goddard, C. C. Huang, E. C. Meng, G. S. Couch, T. I. Croll, J. H. Morris, T. E. Ferrin, *Protein Sci.* **2021**, *30*, 79–82.
[26] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
[27] V. van Houten, F. Denkers, M. van Dijk, M. van den Brekel, R. Brakenhoff, *Anal. Biochem.* **1998**, *265*, 386–389.
[28] C.-X. Liu, L.-L. Chen, *Cell* **2022**, *185*, 2016–2034.
[29] M. S. Xiao, J. E. Wilusz, *Nucleic Acids Res.* **2019**, *47*, 8755–8769.
[30] J. Li, M. Mohammed-Elsabagh, F. Paczkowski, Y. Li, *ChemBioChem* **2020**, *21*, 1547–1566.

Manuscript received: October 13, 2023
Accepted manuscript online: October 20, 2023
Version of record online: ■■, ■■



E. S. Schönegger, Dr. A. Crisp, Dr. M. Radukic, J. Burmester, Dr. T. Frischmuth, Prof. Dr. T. Carell**

1 – 7

Orthogonal End Labelling of Oligonucleotides through Dual Incorporation of Click-Reactive NTP Analogues

A new chemoenzymatic method involving dual incorporation of γ- and 3′-modified NTPs allows sequential 5′- and 3′-end labelling of DNA and RNA molecules.