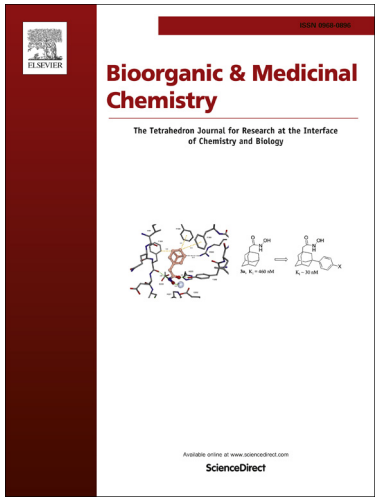

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Graphical Abstract

Enzymatic Incorporation and Fluorescent Labelling of Cyclooctyne-Modified Deoxyuridine Triphosphates in DNA

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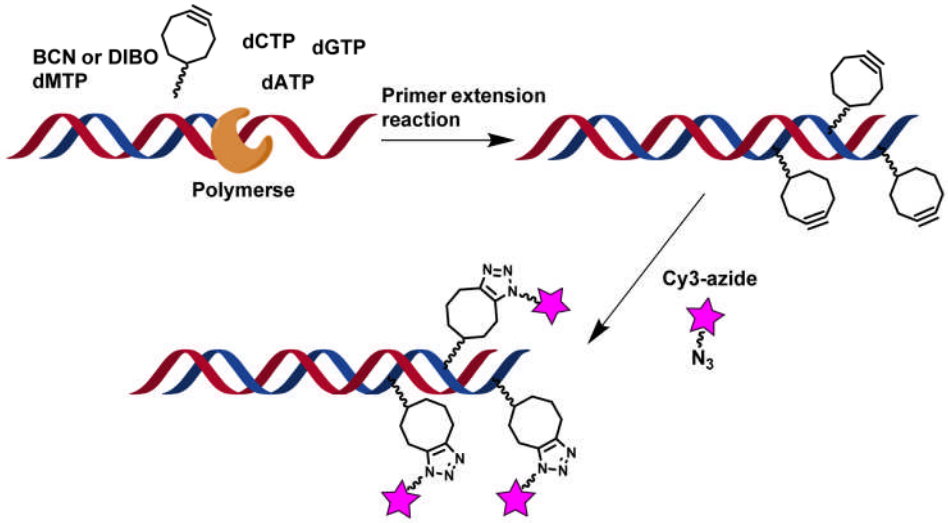

BCN and DIBO modified deoxyuridine triphosphates were successfully incorporated into DNA at multiple sites by enzyme-mediated primer extension and the polymerase chain reaction (PCR). Efficient fluorescent labelling of the BCN-DNA and DIBO-DNA with Cy3-azide was demonstrated.

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ABSTRACT

The amino group of 5-aminopropargyl-2'-deoxyuridine-5'-triphosphate was labelled with dibenzocyclooctyne (DIBO) and two derivatives of bicyclo [6.1.0] non-4-yne (BCN) with short and long linkers to produce three different cycloalkyne-modified deoxyuridine triphosphates. BCN was successfully incorporated into DNA at multiple sites by enzyme-mediated primer extension and the polymerase chain reaction (PCR). Efficient fluorescent labelling of the BCN-DNA and DIBO-DNA with Cy3-azide was demonstrated.

1. Introduction

Chemically modified DNA is used extensively in biology, diagnostics, and nanotechnology. Recently the field of nucleic acid chemistry underwent a revolution when click chemistry [1] and in particular the Cu^{I} catalyzed [3+2] azide-alkyne cycloaddition reaction (CuAAC reaction)[2, 3] was discovered. This reaction has the considerable advantage in the context of nucleic acids that the reacting alkyne and azide groups are stable in aqueous buffer and do not cross-react with other functional groups. The CuAAC reaction has been utilized extensively[4] as a method of joining together single strands of DNA,[5] cross-linking complementary strands,[6] cyclizing single and double strands,[5, 7] labelling oligonucleotides with reporter groups,[8-10] immobilizing DNA on surfaces,[11] constructing nanowires from DNA templates,[12] building DNA nanostructures,[13] producing analogues of DNA with modified nucleobases[14, 15] and backbones,[16-21] synthesizing chemically modified RNA constructs,[22, 23] monitoring DNA synthesis *in vivo*[24] and creating chemically modified PCR templates.[8, 25, 26]

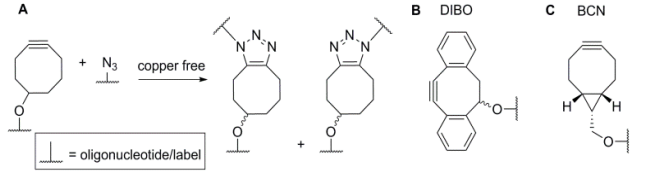


Figure 1. A: Oligonucleotide ligation and labelling by the ring-strain promoted alkyne-azide cycloaddition reaction (SPAAC). **B:** DIBO, **C:** BCN.

However, despite the spectacular success of the CuAAC reaction in the nucleic acid field, Cu^{I} has one particular limitation. It is cytotoxic, and is therefore not compatible with *in vivo* applications of nucleic acids, or for use in experiments that involve living cells.[27-30] Unfortunately the uncatalyzed copper-free DNA-templated AAC Huisgen reaction is very slow[31] unless highly activated terminal alkynes are used, but these are unstable in aqueous media so they cannot be used in biology. For carbohydrates and other biomolecules the issue of biocompatibility has been elegantly solved by the development of the ring strain-promoted azide-alkyne [3+2] cycloaddition reaction (SPAAC) (Figure 1.A).[32-35] This involves the uncatalyzed reaction between an azide and a strained alkyne, normally a cyclooctyne derivative, and is promoted by distortion of the sp-orbitals of the alkyne. Reaction to form a triazole (sp^2 hybridisation) allows release of energy accompanied by the formation of a stable product. Analogues of cyclooctyne have been synthesized which are modified to increase reactivity.[32, 33], and the SPAAC reaction using these has recently been adopted for nucleic acids, both in oligonucleotide labelling[36, 37] and copper-free DNA strand ligation.[38] Phosphoramidite monomers have been synthesized for internal[38] and terminal labelling[39] of DNA with cyclooctynes such as DIBO (dibenzocyclooctyne, Figure 1.B).[39, 40] For applications in which diastereoisomers of the product of the SPAAC reaction might be undesirable, achiral bicycle [6.1.0] nonyne (BCN,

Figure 1.C)[41, 42] has been employed.[43, 44] Due to its symmetry, BCN produces only enantiomeric products.

