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Synthesis of Highly Modified DNA by a Combination of PCR with Alkyne-Bearing Triphosphates and Click Chemistry

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Abstract: We report the combination of "click chemistry" with PCR by using alkyne-modified triphosphates for efficient and homogeneous labeling of DNA. A series of modified PCR products of different lengths (300, 900, and 2000 base pairs) were prepared by using a variety of alkyne- and azide-containing triphosphates and different polymerases. After intensive screening of real-time PCR methods, protocols were developed that allow the amplification of genes by using these modified triphosphates with similar efficiency to that of standard PCR. The click reaction on the highly modified PCR fragments provided conversion rates above 90% and resulted in the functionalization of hundreds of alkynes on large DNA fragments with superb selectivity and efficiency.

Keywords: click chemistry · cycloaddition · DNA labeling · nucleotides · polymerase chain reaction

Introduction

The DNA molecule is currently of outstanding interest for the synthesis of self-assembling nanoarchitectures.[1] The primary sequence and secondary structure of DNA are also used as a general marker in many diagnostic applications. In both areas, the functionalization of DNA is of paramount importance. In nanotechnology, the preparation of functional nanostructures requires the addition of functions such as magnetic properties[2,3] or electrical conductivity[2,4] to the DNA molecule, whereas the modification of DNA with fluorophores or other functions, like reducing groups to enable Ag staining of DNA, is highly desirable for diagnostic applications.[5] One efficient method for the functionalization of long DNA strands is achieved through PCR amplification with unnatural triphosphates carrying the desired function.[6–9] This methodology has received significant attention, especially for the synthesis of highly functionalized aptamers.[8,10] Unfortunately, the type of modification accepted by the PCR enzymes is limited and it is frequently unpredictable which modifications will finally be tolerated.[7,9,11,12] We have therefore started to develop a generally applicable method for the dense functionalization of DNA in a highly reliable and flexible way. Recently we used a protocol in which DNA strands are modified by using the Cu^{I}-catalyzed version of the azide–alkyne cycloaddition reaction[13] ("click" reaction[14]), which was discovered by the groups of Meldal[15] and Sharpless[16] and is now used in many different areas.[17] From the two possible isomer products of the cycloaddition, the 1,4-adduct is exclusively formed under these conditions. The reaction has been used successfully on DNA strands with both single[18] and multiple alkyne or azide functions.[5,19] In order to generate large and highly modified DNA strands, an alkyne-modified triphosphate was used in the PCR, for example, 5-modified deoxyuridine triphosphate (dU*TP) instead of the natural thymidine triphosphate (TTP). The densely alkyne-modified DNA obtained was subsequently further derivatized by means of the click reaction, as depicted in Scheme 1.[5,19]

Recently we described the functionalization of DNA with azides such as **1**, which allowed us to direct silver deposition towards alkyne-modified DNA strands through the Tollens reaction.[5,20] The protocol enables specific Ag staining of

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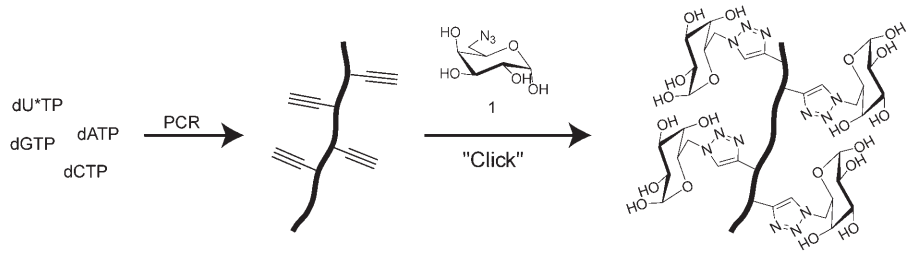


Scheme 1. Schematic depiction of the postsynthetic functionalization of DNA strands by using click chemistry. dATP: deoxyadenosine triphosphate; dCTP: deoxycytidine triphosphate; dGTP: deoxyguanosine triphosphate; dU*TP: alkyne triphosphate.

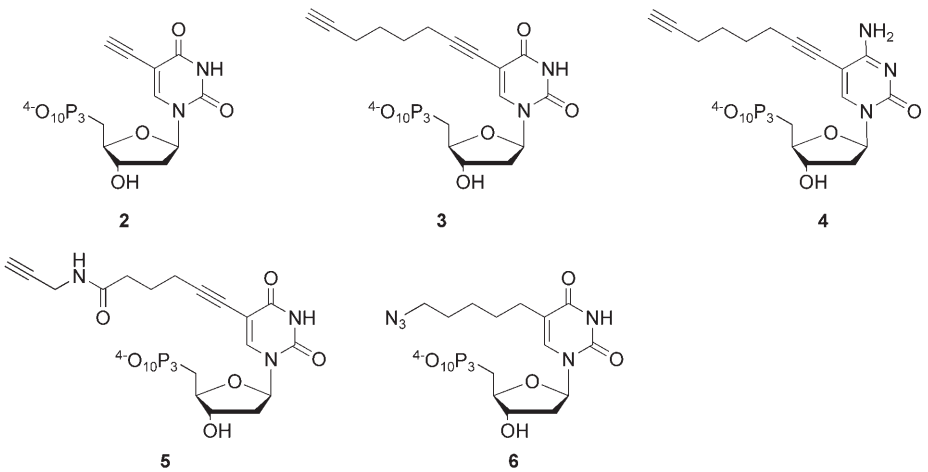
alkyne-labeled DNA. This is of great interest because Ag staining can be more sensitive than fluorescence labeling, while no expensive fluorescence detector is required to visualize the analytical result. Herein we report an investigation of the efficiency of the click reaction on long and complex DNA strands. We describe how the PCR followed by the click reaction depends on the DNA length and sequence and on the kind of triphosphate used. A detailed overview of how the various alkyne-modified triphosphates influence the PCR is given. In order to determine the most efficient PCR conditions, we report the utilization of real-time PCR (RT-PCR) as a facile screening method. Furthermore, UV/Vis and CD data revealing the secondary-structure characteristics of the resulting densely modified DNA duplexes are presented.

