DNA Protection

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Transient and Switchable (Triethylsilyl)ethynyl Protection of DNA against Cleavage by Restriction Endonucleases**

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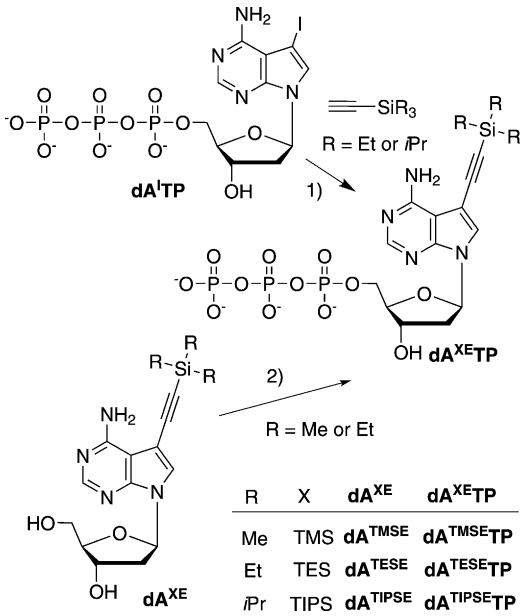
The sequence-specific cleavage of DNA by type II restriction endonucleases (REs) is of paramount importance in DNA manipulations, such as recombination and cloning. Hundreds of REs are commercially available and used in molecular biology.[1] Some of them are sensitive to DNA methylation, and in some bacteria REs cleave only non-methylated sequences as a defense against foreign DNA. When manipulations of large DNA sequences are required, the possibility of the selective protection of certain sequences against cleavage might be useful, especially if the recognition sequence for a particular RE is present in several copies. Base-modified nucleic acids are nowadays frequently applied in diverse areas of chemical biology, but their cleavage by REs has been only scarcely studied. DNA containing 7-deazaadenine[2] (7-deazaA) or 7-deazaguanine[2e] in a recognition sequence was repeatedly reported to be resistant to some endonucleases, apparently because the N7 atom cannot form hydrogen bonds in the major groove. The incorporation of 7-deazaA was used[3] as a means of permanently protecting DNA against cleavage by certain REs. Recently, we reported a systematic study of the cleavage of DNA containing 7-substituted 7-deazaadenines[4] and 5-substituted pyrimidines;[5] we found a surprising tolerance of several REs to the presence of not only 7-deazaadenine but also some 7-substituted derivatives. In these studies,[4,5] permanent protection against cleavage by REs was also demonstrated. Herein, we report on the first transient and switchable protection of DNA against REs.

In our previous study,[4] we noticed that several REs (RsaI,[2a] KpnI,[6] and SacI[7]) showed high tolerance to the presence of an alkynyl group but not to a more bulky phenyl group at position 7 of 7-deazaA. Therefore, we envisaged that some silyl-substituted 7-ethynyl-7-deazaadenines might be used as transient protection against REs (analogous to the (trialkylsilyl)alkyne protection[8] for the triple click labeling of DNA by triazole formation, a method that was recently developed by the Carell group).

Therefore, we tested three trialkylsilyl groups (trimethylsilyl (TMS), triethylsilyl (TES), and triisopropylsilyl (TIPS)) as protection for the acetylene at position 7 of 7-deaza-2′-deoxyadenosine.[9] The synthesis and deprotection was first studied on model nucleoside monophosphates (see the Supporting Information for details). This study showed that the TMS- and TES-protected 7-ethynyl-dAMP could be easily deprotected by simple treatment with ammonia, whereas the TIPS group must be cleaved by treatment with tetra-*n*-butylammonium fluoride (TBAF). The next task was the preparation of (trialkylsilyl)ethynyl-modified deoxyribonucleoside triphosphates (dNTPs) as substrates for the polymerase synthesis of DNA. The polymerase construction of base-modified DNA[10] is now an established and widely used procedure. In our group, we combined this procedure with the aqueous cross-coupling of halogenated dNTPs[11] into an efficient two-step synthesis of DNA bearing redox labels[12] or reactive functional groups.[13] Therefore, we first tried the aqueous Sonogashira cross-coupling reactions of **dA^{I}TP** with (trialkylsilyl)acetylenes (Scheme 1). Similarly to the deoxy-



R	X	dA^{XE}	dA^{XE}TP
Me	TMS	dA^{TMSE}	dA^{TMSE}TP
Et	TES	dA^{TESE}	dA^{TESE}TP
*i*Pr	TIPS	dA^{TIPSE}	dA^{TIPSE}TP

Scheme 1. Reagents and conditions: 1) $Pd(OAc)_2$, TPPTS, CuI, iPr_2NEt, $CH_3CN/H_2O = 1:2$, 30 min, 80 °C (11% yield of **dA^{TESE}TP**, 30% yield of **dA^{TIPSE}TP**). 2) 1. $POCl_3$, $PO(OMe)_3$, 1 h, 0 °C, 2. $(NHBu_3)_2H_2P_2O_7$, DMF, 1 h, 0 °C (26% yield of **dA^{TMSE}TP**, 47% yield of **dA^{TESE}TP**). DMF = *N*,*N*-dimethylformamide, TPPTS = 3,3′,3″-phosphanetriyltris(benzenesulfonic acid) trisodium salt.