Enzymatic incorporation of terminal alkyne and azide modifications into DNA by polymerase enzymes is an efficient and widely employed method of DNA labelling.[10, 25, 45-48] Clearly it would be desirable if this methodology could be used to incorporate reactive cyclooctyne derivatives for applications such as the synthesis of fluorescent probes. Here we report the synthesis of three new DIBO and BCN-modified deoxyuridine triphosphates. We describe their incorporation into DNA by enzymatic primer extension and polymerase chain reactions, and demonstrate efficient fluorescent labelling of the BCN and DIBO-modified DNA with Cy3 hexylazide *via* the SPAAC reaction (Figure 2).

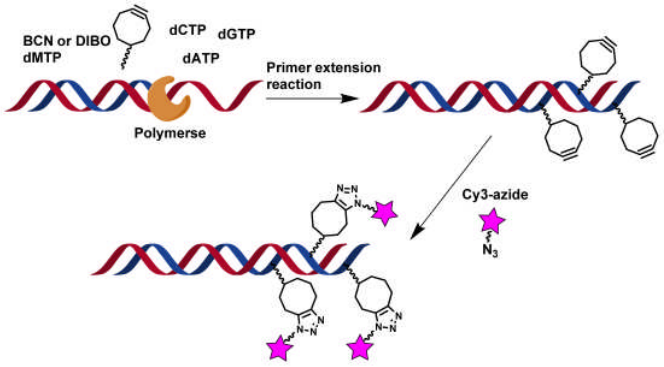
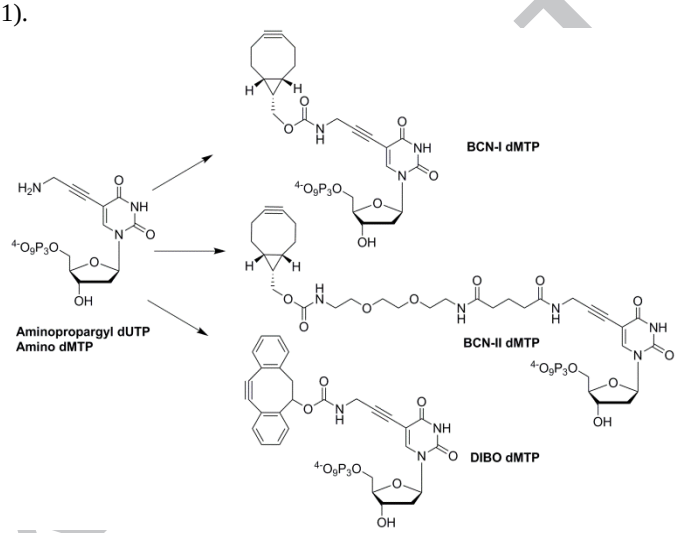


Figure 2. Enzyme-mediated primer extension reaction using DIBO and BCN-modified triphosphates followed by labelling with Cy3-azide.

2. Results and discussion

2.1. Preparation of cycloalkyne modified deoxyuridine triphosphates

Deoxyuridine triphosphate is the simplest of the natural dNTPs to modify chemically. The C5-position of uracil is remote from the H-bonding residues that are involved in Watson-Crick base-pairing, and functional groups attached at this site protrude into space in the major groove of the DNA duplex, without disruption of the normal B-conformation. Hence 5-modified dUTPs (dMTPs) are likely to be compatible with enzymatic incorporation by DNA polymerases, provided that the modification does not inhibit polymerase binding.[49-51] The aminopropargyl linkage is a particularly useful functionality because the ethynyl moiety is known to be compatible with DNA polymerases, and the primary amine is a suitable attachment point for carboxylic acid derivatives of reporter groups, thereby enabling the synthesis of various functionalized dMTPs from a common intermediate.[49, 52, 53] In fact aminopropargyl dUTP is an interesting dMTP in its own right, as it has a short linker in the position normally occupied by the methyl group of dTTP, so it is likely to be a good substrate for various DNA polymerase enzymes.[54] We chose to synthesize three different cyclooctyne dUTP derivatives; a DIBO derivative which has a short linker between the cyclooctyne and the uracil base, and two different BCN dUTP derivatives with short and long linkers (BCN-I and BCN-II, Scheme 1). We expected that the presence of the bulky DIBO ring might lead to less efficient incorporation by DNA polymerases than BCN, and that placing the BCN ring further from the uracil base and away from the enzyme active site would lead to more efficient enzymatic incorporation. In order to synthesize these cyclooctyne deoxyuridine triphosphates we first required the common intermediate, aminopropargyl dUTP. The Sonogashira reaction was used to attach aminoprop-2-yne to 5-iododeoxyuridine[53, 55] which was then converted to its 5'-triphosphate by the Yoshikawa procedure.[56] Phosphorus oxychloride ($POCl_3$) in trimethylphosphate was employed to generate the 5'-dichlorophosphate, and the reaction mixture was treated with a tetrabutylammonium salt of pyrophosphate to produce the required triphosphate. In order to minimize the production of 3', 5'-diphosphate by-products, the reaction was conducted at –15 ºC. 5-Aminopropargyl-dUTP was then labelled with the active esters of DIBO, BCN-I and BCN-II to yield three activated cyclooctyne-modified triphosphates (dMTPs) (Scheme 1).



Scheme 1. Labelling of aminopropargyl-dUTP with DIBO, BCN-I and BCN-II active esters to produce 5-cyclooctyne-modified dUTPs. Reagents: DMF and triethylammonium bicarbonate buffer, 4 h, 55 ˚C.

2.2. Primer extension reactions and PCR

Table 1. Oligonucleotide sequences used in primer extension, reverse transcription and PCR.