Results

Synthesis of 5-modified pyrimidine triphosphates: In order to determine the parameters which govern the efficient incorporation of alkyne-modified triphosphates into DNA by PCR, we prepared and screened the incorporation of a set of 5-alkyne- or 5-azide-modified pyrimidine triphosphates, **2**–**6**, depicted in Scheme 2. The syntheses of the previously undescribed compounds **3**, **5**, and **6** are based on Sonogashira coupling of an appropriate 5-iodopyrimidine nucleoside with an appropriate terminal alkyne, followed by the conversion of the free bases into the corresponding triphosphates, as shown in Scheme 3. The synthesis of compounds **2**[21] and **4**[20] has been described previously.

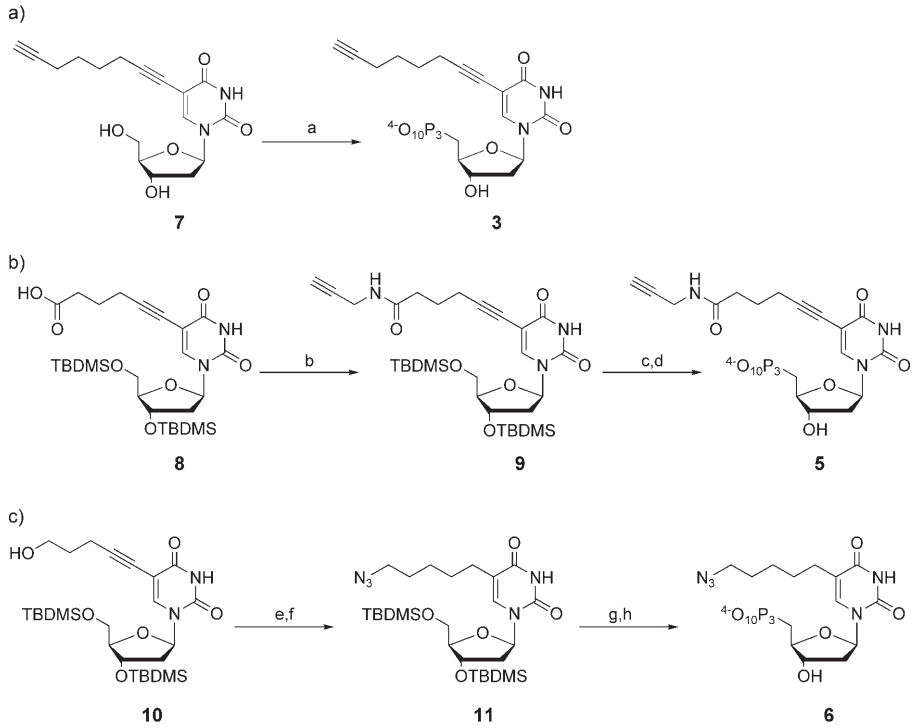
Triphosphate **3** was prepared from the known monomer **7** (Scheme 3).[19] The amide-containing triphosphate **5** was synthesized by coupling the acid-modified uridine **8** with propargylamine. The synthesis of compound **6** begins with the hydrogenation of the triple bond in **10**. The azide is introduced by mesylation and subsequent nucleophilic substitution (see the Supporting Information). The 5-modified pyrimidine triphosphates possess either free alkynes (**2**–**5**) or a free azide (**6**) to allow direct functionalization through the click reaction. Triphosphate **2** features a terminal alkyne in direct conjugation with the uridine nucleobase. In the uridine- or cytidine-based triphosphates **3**–**5**, the terminal alkyne function is situated remotely from the base, with the rationale being that a remote alkyne would result in higher click yields due to reduced steric hindrance.[19]

Triphosphates **3**–**5** possess an internal alkyne in direct conjugation with the pyrimidine ring, in addition to the terminal alkyne function. Based on previous studies, which had shown that an extended conjugation can facilitate polymerase incorporation, we hoped that these alkynes would also improve the acceptance of our building blocks by polymerases in the PCR.[8,11] In order to investigate whether we could reverse the click reaction, we also investigated the incorporation of azide **6** into a DNA strand instead of an alkyne. However, in order to create a stable azide-modified triphosphate, we were forced to utilize a saturated carbon chain to link the azide function to the nucleobase in order to avoid a thermally induced intramolecular cycloaddition reaction between the azide and the internal alkyne resulting in a triazole product.[22] Such saturated linkers, however, are known to be somewhat troublesome substrates for polymerases.[11,23]



Scheme 2. 5-Modified pyrimidine triphosphates used in this study.

