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COMMUNICATION

Synthesis of γ -Labeled Nucleoside 5'-Triphosphates using Click Chemistry

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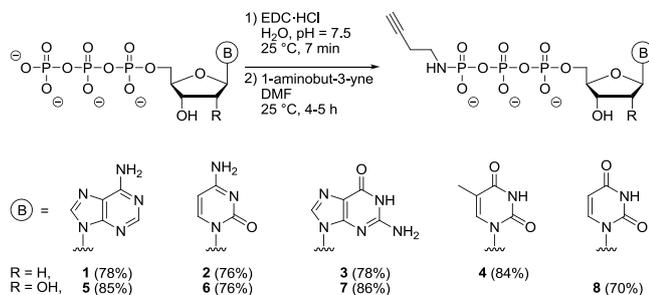
Real-time enzymatic studies are gaining importance as their chemical and technical instrumentation improves. Here we report the efficient synthesis of γ -alkyne modified triphosphate amidates that are converted into a variety of γ -fluorophore labeled triphosphates by Cu(I) catalyzed alkyne/azide click reactions. The synthesized triphosphates are incorporated into DNA by DNA polymerases.

With the advancement of technical instrumentation, real-time and single molecule enzymatic studies are becoming feasible tools.¹ In the centre of these technologies are chemical probes that are for example modified substrates or cofactors needed for the enzyme of interest. To enable direct monitoring of the reaction, these molecules are usually equipped with fluorophores at positions of the substrates that barely interfere with enzyme binding or activity.² Recently, an ATP probe containing a FRET donor-acceptor pair was reported which was used to observe ubiquitin activation in real-time.³ Finally, single molecule real-time (SMRT) DNA sequencing has become a powerful application of these technologies. SMRT sequencing in principle allows to obtain sequence information of non-canonical bases such as 5-methyl-dC and its oxidized derivatives in addition to the canonical bases (dA, dC, dG and dT).^{4,5} This method and other DNA based processes require deoxynucleoside triphosphates with different fluorophores linked to the respective γ -phosphate, which are released when the nucleoside triphosphate is consumed, e.g. by attachment to the growing primer strand.⁵ The synthesis of these labeled compounds involves a multistep protocol which furnishes low overall yields and requires anhydrous conditions, which are interfering with the solubility of the triphosphates.⁶ Consequently, there is a need for a straightforward synthesis of γ -labeled nucleotides. Herein we report a practical method for the preparation of nucleoside triphosphate analogs carrying an alkyne group linked

to the γ -phosphate. The attachment of a fluorophore group can efficiently be performed via Cu(I) catalyzed click reaction between this alkyne triphosphate and a fluorophore azide in aqueous mixtures. The generality of our two-step method is demonstrated by applying it to all eight (deoxy)nucleotides using four fluorophore azides from different dye classes (rhodamine, fluorescein, BODIPY and coumarin). Finally, we show that these fluorescently labeled triphosphates are accepted as substrates by DNA polymerases in primer extension experiments.

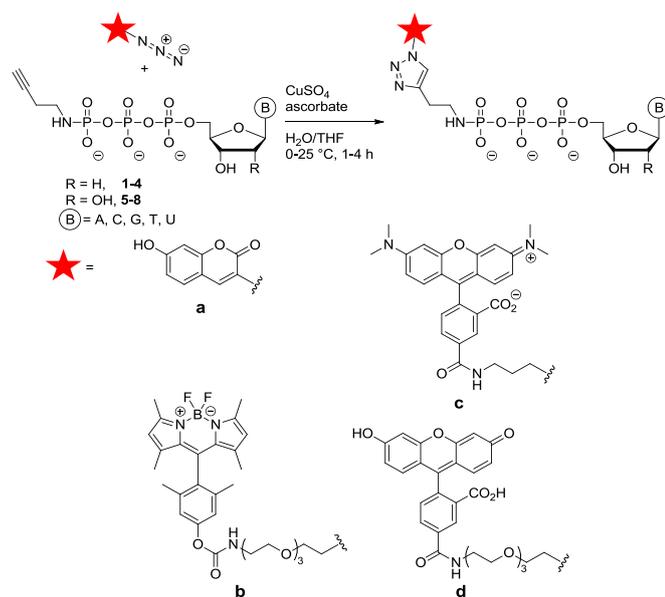
Starting point for the synthesis were the commercially available deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP) and ribonucleoside triphosphates (ATP, CTP, GTP and UTP) in their sodium salt form. In contrast to existing methods for the γ -modification of triphosphates, it was not necessary to prepare the tetrabutylammonium salt of the nucleotides or to use dry solvents.^{5,7} Instead, the triphosphates were directly converted into the alkyne-carrying γ -phosphoramidates **1-4** and **5-8** using commercially available 1-aminobut-3-yne and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC·HCl) as the coupling reagent (Scheme 1). When performing the reaction in a water/DMF (1:1) mixture at pH = 7.5, isolated yields from 70% to 86% of the γ -alkyne labeled triphosphates were obtained as their triethylammonium salts (Scheme 1). RP-HPLC analysis of aliquots from the crude reaction mixture proved that the process itself proceeded with conversion yields of $\geq 90\%$ in most cases (S1, S2 and S3). Carrying out the reaction at lower or higher pH values or using other coupling reagents like carbonyldiimidazole afforded lower yields. This is in accordance with existing similar protocols, to which our present procedure has an almost 2-4-fold improvement in yield.^{7,8} Moreover, we illustrate the generality of the method, as it was successfully applied to all major natural nucleotides without significant decreases in yield. It is also noteworthy that no protecting

reagents are needed. The coupling reaction is taking place with complete selectivity at the γ -phosphate unit as has been proved by ^{31}P - ^1H HMBC NMR spectroscopy (S6).