Code	Sequences(5'-3')	Mass Calc.	Mass Found
T1	C**A**GTC**A**CTGT**A**CTGCCGACACACATAA CC (DNA template)	8776	8775
T2	C**A**GTC**A**C**AAAA**CTGCCGACACACATAA CC (DNA template)	8778	8777
P3	FAM-GGTTATGTGTGTCGGCAG (primer)	6138	6138
T4	C**A**GUC**A**CUGU**A**CUGCCGACACACAUAA CC (RNA template)	9169	9169
P5	GCATTCGAGCAACGTAAG (PCR primer)	6548	6548
P6	GGTTATGTGTGTCGGCAG (PCR primer)	7040	7040
T7	FAM-GG**TTAT**GT**G**T**G**TCGGC**A**G**TATT**GT**C**A**G **T**GT**G**A**ATT**CC**A**G**A**GT**G**T**G**A**G**A**TT**GT**G**T GC**T**GGCG**AT**C**TTA**CG**TT**GC**T**CG**AAT**GC (PCR template)		

FAM is 6-carboxyfluorescein. The RNA template has the equivalent sequence to the DNA template T1. The bold A (A/T for PCR) shows the site to incorporate the modified triphosphates.

Primer extension is a biochemical approach to the synthesis of chemically modified DNA of defined sequence and substitution pattern. Primer extension reactions utilizing amino, DIBO, BCN-I and BCN-II dMTPs were carried out with family A (Klenow large fragment) and family B polymerases (Therminator II, Gotaq and KOD). The sequences of primers and templates are shown in Table 1. The 5'-end of the primer was labelled with 6-carboxyfluorescein (FAM) for ease of visualization on analytical polyacrylamide gels. Two templates were used with different numbers and arrangements of adenine bases after the starting point of linear copying. Template (T1) begins with one A, and contains just three adenines for replication while the “highly demanding” template (T2) starts with four consecutive adenines (A) and contains six adenine bases to be replicated. In each case

the primer must be extended by 11 bases for complete replication to give a 29-mer product. Alternatively the primer can be extended using only the modified triphosphate to give shorter products, a 19-mer with template T1 and a 22-mer with template T2. The four natural dNTPs (dATP, dCTP, dGTP, dTTP) were used in control experiments.

Polyacrylamide gel electrophoresis (PAGE) and mass spectrometry confirmed that KOD, Therminator II, Gotaq and Klenow large fragment polymerases successfully incorporated all four dMTPs into the template T1 (Figures 3 and SI). All the copies of the templates containing dMTPs (Figure 3.A, lanes 1, 2) exhibited lower electrophoretic mobility compared to the natural counterparts (Figure 3.A, lane 4, 5). This is due to the extra bulk of the modified uracil bases, particularly in the case of BCN-II dMTP with a long linker which showed a large difference in mobility compared to the natural counterparts. The polymerases incorporated one or two additional nucleotides at the 3'-end of the linear copy of the template when using only dTTP or dMTPs (Figure 3, lane 4 and SI). When modified dNTPs are incorporated into DNA enzymatically the modified dNTP is usually doped with its natural counterpart to avoid premature termination of replication. We therefore explored the effect of mixing the individual dMTPs with dTTP (Figure 3.A, lane 3) and found that the modified triphosphates were all able to compete effectively, producing fully extended primers with a mixture of dTTP and dMTP opposite to the dA sites in the template. This is evident from the multiple or broader bands of the fully extended mixed products of dMTP with dTTP, dATP, dCTP, dGTP (Figure 3.A, lane 3) compared to the control dNTP (lane 5).

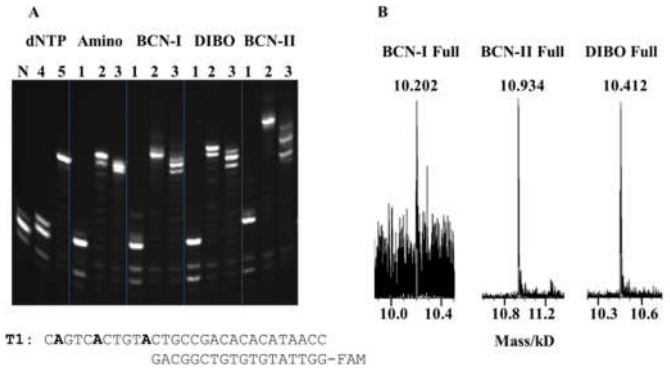


Figure 3. Primer extension reactions using Klenow polymerase (1 unit) with P3 (66 pmol) and T1 (132 pmol). 3.2 nmol of dMTPs or dNTPs for each triphosphate were used unless otherwise stated. **A:** Lane N, P3 + T1 without triphosphates; lane 1, dMTP; lane 2, dMTP + dATP + dCTP + dGTP; lane 3, dMTP (2.1 nmol) + dTTP (1.1 nmol) + dATP + dCTP + dGTP; lane 4, dTTP; lane 5, dTTP + dATP + dCTP + dGTP (20% PAGE gel). **B:** Mass spectra of fully extended primers: BCN-I Full is produced from BCN-I dMTP + dATP + dCTP + dGTP (calc. 10202, found 10202); BCN-II Full is produced from BCN-II dMTP + dATP + dCTP + dGTP (calc. 10934, found 10934); DIBO Full is produced from DIBO dMTP + dATP + dCTP + dGTP (calc. mass 10412, found 10412).

Next template T2 with four consecutive adenines was used to evaluate the incorporation of the various dMTPs in a highly demanding case. Encouragingly dMTP containing short chain BCN-I was successfully incorporated by KOD, Therminator II and Klenow polymerases (Figure 4). The dMTP containing long chain BCN-II gave fully and partially extended products, whereas the more bulky DIBO did not produce a fully extended product with any of the polymerases used (see SI). It is noteworthy that the most suitable cyclooctyne dUTP in this study is the one with the smallest mass attached to the 5-position of the uracil base (BCN-I), even though this has a much shorter linker than BCN-II.

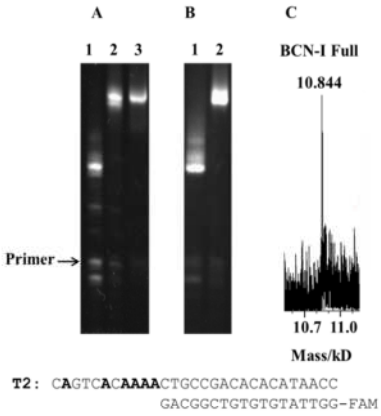


Figure 4. Primer extension reactions with P3 (66 pmol), T2 (132 pmol), BCN-I dMTP (3.2 nmol for each triphosphate). **A:** Lane 1, BCN-I dMTP using Klenow polymerase (2 units); lane 2, dMTP + dATP + dCTP + dGTP using Klenow polymerase (2 units); lane 3, dMTP + dATP + dCTP + dGTP using Therminator II polymerase (1 unit). (20 % PAGE gel) **B:** Reactions using KOD polymerase (2 units, 0.4 mM $MgCl_2$). Lane 1, BCN-I dMTP; lane 2, BCN-I dMTP +dATP + dCTP + dGTP. (20 % PAGE gel) **C:** Mass spectrum of fully extended primer (BCN-I Full) from **B**-lane 2 (calc. mass 10845, found 10844).