Primer extension studies: To explore the acceptance of the triphosphates by different polymerases, we initially performed primer extension studies with a range of polymerases, including



Scheme 3. Syntheses of the triphosphates **3**, **5**, and **6**: a) 1) $POCl_3$, proton sponge; 2) tributylammonium pyrophosphate; 3) TEAB; b) HOBT, HBTU, HATU, propargylamine, DCM, 75%; c) TBAF, pyridine, 72%; d) 1) $POCl_3$, proton sponge; 2) tributylammonium pyrophosphate; 3) TEAB; e) Pd/C, H_2, 73%; f) 1) MsCl, DIEA, DCM, 2) NaN_3, DMF, 82%; g) TBAF, THF, 91%; h) 1) $POCl_3$, proton sponge; 2) tributylammonium pyrophosphate; 3) TEAB. TBDMS: *tert*-butyldimethylsilyl; TEAB: tetraethylammonium bromide; HOBT: 1-hydroxy-1*H*-benzotriazole; HBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate; DCM: dichloromethane; TBAF: tetrabutylammonium fluoride; Ms: methanesulfonyl; DIEA: *N*,*N*-diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; THF: tetrahydrofuran.

the family A polymerase from *Thermus aquaticus* (Taq) and the family B polymerases from *Pyrococcus woesi* (Pwo), *Thermococcus litoralis* (Deep Vent *exo*$^-$), and *Thermococcus kodakaraensis* (KOD XL), which is a mixture of the natural form and an *exo*$^-$ mutant. The original assay developed by Held and Benner was utilized; this uses different templates of increasing difficulty (**T1**–**T3**).[23] While for **T1** only the incorporation of one modified uridine is needed, **T2** and **T3** demand the polymerase to incorporate two or more consecutive modifications. The sequences of the 5′-fluorescein-labeled primer **P** and the three different templates **T1**–**T3** are listed in Table 1. The primer extensions were conducted at 72 °C with a reaction time of only 10 minutes.

Figure 1 summarizes the results obtained with the most successful polymerase Pwo, which has full exonuclease activity. The experiments with the triphosphates **2**–**6** revealed efficient incorporation of **2**–**4**, while only inefficient incorporation of the triphosphates **5** and **6** was observed (see also the Supporting Information).

To our surprise triphosphate **5** was not accepted as a substrate by the polymerases tested. This is difficult to explain because other triphosphates containing an amide bond, albeit in a different direction, and a triple bond adjacent to the base were efficiently accepted.[8,11] In the case of triphosphate **6**, the inefficient incorporation can most likely be attributed to the saturation of the carbon chain directly attached to the pyrimidine ring because similar results were obtained with other 5-modified pyrimidine triphosphates with saturated carbon linkers.[23] Sawai and co-workers recently reported the efficient incor-

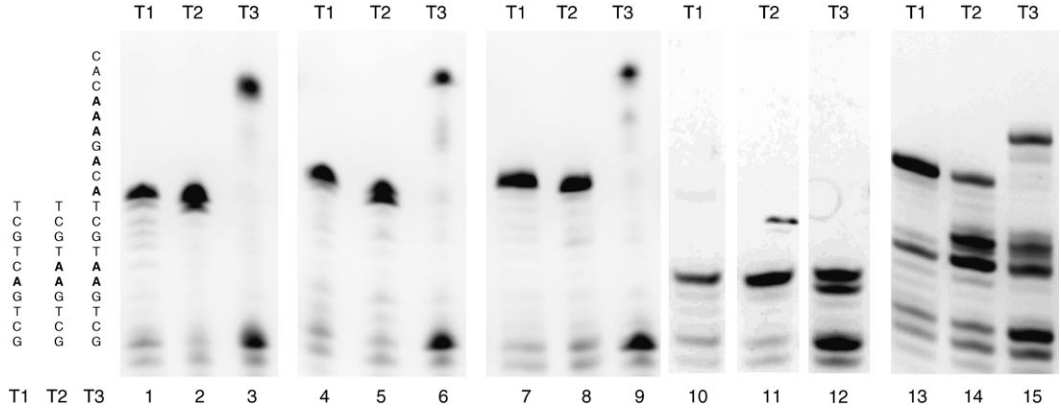


Figure 1. Primer extension with Pwo polymerase. Lanes 1–3: dATP, dCTP, dGTP, **2**; lanes 4–6: dATP, dCTP, dGTP, **3**; lanes 7–9: dATP, **4**, dGTP, **3**; lanes 10–12: dATP, dCTP, dGTP, **5**; lanes 13–15: dATP, dCTP, dGTP, **6**.

Table 1. Sequences of the primer and the templates used in the primer extension experiments. The bold **A** marks the position where the polymerase has to insert a modified dU*TP.