Scheme 1. Synthesis of alkyne nucleotides labeled at the γ -phosphate. HPLC-purification yielded the labeled triphosphates **1-8** as their tris-triethylammonium salts.

We next attempted the coupling of the fluorophore group by using the Cu(I) catalyzed reaction between alkynes and azides.^{9,10} Thus, we first optimized the cycloaddition protocol of the already prepared alkyne nucleotide **4** towards fluorophore-containing azides. In order to find the initial click conditions we took advantage of the specific properties of coumarin azide **a** (Scheme 2), which starts to fluoresce upon formation of the triazole and moreover is easily synthesized.¹¹ We observed that the best yields were obtained when we used freshly prepared Cu(I) catalyst by *in situ* reduction of CuSO_4 with sodium ascorbate in a water/THF mixture from 0-25 °C. Under these conditions, the triazole products **4a** and **5a** were synthesized in 70% and 77% isolated yield, respectively (Table 1, entries 5 and 8). Full characterization includes ^1H , ^{13}C and ^{31}P NMR spectra (S7). Through the adjustment of the THF content according to the dye azide solubility, we could successfully extend this method to other fluorophores.



Scheme 2. Synthesis of fluorophore γ -labeled nucleoside 5'-triphosphates using Cu(I) catalyzed Huisgen-cycloadditions. HPLC-purification afforded the γ -fluorophore triphosphates as their tris-triethylammonium salts.

To exemplify its generality we reacted the alkyne containing nucleotide **4** with the four different dye azides **a-d** (Scheme 2). Reaction progress was monitored using analytical RP-HPLC proving conversion yields of $\geq 90\%$ for the click products **4a-4d** (Table 1, entries 4-7) in the presence of only 1.5 equivalents of fluorophore azide. Moreover, no Cu(I)-stabilizing ligand like TBTA or THPTA was necessary to achieve good yields in a reaction time of 1-4 h.¹² Due to the high cost of fluorophore dyes, especially the ones that are routinely used in SMRT sequencing,^[6a] we simply synthesized three easy accessible azides (**a, b, d**).

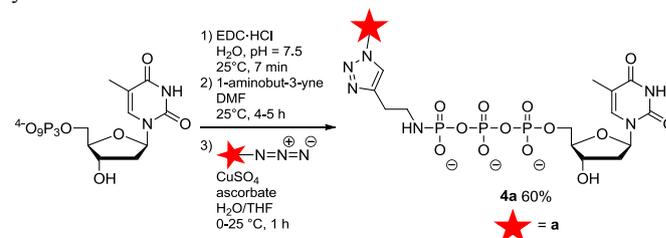
The general applicability of the protocol is demonstrated by the treatment of all nucleotide phosphoramidates (**1-8**) with fluorophore azide **d** in a 400 nmol setup. Again, analytical RP-HPLC indicated conversion yields of $\geq 90\%$ for the click products **1d-8d** (S4 and Table 1, entries 1-4 and 9-12). All click products were characterized by HPLC high resolution ESI-MS to analyze the purity and prove the formation of the correct product.

Table 1. Click reactions overview.

Entry	γ -NTP	azide	yield (%) ¹⁾	chemical formula [M-H] ⁻	MS calc. m/z	MS found m/z
1	dATP	d	≥ 90	$\text{C}_{43}\text{H}_{48}\text{N}_{10}\text{O}_{20}\text{P}_3^-$	1117.2265	1117.2221
2	dCTP	d	≥ 90	$\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_{21}\text{P}_3^-$	1093.2152	1093.2110
3	dGTP	d	≥ 90	$\text{C}_{43}\text{H}_{48}\text{N}_{10}\text{O}_{21}\text{P}_3^-$	1133.2214	1133.2168
4	dTTP	d	≥ 90	$\text{C}_{43}\text{H}_{49}\text{N}_7\text{O}_{22}\text{P}_3^-$	1108.2149	1108.2113
5	dTTP	a	90, 70 ²⁾	$\text{C}_{23}\text{H}_{26}\text{N}_6\text{O}_{16}\text{P}_3^-$	735.0624	735.0620
6	dTTP	b	≥ 90	$\text{C}_{44}\text{H}_{60}\text{BF}_2\text{N}_9\text{O}_{18}\text{P}_3^-$	1144.3336	1144.3344
7	dTTP	c	≥ 90	$\text{C}_{42}\text{H}_{49}\text{N}_9\text{O}_{17}\text{P}_3^-$	1044.2465	1044.2438
8	ATP	a	90, 77 ²⁾	$\text{C}_{23}\text{H}_{25}\text{N}_9\text{O}_{15}\text{P}_3^-$	760.0688	760.0687
9	ATP	d	≥ 90	$\text{C}_{43}\text{H}_{48}\text{N}_{10}\text{O}_{21}\text{P}_3^-$	1133.2214	1133.2224
10	CTP	d	≥ 90	$\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_{22}\text{P}_3^-$	1109.2101	1109.2122
11	GTP	d	≥ 90	$\text{C}_{43}\text{H}_{48}\text{N}_{10}\text{O}_{22}\text{P}_3^-$	1149.2122	1149.2163
12	UTP	d	≥ 90	$\text{C}_{42}\text{H}_{47}\text{N}_7\text{O}_{23}\text{P}_3^-$	1110.1942	1110.1913