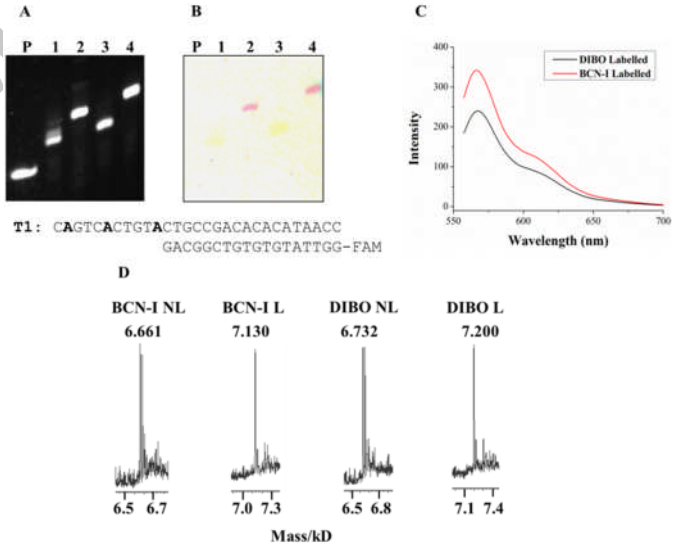


Figure 5. Cy3-azide labelling of primer extension products from P3, T1 and dMTPs using Gotaq polymerase (1 unit). Lane P, P3; lane 1, BCN-I dMTP (non-labelled); lane 2, BCN-I dMTP labelled with Cy3-azide; lane 3, DIBO dMTP (non-labelled); lane 4, DIBO dMTP labelled with Cy3-azide. (20 % PAGE gel) **A:** Picture was taken using transilluminator light and UV filter. **B:** Picture was taken using normal digital camera. **C:** Fluorescent spectra of lane 2 (BCN-I labelled) and lane 4 (DIBO labelled) products (Excited at 540 nm on PE LS50B fluorimeter). **D:** Mass spectra of one base extended primers, non-labelled product (NL) and Cy3-azide labelled product (L). BCN-I NL (lane 1 in PAGE): calc.6657 [M], found 6661[M] and 6683 [M+Na]; BCN-I L (lane 2 in PAGE): calc. 7125, found 7130; DIBO NL (lane 3 in PAGE), calc. 6727 [M], found 6732 [M] and 6754 [M+Na]; DIBO L (lane 4 in PAGE), calc.7195, found 7200.

2.3. Fluorescent labelling

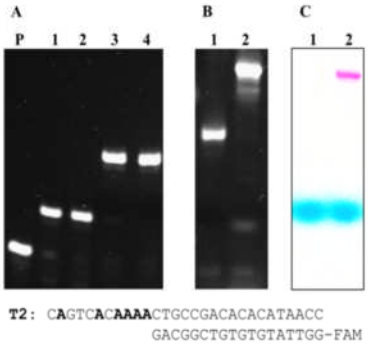


Figure 6. Cy3-azide labelling of primer extension products from P3, T2 and BCN-I dMTP + dATP + dCTP + dGTP using KOD polymerase (1 unit, 1.0 mM $MgCl_2$). **A:** Lane P, P3 + T2; lane 1, dTTP; lane 2, lane 1 with Cy3 azide; lane 3, dATP + dCTP + dGTP + dTTP; lane 4, lane 3 with Cy3-azide. **B:** Lane 1, BCN-I dMTP + dATP + dCTP + dGTP(non-labelled); lane 2, lane 1 labelled with Cy3-azide. Pictures were taken using transilluminator light and UV filter. **C:** Picture of gel B was taken using a digital camera. (20 % PAGE gel)

Cy3 is commonly used in the synthesis of fluorescent DNA probes.[57] In this context it has a number of favorable properties which make it suitable for the multiple labelling of DNA strands. It is very bright, sterically undemanding and not excessively hydrophobic. After primer extension with template T1 and ethanol precipitation to remove unincorporated triphosphates, the BCN-I and DIBO-functionalized linear extension products were labelled efficiently with Cy3 hexylazide *via* the SPAAC reaction (Figure 5 and 6) (see SI for the structure and synthesis of the Cy3 hexylazide). Natural dTTP and dNTP were used as negative controls. The labelled products exhibited high fluorescence and much lower electrophoretic mobility than the unlabeled controls. This confirms that the BCN and DIBO moieties are stable to the conditions of their incorporation and labelling.

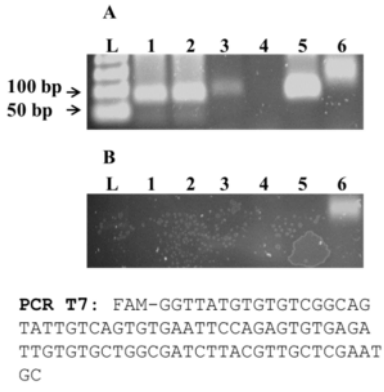


Figure 7. Cy3-azide labelling of polymerase chain reaction product from P5, P6, T7 and BCN-I dMTP with dTTP + dATP + dGTP + dCTP using KOD polymerase (0.5 unit, 1.0 mM $MgCl_2$). Unless otherwise stated 4 nmol of each triphosphate was used. Lane L, 50 bp ladder; lane 1, dTTP + dATP + dGTP + dCTP (non-labelled); lane 2, lane 1 with Cy3 azide; lane 3, BCN-I dMTP (2 nmol) + dTTP (2 nmol) + dATP + dGTP + dCTP (non-labelled); lane 4, lane 3 labelled with Cy3-azide; lane 5, BCN-I dMTP (1 nmol) + dTTP (3 nmol) + dATP + dGTP + dCTP (non-labelled); lane 6, lane 5 labelled with Cy3-azide. **A:** Gel after staining with ethidium bromide. **B:** Gel before staining. (2% agarose gel)

Efficient incorporation of cyclooctynes into DNA during PCR amplification and subsequent fluorescence labelling by the SPAAC reaction would enable the synthesis of long fluorescent probes in practical quantities. To this end PCR was carried out with BCN-I dMTP using KOD polymerase and 81-mer DNA template T7. The modified triphosphate was successfully incorporated when it was mixed with the four natural triphosphates, using a 1:3 ratio of dMTP to dTTP. Labelling of the PCR product created from this mixture with Cy3 hexylazide gave a fluorescent band (Figure 7.B, lane 6). As expected, subsequent staining with ethidium bromide revealed slower migration of this band compared with the unlabeled amplicon (Figure 7.A, lane 5 and 6).