P	CATGGGCCAAGCTTCTTCGG-Fl-5′[a]
T1	TCGTC**A**GTCGGTACCCGGTTCGAAGAAGCC-3′
T2	TCGT**AA**GTCGGTACCCGGTTCGAAGAAGCC-3′
T3	C**A**C**AAA**G**A**C**A**TCGT**AA**TGCGGTACCCGGTTCGAA-GAAGCC-3′

[a] Fl: fluorescein.

poration of 5-modified triphosphates bearing an α-methylamide function by using the KOD polymerase (see reference [9] and references cited therein). With our substrate, KOD XL produced only small amounts of full-length extension product with **6**. Figure 1 shows the results for **6** with the Pwo polymerase, which stops at the double-incorporation site and is not able to elongate over the triple-incorporation site of **T3**. The incorporation of **2**–**4** also occurred with all of the other polymerases tested, a result indicating that these triphosphates should also be substrates for PCR (see the Supporting Information).

RT-PCR investigations: Incorporation of modified triphosphates into long DNA strands through PCR is known to be a rather empirical process. In order to facilitate the finding of the correct conditions, we performed a combinatorial screening of variables such as the cycling time, annealing temperature, chemical additives, and substrate concentrations. To this end an RT-PCR assay with SYBR Green II as a fluorescent intercalator was utilized. We first investigated the polymerases Pwo, Deep Vent *exo*$^-$, KOD XL, and Taq for their ability to amplify a 300-base-pair fragment from the *Polη* gene. In the experiments either TTP or dCTP, depending on the type of modified base investigated, was fully replaced by either **2**, **3**, or **4**. The expected PCR product contains 154 deoxythymidines and 104 deoxycytidines, which are exchanged during the PCR into modified TTP or dCTP, respectively.

Poor amplification was observed in the presence of the triphosphates **2** and **4** with the family A polymerase Taq. The polymerase failed completely to amplify a product with triphosphate **3**. By contrast, full-length amplicons were produced in high yield with all of the triphosphates **2**–**4** if one of the family B polymerases, Pwo, Deep Vent *exo*$^-$, or KOD XL, was employed; this is fully consistent with previous studies by Held and Benner[23] and by Sawai and coworkers,[9] who reported that family B polymerases have a broader substrate acceptance than the members of family A. In agreement with the primer extension studies, we could not, under any circumstances, observe PCR product in the presence of the triphosphates **5** and **6.**

The *Pwo* polymerase produced amplicons of the highest quality compared with all other family B polymerases tested (Figure 2a). The PCR product PCR300·**3** exhibits a slightly reduced mobility on the gel. Figure 2b shows the fluorescence curves obtained from the RT-PCR study with Pwo polymerase. All of the triphosphates, except **3**, provided a

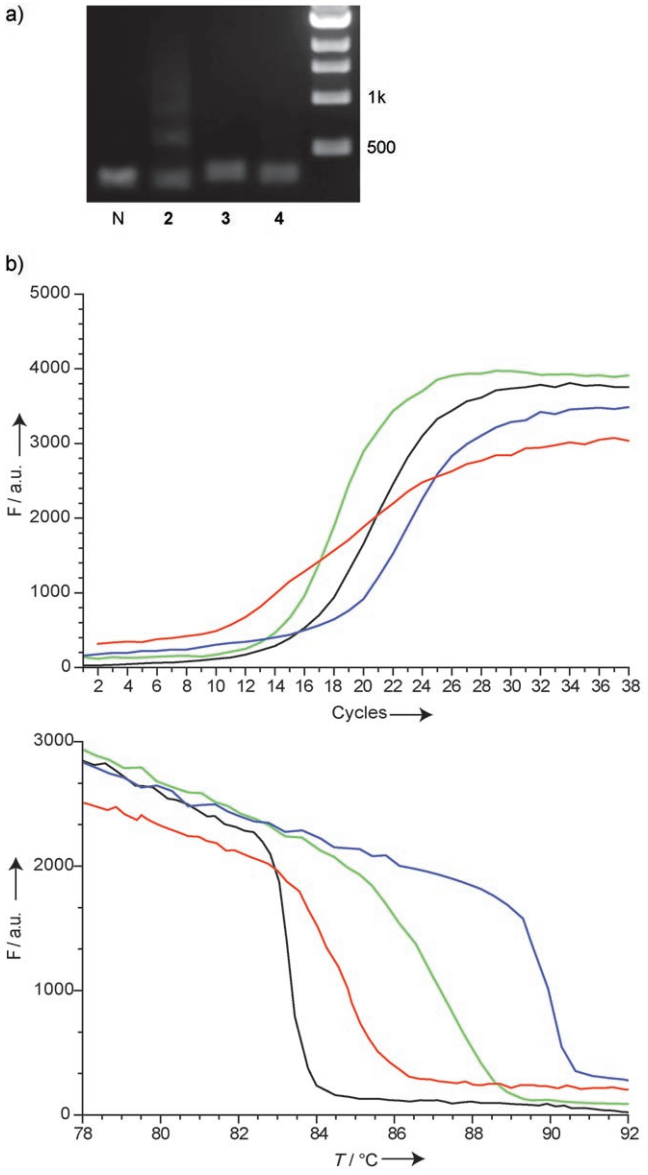


Figure 2. Incorporation of modified triphosphates **2**–**4** into a 300-base-pair fragment by using Pwo for RT-PCR. a) Agarose gels of PCR fragments derived from a 300-base-pair template: lane 1: dATP, dCTP, dGTP, TTP; lane 2: dATP, dCTP, dGTP, **2**; lane 3: dATP, dCTP, dGTP, **3**; lane 4: dATP, **4**, dGTP, TTP; lane 5: DNA ladder. b) Corresponding RT-PCR profiles (top) and melting curves (bottom) of the PCR with Pwo. PCR300·**2**–**4** (**2**: ——, **3**: ——, **4**: ——) and PCR300·**N** (——) are the products with incorporated triphosphates **2**–**4** or with only natural triphosphates, respectively.