1) Conversion yield determined by analytical RP-HPLC; 2) Isolated yield after RP-HPLC purification.

Having established the procedures for both reaction steps, we investigated whether the synthesis could be carried out in a one-pot fashion directly from the commercially available dTTP (Scheme 3). Without isolating the alkyne intermediate by means of time-consuming HPLC-purification, the click procedure was successfully performed. For the reaction of dTTP and the coumarin azide **a**, this one-pot process provided the labeled nucleotide **4a** in 60% overall yield.



Scheme 3. One-pot synthesis of γ -coumarin dTTP **4a** from dTTP.

In order to investigate the acceptance of our dye-labeled nucleotides by DNA polymerases, dTTP labeled with four different fluorophore azides **4a-4d** was incorporated by primer extensions. To this end, the natural dTTP was exchanged by HPLC-purified labeled dTTP derivatives **4a-4d** (Lanes 3-6 in Figure 1B). In an analogous fashion, natural dNTPs were replaced by fluorescein-labeled dNTPs **1d-4d** (Lanes 3-6 in Figure 1C). As shown in Fig. 1, all seven dye-labeled triphosphate analogs were accepted as substrates under standard primer extension conditions to give the fully extended DNA strand. Only fluorescein-labeled dTTP **4d** seemed to be a somewhat more difficult substrate for the DNA polymerase Klenow fragment (exo⁻) (Lane 5, Figure 1C), since fully elongated primer is not exclusively generated. From the data we conclude that the triazole linked dye-labeled nucleoside triphosphate amidates presented here could be useful substrates in studying polymerases and possibly other triphosphate consuming enzymes in real-time. To minimize undesired substrate-enzyme interactions that compromise enzyme function and activity, our modular synthesis could provide easy access to a great variety of terminal-labeled nucleotides. At the same time a polymerase screening or even a directed mutagenesis approach could result in an enzyme which accepts the γ -labeled nucleotides like the canonical ones.

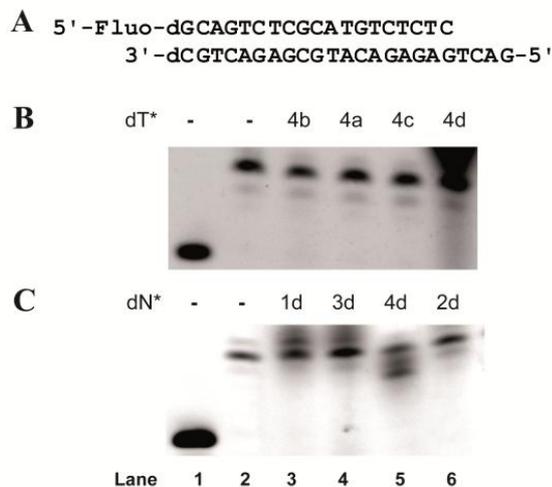


Figure 1. (A) Sequence of the DNA template for the primer extension experiments using Klenow fragment (exo⁻), 0.5 μ M template and 200 μ M nucleotides. The nucleotides are partly replaced by (B) different dye-labeled dTTPs (**4a-4d**) instead of dTTP (Lane 3-6) or by (C) fluorescein-labeled deoxynucleotides (**1d-4d**) for the natural dNTPs (Lane 3-6). In both gels primer (Lane 1) and a control using all natural deoxynucleotides (Lane 2) is given for comparison.

In summary we present a short and high-yielding procedure for the preparation of γ -fluorophore labeled nucleoside 5'-triphosphates. The data show that the triazole containing building blocks synthesized by the click reaction are well accepted by polymerases. Potential applications could be an improved synthesis of substrates for SMRT sequencing. Furthermore, real-time studies as well as labeling experiments involving triphosphate consuming enzymes could benefit from our results.

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Electronic Supplementary Information (ESI) available: Experimental procedures for **1-8**, **a-b**, **d** and click reactions, characterization data of compounds, HPLC-profiles, NMR spectra and primer extension conditions. See DOI: 10.1039/c000000x/

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