2.4. Reverse transcription

Reverse transcriptase (RT) enzymes can be used to copy DNA and RNA templates in order to prepare chemically modified complementary DNA (cDNA). This methodology can efficiently produce functionalized complementary DNA (cDNA) from RNA that has been isolated from natural sources. In the current work we chose to evaluate the cyclooctyne-modified dMTPs with Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT, RNase H^-) which conveniently lacks the catalytic activity necessary to cleave RNA strands. RNA template T4 of the equivalent sequence to the previously used DNA template with dispersed adenines was employed. BCN-II dMTP was incorporated efficiently within 1 h while BCN-I dMTP needed 4 h. Even with this extended period the DIBO reaction was incomplete (Figure 8, lanes 1). The above reverse transcription reactions would clearly benefit from further optimization of the reaction conditions.

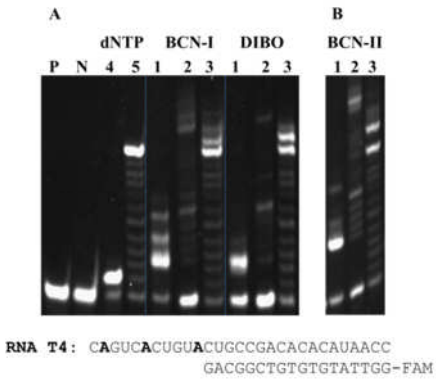


Figure 8. Reverse transcription reactions with P3 (66 pmol), RNA template T4 (132 pmol), dMTPs or dNTPs (3.2 nmol for each triphosphate unless otherwise stated) using M-MuLV (RNase H^-) reverse transcriptase (100 units). **A:** The reaction time was 4 h. Lane P, P3 + T4 without triphosphate; lane N, dATP + dCTP + dGTP; lane 1, dMTP; lane 2, dMTP + dATP + dCTP + dGTP; lane 3, dMTP (1.6 nmol) + dTTP (1.6 nmol) + dATP + dCTP + dGTP; lane 4, dTTP; lane 5, dATP + dCTP + dGTP + dTTP. **B:** The reaction time was 1 h. Lane 1, dMTP; lane 2, dMTP + dATP + dCTP + dGTP; lane 3, dMTP (2.1 nmol) + dTTP (1.1 nmol) + dATP + dCTP + dGTP. (20 % PAGE gel)

3. Conclusions

The strain-promoted alkyne-azide cycloaddition reaction is a valuable tool for DNA labelling, and in this context the efficient incorporation of reactive cyclooctyne derivatives into DNA by enzymatic methods is an important objective. To this end we have synthesized one DIBO and two BCN dUTP analogues from a common aminopropargyl dUTP intermediate and incorporated them into DNA by linear extension using two different templates and a variety of DNA polymerases. All three cyclooctynes (DIBO, BCN-I and BCN-II) were incorporated successfully against template with dispersed adenine bases, whereas BCN was the most successful dMTP when using a template with four

consecutive adenines. Labelling of the modified extension products with Cy3 hexylazide *via* the copper-free SPAAC click reaction demonstrated the efficient incorporation and stability of these modified triphosphates to the enzymatic reaction conditions. The acceptance of cyclooctyne-modified triphosphates as substrates for various DNA polymerases in linear extension and PCR is encouraging in the context of future *in vitro* and *in vivo* applications.

4. Experimental section

4.1. General method for oligonucleotide synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 μmol scale phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0 %. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 40 s. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C.

The building blocks for the RNA analogues were prepared using 2'-TBS protected RNA phosphoramidite monomers with *t*-butylphenoxyacetyl protection of the A, G and C nucleobases and unprotected U (Sigma-Aldrich). A solution of 0.3 M benzylthiotetrazole in acetonitrile (Link Technologies) was used as the coupling agent, *t*-butylphenoxyacetic anhydride was employed as the capping agent and 0.1 M iodine as the oxidizing agent (Sigma-Aldrich). All RNA phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use, and the coupling time for all monomers was 10 min. Stepwise coupling efficiencies were determined by automated trityl cation conductivity monitoring and in all cases were >96 %. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia/ethanol (3/1 v/v) for 2 h at room temperature followed by heating in a sealed tube for 45 min at 55 °C. After cleavage from the solid support and deprotection of the nucleobases and phosphotriesters, RNA oligonucleotides were concentrated to a small volume *in vacuo*, transferred to 15 mL plastic tubes and freeze dried. The residues were dissolved in DMSO (300 μL) and triethylamine trihydrofluoride (300 μL) was added after which the reaction mixtures were kept at 65 °C for 2.5 h. Sodium acetate (3 M, 50 μL) and butanol (3 mL) were added with vortexing and the samples were kept at -80 °C for 30 min then centrifuged at 4 °C at 13,000 rpm for 10 min. The supernatant was decanted and the precipitate was washed twice with ethanol (0.75 mL) then dried under vacuum.

The fully deprotected oligonucleotides (DNA or RNA) were purified by reversed-phase HPLC on a Gilson system using a Luna 10μ C8 100Å pore Phenomenex 10x250 mm column with a gradient of acetonitrile in ammonium acetate, in case of DNA, (0 % to 50 % buffer B over 20 min, flow rate 4 mL/min), (buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate, pH 7.0, with 50 % acetonitrile). Elution was monitored by UV absorption at 295 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 columns (GE Healthcare). For HPLC purification of RNA, triethylammonium bicarbonate buffer was used (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.5, buffer B: 0.1 M triethylammonium bicarbonate, pH 7.5, with 50 % acetonitrile). The fractions from HPLC were evaporated without need for additional desalting.