typical PCR profile. The slower increase of the PCR signal for **3** could indicate a slower incorporation of this triphosphate compared to that of the others. Similar effects were observed with the Deep Vent *exo*$^-$ polymerase. The slightly less effective incorporation of triphosphate **3** may be of importance if longer and/or more demanding templates require amplification (see below). In order to ensure the amplicons created by Pwo possess the correct base sequence, we used the alkyne-containing amplicons as templates for a second PCR with all four natural triphosphates present. These

back-amplified amplicons were subsequently sequenced. To our surprise, we detected no sequence errors, which indicates that the incorporation of triphosphates **2**–**4** proceeds with high fidelity. Restriction assays with different restriction enzymes revealed that the modifications can, in some cases, inhibit these enzymes (see the Supporting Information).

Investigation of RT-PCR fluorescence melting curves shows that incorporation of the modified triphosphates **2**–**4** increased the melting point of the PCR product (Table 2).

Table 2. Melting temperatures of PCR300 amplicons as a function of polymerase buffer.

PCR product	T_m [°C]	
	Pwo	Deep Vent *exo*$^-$
PCR300·**2**	87	85
PCR300·**3**	85	82
PCR300·**4**	91	88
PCR300·**N**	83	81

PCR300·**4** shows the largest stabilization (≈8 °C increase) when compared with a natural control. Intriguingly, PCR300·**3** amplicons are destabilized (≈6–7 °C decrease) relative to PCR300·**4** amplicons, despite having the same bisalkyne addend. The increased linker length of triphosphate **3** compared to **2** seems to lower the melting point by around 2 °C.

Encouraged by the successful incorporation of triphosphates **2**–**4**, we next investigated the PCR amplification of longer DNA fragments with 900 base pairs. The investigated PCR product now comprises 551 exchangeable thymidines and 352 exchangeable cytidines (for the RT-PCR traces, see the Supporting Information). In general, we observed that longer templates were more difficult to produce and careful balancing of the PCR additives and cycling temperatures was required. Besides an elongation of the PCR cycle time, we increased the denaturing temperature to 99 °C and 4 % dimethylsulfoxide (DMSO) was added to decrease the melting temperature of all DNA hybrids formed during the PCR. In the presence of triphosphates **2** and **4**, efficient PCR amplification of the 900-base-pair template could be achieved (Figure 3a). Only KOD XL polymerase was able to generate full-length amplicons with triphosphate **3**. Again, these amplicons show slightly reduced mobility.

Encouraged by these results, we investigated the incorporation of modified triphosphates into DNA strands of 2000 base pairs in length, which comprise 887 exchangeable cytidines (see also the Supporting Information). These studies were only performed with the triphosphate **4**, which had given the best results thus far. Careful optimization of the PCR protocol was required. Figure 3b depicts the results obtained with different annealing temperatures. With a temperature of 57 °C, a PCR product was obtained with natural triphosphates, but only unspecific products were obtained when dCTP was replaced by the modified triphosphate **4**. To reduce the unspecific binding of the primer, 4 % DMSO

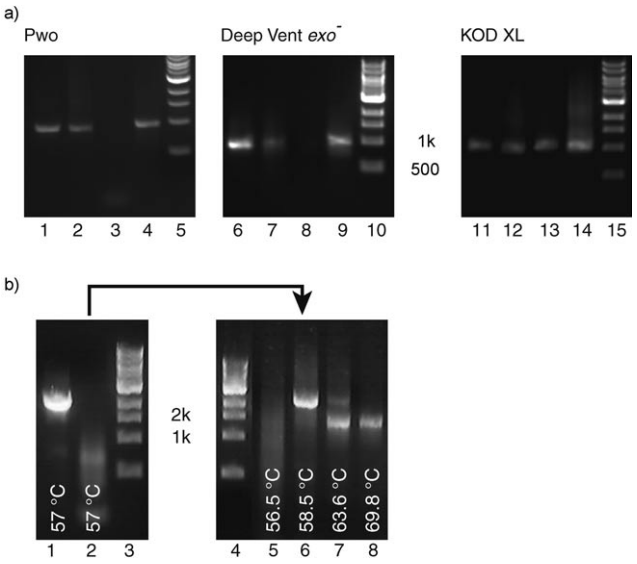


Figure 3. a) Incorporation of modified triphosphates **2**–**4** into a 900-base-pair fragment by using PCR. Agarose gels of the PCR products with the different polymerases: Lanes 1, 6, 11: dATP, dCTP, dGTP, TTP; lanes 2, 7, 12: dATP, dCTP, dGTP, **2**; lanes 3, 8, 13: dATP, dCTP, dGTP, **3**; lanes 4, 9, 14: dATP, **4**, dGTP, TTP; lanes 5, 10, 15: DNA ladder. b) PCR with a 2000-base-pair fragment. Screening of the annealing temperature for the production of PCR2000·**4** with Pwo: comparison between natural (lane 1) and modified (lane 2) triphosphates at a 57 °C annealing temperature; lanes 5–8: screening of different annealing temperatures; lanes 3, 4: DNA ladder.