All oligonucleotides were characterized by negative-mode electrospray HPLC-mass spectrometry, using a Brueker Daltronics micro-TOF mass spectrometer and an Acquity UPLC system with a BEH C18 1.7 μm column (Waters). A gradient of acetonitrile in triethylammonium acetate (TEAA) and hexafluoroisopropanol (HFIP) was employed, increasing from 5 % to 40 % buffer B over 14 min, with a flow rate of 0.1 mL/min (buffer A: 10 mM TEAA, 100 mM HFIP in H_2O; buffer B: 20 mM TEAA in CH_3CN). Raw data were processed and deconvoluted using the Data Analysis function of the Bruker Daltronics Compass™ 1.3 software package.

4.2. Primer extension experiments

In a 20 μL reaction, 66 pmol of FAM labelled DNA primer (P3), 132 pmol of template (T1 or T2) and 3.2 nmol of each dMTP or dNTP were mixed with the polymerase enzyme (1 or 2 units, see figure legends) and 1X buffer (supplied with the enzymes). In the case of KOD polymerase, 0.4 or 1 mM $MgCl_2$ was added separately to the reaction buffer. The reaction mixtures were heated for 1 or 1.5 h (KOD, Therminator II and GoTaq were at 72 °C while Klenow was at 37 °C) and 20 μL formamide was added before analysis by 20 % denaturing polyacrylamide gel electrophoresis under a constant 20 W. For the mass spectrometry analysis, 40 μL reactions were carried out, desalted on a NAP-10 column and concentrated by freeze-drying. KOD was purchased from Merck Millipore, Therminator II and Klenow large fragment from New England Bio and Gotaq from Promega.

4.3. Polymerase chain reaction

Samples containing 10 pmol of two primers (P5, P6), 50 pg of template (T7) and 4 nmol dMTP or dNTP in total were added followed by polymerase, 10X KOD polymerase buffer (pH = 8) and 25 mM $MgCl_2$. The final reaction volume was 20 μL with 1X reaction buffer and 1 mM $MgCl_2$. Amplification was performed using the following procedure: an initial denaturing at 95 ºC for 1 min, followed by 25 cycles of denaturing at 95 ºC for 15 s, primer annealing at 54 ºC for 20 s, and extension at 72 ºC for 30 s, then further extension at 72 ºC for 5 min. The samples were analyzed by 2 % agarose gel electrophoresis in 1X TBE buffer (126 V).

4.4. Reverse transcription

Samples containing 66 pmol of FAM labelled DNA primer (P3), 132 pmol of RNA template (T4) and 3.2 nmol dMTP or dNTP were mixed with M-MuLV (RNase H^-) reverse transcriptase (100 units), 5X buffer and 1X dithiothretitol (DTT, supplied with the enzyme). The final reaction volume was 20 μL. The reaction mixtures were heated at 42 ºC for 1 h or 4 h, 20 μL of formamide was added and samples were analyzed by 20 % denaturing polyacrylamide gel electrophoresis under constant 20 W.

4.5. Fluorescent labelling

Primer extension reactions or PCR (2 X 20 μL + 10 μL water) were prepared as explained above in section 4.2 or 4.3 using BCN-1 dMTP, DIBO dMTP or dNTP negative control. DNA was precipitated by mixing with 5 μl 3M sodium acetate (pH 5.3) followed by 150 μl ethanol and was then left on dry ice for 10 min and at -20 °C for 20 min. This was followed by

centrifugation at 4 °C for 30 min (13,000 rpm). The precipitate was re-dissolved in 40 µl 1X Gotaq Green buffer and 20 µl was reacted with 1.6 µl Cy3 hexylazide (10 mM DMSO solution) for 1 h. for primer extensions, 20 µl of formamide was added to the labeled DNA and the unlabeled primer extension reactions (20 µl) followed by analysis on a 20 % PAGE gel at 20 W. for PCR, after the labelling reactions, a second ethanol precipitation was carried out using 2 µL of 3M sodium acetate (pH 5.3) and 60 µL of ethanol. The samples were analyzed by 2 % agarose gel electrophoresis in 1X TBE buffer (126 V) For mass spectrometry and fluorescent spectra analysis, 40 µL of non-labelled and labelled reactions were carried out as above followed by desalting on NAP-10 columns.

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Supplementary data

Supplementary data associated with this article includes chemical synthesis, primer extension experiments and fluorescent labelling reactions. This can be found in the online version.

References

[1] H.C. Kolb, M.G. Finn, K.B. Sharpless, Click chemistry: Diverse chemical function from a few good reactions, Angew. Chem. Int. Edit., 40 (2001) 2004-2021.
[2] C.W. Tornoe, C. Christensen, M. Meldal, Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, J. Org. Chem., 67 (2002) 3057-3064.
[3] V.V. Rostovtsev, L.G. Green, V.V. Fokin, K.B. Sharpless, A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, Angew. Chem. Int. Edit., 41 (2002) 2596-2599.
[4] A.H. El-Sagheer, T. Brown, Click chemistry with DNA, Chem. Soc. Rev., 39 (2010) 1388-1405.
[5] R. Kumar, A.H. El-Sagheer, J. Tumpane, P. Lincoln, L.M. Wilhelmsson, T. Brown, Template-directed oligonucleotide strand ligation, covalent intramolecular DNA circularization and catenation using click chemistry, J. Am. Chem. Soc., 129 (2007) 6859-6864.
[6] P. Kocalka, A.H. El-Sagheer, T. Brown, Rapid and efficient DNA strand cross-linking by click chemistry, Chembiochem, 9 (2008) 1280-1285.
[7] A.H. El-Sagheer, R. Kumar, S. Findlow, J.M. Werner, A.N. Lane, T. Brown, A very stable cyclic DNA miniduplex with just two base pairs, Chembiochem, 9 (2008) 50-52.
[8] J. Gierlich, G.A. Burley, P.M.E. Gramlich, D.M. Hammond, T. Carell, Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA, Org. Lett., 8 (2006) 3639-3642.
[9] E.A. Motea, I. Lee, A.J. Berdis, Development of a 'clickable' non-natural nucleotide to visualize the replication of non-instructional DNA lesions, Nucleic Acids Res. , 40 (2012) 2357-2367.
[10] U. Wenge, T. Ehrenschwender, H.-A. Wagenknecht, Synthesis of 2'-O-propargyl nucleoside triphosphates for enzymatic oligonucleotide preparation and "click" modification of DNA with nile red as fluorescent probe, Bioconjugate Chem. , 24 (2013) 301-304.
[11] D.I. Rozkiewicz, J. Gierlich, G.A. Burley, K. Gutsmiedl, T. Carell, B.J. Ravoo, D.N. Reinhoudt, Transfer printing of DNA by "click" chemistry, Chembiochem, 8 (2007) 1997-2002.
[12] M. Fischler, U. Simon, H. Nir, Y. Eichen, G.A. Burley, J. Gierlich, P.M.E. Gramlich, T. Carell, Formation of bimetallic Ag-Au nanowires by metallization of artificial DNA duplexes, Small, 3 (2007) 1049-1055.
[13] E.P. Lundberg, A.H. El-Sagheer, P. Kocalka, L.M. Wilhelmsson, T. Brown, B. Norden, A new fixation strategy for addressable nano-network building blocks, Chem. Commun., 46 (2010) 3714-3716.
[14] F. Seela, X. Ming, Oligonucleotides containing 7-deaza-2'-deoxyinosine as universal nucleoside: Synthesis of 7-halogenated and 7-alkynylated derivatives, ambiguous base pairing, and dye functionalization by the alkyne - azide 'click' reaction, Helv. Chim. Acta, 91 (2008) 1181-1200.
[15] P. Kocalka, N.K. Andersen, F. Jensen, P. Nielsen, Synthesis of 5-(1,2,3-triazol-4-yl)-2'deoxyuridines by a click chemistry approach: Stacking of triazoles in the major groove gives increased nucleic acid duplex stability, Chembiochem, 8 (2007) 2106-2116.
[16] T. Fujino, N. Yamazaki, H. Isobe, Convergent synthesis of oligomers of triazole-linked DNA analogue ((TL)DNA) in solution phase, Tetrahedron Lett., 50 (2009) 4101-4103.
[17] H. Isobe, T. Fujino, N. Yamazaki, M. Guillot-Nieckowski, E. Nakamura, Triazole-linked analogue of deoxyribonucleic acid (^{TL}DNA): Design, synthesis, and double-strand formation with natural DNA, Org. Lett., 10 (2008) 3729-3732.
[18] A. Nuzzi, A. Massi, A. Dondoni, Model studies toward the synthesis of thymidine oligonucleotides with triazole internucleosidic linkages via iterative Cu(I)-promoted azide-alkyne ligation chemistry, Qsar & Combinatorial Sci., 26 (2007) 1191-1199.
[19] R. Lucas, P.H. Elchinger, P.A. Faugeras, R. Zerrouki, Pyrimidine-purine and pyrimidine heterodinucleosides synthesis containing a triazole linkage, Nucleoside, Nucleotide & Nucleic Acid, 29 (2010) 168-177.
[20] R. Lucas, R. Zerrouki, R. Granet, P. Krausz, Y. Champavier, A rapid efficient microwave-assisted synthesis of a 3',5'-pentathymidine by copper(I)-catalyzed [3 + 2] cycloaddition, Tetrahedron, 64 (2008) 5467-5471.
[21] J. Vergnaud, P.-. Faugeras, V. Chaleix, Y. Champavier, R. Zerrouki, Design of a new oligotriazole peptide nucleic acid analogue (oT-PNA), Tetrahedron Lett., 52 (2011) 6185-6189.
[22] A.H. El-Sagheer, T. Brown, New strategy for the synthesis of chemically modified RNA constructs exemplified by hairpin and hammerhead ribozymes, Proc. Natl. Acad. Sci. U. S. A., 107 (2010) 15329-15334.
[23] E. Paredes, S.R. Das, Click chemistry for rapid labeling and ligation of RNA, ChemBioChem 12 (2011) 125-131.
[24] A. Salic, T.J. Mitchison, A chemical method for fast and sensitive detection of DNA synthesis *in vivo*, Proc. Natl. Acad. Sci. U. S. A., 105 (2008) 2415-2420.
[25] J. Gierlich, K. Gutsmiedl, P.M.E. Gramlich, A. Schmidt, G.A. Burley, T. Carell, Synthesis of highly modified DNA by a combination of PCR with alkyne-bearing triphosphates and click chemistry, Chem. Eur. J., 13 (2007) 9486-9494.
[26] A.H. El-Sagheer, A.P. Sanzone, R. Gao, A. Tavassoli, T. Brown, Biocompatible artificial DNA linker that is read through by DNA polymerases and is functional in Escherichia coli, Proc. Nat. Acad. Sci. U.S.A., 108 (2011) 11338-11343.
[27] S.H. Chiou, DNA-Scission and protein-scission activities of ascorbate in the presence of copper-ion and a copper-peptide complex, J. Biochem. , 94 (1983) 1259-1267.
[28] R. Stoewe, W.A. Prutz, Copper-catalyzed DNA damage by ascorbate and hydrogen-peroxide - kinetics and yield, Free Radical Biol. Med. , 3 (1987) 97-105.