was added and the denaturing temperature was set to 99 °C. An annealing temperature screen showed that the correct product was only formed in a small range of temperatures at around 58.5 °C. At higher temperatures, shorter amplicons were synthesized. In the final protocol, an annealing temperature of 59.3 °C was used, which allowed us to create a 2000-base-pair PCR product with 887 alkynes attached to the modified cytidines.

UV/Vis and CD spectroscopy of the modified PCR amplicons: The incorporation of modified triphosphates **2**–**4** influences the UV spectra of the resulting PCR amplicons as a consequence of the increased π conjugation of the pyrimidine ring due to the presence of the alkyne group. This results in new absorbances in the 300–320 nm region (Figure 4a). The UV-absorption spectra features a small hypsochromic shift of about 5–10 nm for the 260 nm band. The CD spectra reveal an overall B-type secondary structure for all of the generated DNA amplicons (Figure 4b). The CD spectra of PCR300·**N** and PCR300·**4** show positive maxima between 230–250 nm and 270–290 nm and minima in the 250–260 nm region. Interestingly, the CD spectra of the modified amplicons feature an additional minimum between 280–300 nm due to the additional absorbance in this region.

Analysis of the click efficiency: As the alkynes present anchor groups onto which we intended to click various functional molecules, we next investigated the efficiency of the

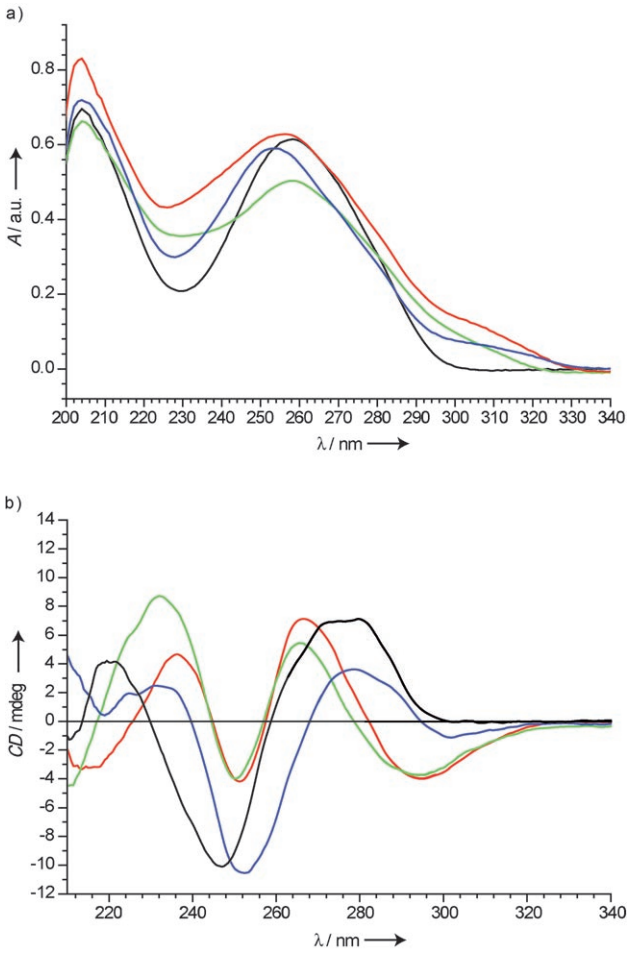


Figure 4. a) UV/Vis and b) CD spectra of the 300-base-pair PCR amplicons PCR300·**2** (——), PCR300·**3** (——), and PCR300·**4** (——). The spectra of the control amplicon PCR300·**N** (——) are shown in black.

click reaction on DNA strands with hundreds of alkynes. For this investigation, we first performed the click reaction on the amplicons and subsequently digested the obtained DNA products by using a mixture of DNA degrading enzymes (*Penicillium citrinum* nuclease P1, calf spleen phosphodiesterase II, alkaline phosphatase, and *Crotalus adamanteus* snake venom phosphodiesterase I). The mixture of nucleotides obtained was subsequently analyzed by using HPLC-MS/MS. For the investigation we used the shorter PCR300·**2**–**4** amplicons and the large PCR2000·**4** amplicon as examples and the sugar azide **1** as the clicking partner. The reaction was conducted by using a copper(I)-stabilizing ligand and CuBr. After a reaction time of 2 h, the DNA was purified by ethanol precipitation.

The results are depicted in Figure 5. Enzymatic digests of the amplicons before the reaction provide four peaks corresponding to the monomers of the triphosphates used for the PCR, plus a small amount of inosine which is formed due to deamination of dA under the reaction conditions. Analysis of the amplicons after the click reaction gave clean chromatograms for all of the modified amplicons.

In addition, HPLC analysis of the enzymatic digestion of the products arising from PCR300·**2** and PCR300·**3** revealed a surprisingly high clicking efficiency of ≈95%, according to integration of the residual starting-material peaks (22.2 min for PCR300·**2**, 42.5 min for PCR300·**3**, Figure 5a) relative to the product peaks (23.2 min and 24.0 min for PCR300·**2**; 30.6 min and 31.2 min for 300·**3**, Figure 5b) at the diagnostic wavelength of 310 nm for 5-modified pyrimidines (see the UV spectra of the monomers in the Supporting Information). An even higher efficiency was observed with the construct PCR300·**4**. Here, no alkyne starting nucleoside could be detected after the click reaction. The double peaks obtained for the clicked-on sugar nucleosides arise because of the rapid anomerization of the sugar moiety. This was confirmed by HPLC-MS/MS measurements, which showed the same expected molecular weight for both peaks (see also the Supporting Information). The same almost-quantitative conversion was also observed for the click reaction with the PCR2000·**4** construct containing 887 alkyne modifications. They all were converted into the corresponding product, a result showing the amazing efficiency of the click reaction. To the best of our knowledge, the simultaneous high-yielding modification of more than 800 reaction sites in a single process is unprecedented in the literature.

Discussion and Conclusion

The incorporation of the different triphosphates **2**–**6** confirms the overall trends also observed in other studies. An extended π system improves the acceptance of a triphosphate by a DNA polymerase. The failed incorporation of **6** shows that a small modification of the structure of the monomer can induce massive incorporation problems. The reason for this is still not clear and will require more extensive investigation. The stability of the PCR products obtained with the triphosphates **2**–**4** gives more insight into how they influence the DNA strand structure. All of the alkyne modifications stabilize the DNA with respect to the natural bases. The alkyne content of PCR300 is around 52% (153 alkyne base pairs) for the thymidines and 35% (104 alkyne base pairs) for the cytidines. Consequently, there are about 1.5 times more alkyne base pairs in PCR300·**2**/**3** than in PCR300·**4**. The destabilization of PCR300·**3** by about 3 °C compared to PCR300·**2** can be attributed to the increased steric clash of the longer alkyl chains in the major groove and the exclusion of water interactions in the major groove.[24] Although PCR300·**4** contains the same alkyl modifications, it is more stable than PCR300·**3**, possibly because of the decreased density of modifications and the increased stabilization induced by alkyne cytidines compared to alkyne thymidines.[25] Since the same trends are observed in the 900-base-pair series, the stability differences seem to be a general property of PCR products with similar base composition (see the Supporting Information). The increased thermal stability of the modified DNA seems to be the problem when the PCR reaction with triphosphate **4** is