[29] S. Kobayashi, K. Ueda, J. Morita, H. Sakai, T. Komano, DNA damage induced by ascorbate in the presence of Cu-2+, BBA 949 (1988) 143-147.
[30] D.E. Won, C.S. Lee, Y.K. Shin, K.S. Lee, DNA degradation by mitomycin c in the presence of copper and epinephrine, Chung-Ang Journal of Medicine, 14 (1989) 59-74.
[31] A.H. El-Sagheer, T. Brown, Factors influencing hairpin oligonucleotide cyclization by the uncatalyzed alkyne-azide cycloaddition (AAC) reaction, Pure & Applied Chem, 82 (2010) 1599-1607.
[32] J.C. Jewett, E.M. Sletten, C.R. Bertozzi, Rapid Cu-free click chemistry with readily synthesized biarylazacyclooctynones, J. Am. Chem. Soc., 132 (2010) 3688-3690.
[33] P.V. Chang, J.A. Prescher, E.M. Sletten, J.M. Baskin, I.A. Miller, N.J. Agard, A. Lo, C.R. Bertozzi, Copper-free click chemistry in living animals, Proc. Nat. Acad. Sci. U.S.A. , 107 (2010) 1821-1826.
[34] J.C. Jewett, C.R. Bertozzi, Cu-free click cycloaddition reactions in chemical biology, Chem. Soc. Rev. , 39 (2010) 1272-1279.
[35] E.M. Sletten, C.R. Bertozzi, A hydrophilic azacyclooctyne for Cu-free click chemistry, Org. Lett., 10 (2008) 3097-3099.
[36] P. van Delft, N.J. Meeuwenoord, S. Hoogendoorn, J. Dinkelaar, H.S. Overkleeft, G.A. van der Marel, D.V. Filippov, Synthesis of oligoribonucleic acid conjugates using a cyclooctyne phosphoramidite, Org. Lett., 12 (2010) 5486-5489.
[37] K.N. Jayaprakash, C.G. Peng, D. Butler, J.P. Varghese, M.A. Maier, K.G. Rajeev, M. Manoharan, Non-nucleoside building blocks for copper-assisted and copper-free click chemistry for the efficient synthesis of RNA conjugates, Org. Lett., 12 (2010) 5410-5413.
[38] M. Shelbourne, X. Chen, T. Brown, A.H. El-Sagheer, Fast copper-free click DNA ligation by the ring-strain promoted alkyne-azide cycloaddition reaction, Chem. Commun., 47 (2011) 6257-6259.
[39] M. Shelbourne, T. Brown, A.H. El-Sagheer, T. Brown, Fast and efficient DNA crosslinking and multiple orthogonal labelling by copper-free click chemistry, Chem. Commun., 48 (2012) 11184-11186.
[40] I.S. Marks, J.S. Kang, B.T. Jones, K.J. Landmark, A.J. Cleland, T.A. Taton, Strain-promoted "click" chemistry for terminal labeling of DNA, Bioconjugate Chem., 22 (2011) 1259-1263.
[41] M.F. Debets, S.S. Van Berkel, J. Dommerholt, A.J. Dirks, F. Rutjes, F.L. Van Delft, Bioconjugation with strained alkenes and alkynes, Accounts Chem. Res., 44 (2011) 805-815.
[42] A. Borrmann, S. Milles, T. Plass, J. Dommerholt, J.M.M. Verkade, M. Wiessler, C. Schultz, J.C.M. van Hest, F.L. van Delft, E.A. Lemke, Genetic encoding of a bicyclo 6.1.0 nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation, ChemBioChem, 13 (2012) 2094-2099.
[43] J. Dommerholt, S. Schmidt, R. Temming, L.J.A. Hendriks, F. Rutjes, J.C.M. van Hest, D.J. Lefeber, P. Friedl, F.L. van Delft, Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells, Angew. Chem. Int. Edit., 49 (2010) 9422-9425.
[44] A.M. Jawalekar, S. Malik, J.M.M. Verkade, B. Gibson, N.S. Barta, J.C. Hodges, A. Rowan, F.L.v. Delft, Oligonucleotide tagging for copper-free click conjugation, Molecules, 18 (2013) 7346-7363.
[45] K. Gutsmiedl, D. Fazio, T. Carell, High-Density DNA Functionalization by a Combination of Cu-Catalyzed and Cu-Free Click Chemistry, Chem. Eur. J., 16 (2010) 6877-6883.
[46] S.H. Weisbrod, A. Marx, A nucleoside triphosphate for site-specific labelling of DNA by the Staudinger ligation, Chem. Commun. , (2007) 1828-1830.
[47] H. Rao, A.A. Sawant, A.A. Tanpure, S.G. Srivatsan, Posttranscriptional chemical functionalization of azide-modified oligoribonucleotides by bioorthogonal click and Staudinger reactions, Chem. Commun. , 48 (2012) 498-500.
[48] H. Rao, A.A. Tanpure, A.A. Sawant, S.G. Srivatsan, Enzymatic incorporation of an azide-modified UTP analog into oligoribonucleotides for post-transcriptional chemical functionalization, Nat. Protoc. , 7 (2012) 1097-1112.
[49] S. Jager, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, A versatile toolbox for variable DNA functionalization at high density, J. Am. Chem. Soc. , 127 (2005) 15071-15082.
[50] J. Sagi, A. Szemzo, K. Ebinger, A. Szabolcs, G. Sagi, E. Ruff, L. Otvos, Base-modified oligodeoxynucleotides .1. Effect of 5-alkyl, 5-(1-alkenyl) and 5-(1-alkynyl) substitution of the pyrimidines on duplex stability and hydrophobicity, Tetrahedron Lett. , 34 (1993) 2191-2194.
[51] B.M. Znosko, T.W. Barnes, T.R. Krugh, D.H. Turner, NMR studies of DNA single strands and DNA : RNA hybrids with and without 1-propynylation at C5 of oligopyrimidines, J. Am. Chem. Soc. , 125 (2003) 6090-6097.
[52] G. Giller, T. Tasara, B. Angerer, K. Muhlegger, M. Amacker, H. Winter, Incorporation of reporter molecule-labeled nucleotides by DNA polymerases. I. Chemical synthesis of various reporter group-labeled 2'-deoxyribonucleoside-5'-triphosphates, Nucleic Acids Res., 31 (2003) 2630-2635.
[53] V. Borsenberger, M. Kukwikila, S. Howorka, Synthesis and enzymatic incorporation of modified deoxyuridine triphosphates, Org. Biomol. Chem. , 7 (2009) 3826-3835.
[54] S.E. Lee, A. Sidorov, T. Gourlain, N. Mignet, S.J. Thorpe, J.A. Brazier, M.J. Dickman, D.P. Hornby, J.A. Grasby, D.M. Williams, Enhancing the catalytic repertoire of nucleic acids: a systematic study of linker length and rigidity, Nucleic Acids Res. , 29 (2001) 1565-1573.
[55] F.W. Hobbs, Palladium-catalyzed synthesis of alkynylamino nucleosides - a universal linker for nucleic-acids, J. Org. Chem. , 54 (1989) 3420-3422.
[56] M. Yoshikawa, T. Kato, T. Takenishi, A novel method for phosphorylation of nucleosides to 5-nucleotigei, Tetrahedron Lett. , 50 (1967) 5065-5068.
[57] L.M. Hall, M. Gerowska, T. Brown, A highly fluorescent DNA toolkit: synthesis and properties of oligonucleotides containing new Cy3, Cy5 and Cy3B monomers, Nucleic Acids Res. , 40 (2012).