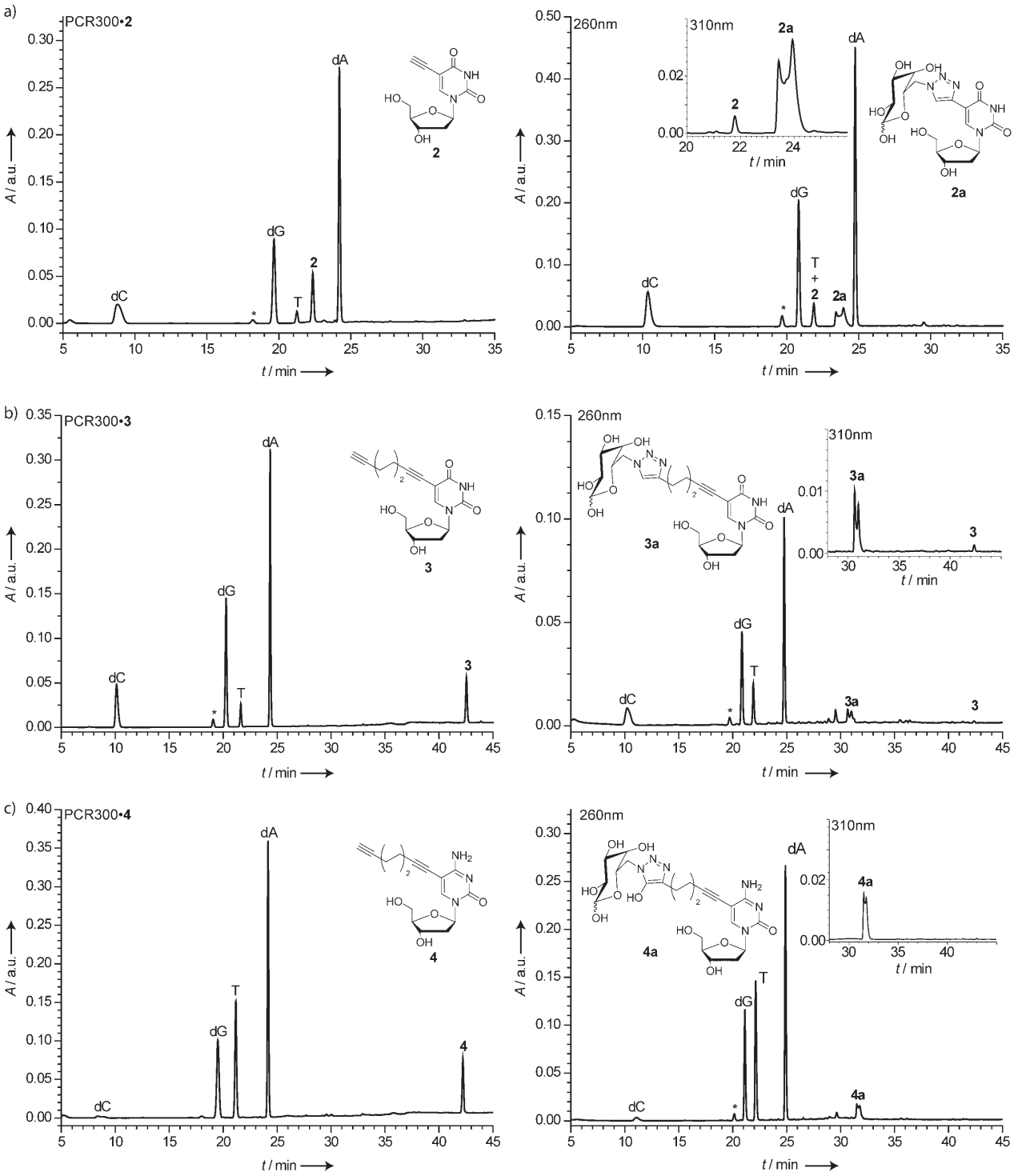


Figure 5. HPLC chromatograms of the enzymatic digests of PCR products a) PCR300·**2**, b) PCR300·**3**, and c) PCR300·**4** before (left) and after (right) the click reaction with **1** to form the corresponding products **2**–**4a**. (In the chromatogram for **2a**, the starting material **2** lies under the thymidine peak.) The insets show the HPLC traces at 310 nm. * marks the peaks assigned to inosin, which is formed due to deamination during the assay. dA: deoxyadenosine; dC: deoxycytidine; dG: deoxyguanosine; T: thymidine.

used for longer PCR products, while the higher density or the sequence of the template seems to be the problem for triphosphate **3**.

The differences observed in the CD spectra of PCR300·**N** and PCR300·**2**–**4** can most likely be attributed to slightly altered base stacking as a consequence of the presence of the alkyne modifications in the 5-position of the pyrimidine nu-

cleobases. The absorptions of the bases at around 300 nm cause an additional CD anomaly. Similar effects were observed by Famulok and co-workers[8] and by He and Seela[26] for either shorter or synthetic DNA strands. Due to the length of the PCR products measured in this study, the unmodified primer regions should not influence the result. What can be deduced from the spectra with reasonable confidence is that the modified DNA still has a B-like conformation. Since the spectra of the longer PCR products are almost identical to the PCR300 series, the measured effects seem to be averaged properties of PCR products with a similar base composition (see the Supporting Information).

The unusually high clicking efficiency observed for PCR300·**2** was unexpected considering the less than optimal yields observed in our previous oligonucleotide study.[19] One could speculate that this deviation is a consequence of the secondary structures of the two systems. In the short oligonucleotide series, we utilized single-stranded 16-mers, whereas double-stranded PCR fragments were used for the click reaction in this study. The single-stranded oligonucleotides have greater flexibility and may adopt a variety of secondary structures in solution; this, in turn, might sequester certain alkyne groups. This would be less likely in a PCR fragment where the alkyne functions are pointing out of the major groove in a rather stiff double-helix environment.

An additional explanation of this observed result may be the density of alkyne modifications found in the primary sequence of the PCR fragments. Our preliminary oligonucleotide study revealed that consecutive stretches of truncated alkyne functions decreased the clicking efficiency markedly, yet the primary sequence of PCR300 only contains six consecutive stretches of three thymidines and three consecutive stretches of four thymidines. Thus, the DNA templates used in this study are less demanding, although they are much longer.

The analysis of the products showed that the click conditions used do not induce any DNA damage, for example, no oxidized DNA bases like 8-oxo-dG were found in the enzymatic digest. Only when the reaction mixtures were heated or when they were concentrated at higher temperatures, minor side products were observed. As concluded from the mass spectrometry data, it is most likely that oxidized triazole species are only formed, if at all, through oxidation of the copper triazole species arising during the click reaction (see the Supporting Information).

This study establishes that PCR in combination with the click reaction provides a modular tool for the synthesis of highly modified DNA strands. The optimization of the PCR conditions shows that the stability of the resulting strands may require the design of special PCR programs. In the template series used, the incorporation of 5-modified cytidine triphosphates was found to be more efficient than that of 5-modified uridine triphosphates. The reason for this observation is not yet clear. Polymerases of the B family, like Pwo and KOD XL, are highly superior compared to the other polymerases tested when efficient incorporation of the modified triphosphates is desired. The click reaction is an extremely facile, simple, and efficient (yields of $>90\,\%$ up to quantitative) method to convert the introduced alkynes, even on long DNA strands containing hundreds of modifications, into the click product of choice. The combination of PCR and the copper(I)-catalyzed reaction of alkynes with azides is a perfect and powerful marriage if the synthesis of highly modified oligonucleotides is desired. The influence of the single or double strand on the click reaction, especially for alkyne **2**, will be the subject of further studies.

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