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ribonucleoside monophosphate (dNMPs), the reaction of **dA^{I}TP** with (trimethylsilyl)acetylene under aqueous conditions did not proceed. On the other hand, the reaction of **dA^{I}TP** with (triethylsilyl)- and (triisopropylsilyl)acetylenes proceeded reasonably well to give the desired dNTPs (**dA^{TESE}TP** and **dA^{TIPSE}TP**) in 15 and 30% yields, respectively (Scheme 1). The TMSE derivative (**dA^{TMSE}TP**) was prepared by triphosphorylation of the nucleoside **dA^{TMSE}** in 26% yield (diminished by partial desilylation during the isolation). This alternative approach was also used for a more efficient synthesis of **dA^{TESE}TP** (47% yield after triphosphorylation of **dA^{TESE}**).

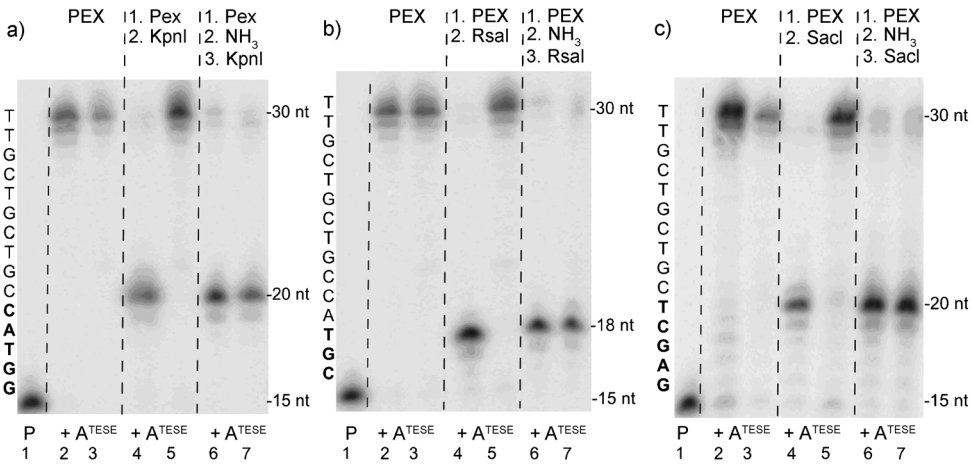

Figure 1. PAGE analyses of PEX, deprotection, and cleavage experiments: a) PEX with temp^{Kp}, cleavage by KpnI; b) PEX with temp^{Rs}, cleavage by RsaI; c) PEX with temp^{Sa}, cleavage by SacI. P: primer; +: natural (unmodified) dNTPs; A^{TESE}: modified dNTPs. Lane 1: primer; lane 2: product of PEX with natural dNTPs; lane 3: product of PEX with dTTP, dCTP, dGTP, **dA^{TESE}TP**; lane 4: cleavage of the unmodified PEX product with the RE; lane 5: treatment of the TESE-modified PEX product with the RE (no cleavage); lane 6: treatment of the unmodified PEX product with NH_3 followed by cleavage with the RE; lane 7: treatment of the TESE-modified PEX product with NH_3 followed by cleavage with the RE (full cleavage).

All three **dA^{XE}TP**s were tested as substrates for DNA polymerases in primer extension (PEX) experiments using temp^{4base} (incorporation of four modified **dA^{XE}** units into a 31 nucleotide (nt) DNA). Both tested polymerases (Vent(*exo*$^-$) and KOD XL) efficiently incorporated the four modified nucleotides into the full-length oligonucleotides (see Figure S1 in the Supporting Information). Therefore, each modified **dA^{XE}** was then incorporated into a double-stranded DNA (dsDNA) containing a recognition sequence for RsaI, KpnI, or SacI REs (for sequences, see Table S1 in the Supporting Information) using Vent(*exo*$^-$) polymerase. The PEX products (both positive-control unmodified DNA and the DNA containing a **dA^{XE}** in the recognition sequence) were treated with the corresponding RE (Figure 1 and Figures S2 and S3 in Supporting Information). In all cases, the unmodified DNA was cleaved, whereas the (trialkylsilyl)ethynyl-modified DNA (containing a **dA^{TESE}** or **dA^{TIPSE}** modification) completely resisted the cleavage and the (trimethylsilyl)ethynyl-modified DNA (**dA^{TMSE}** modification) was partly cleaved by RsaI and KpnI REs (see Figure S2 in the Supporting Information). Therefore, the TMS protection group was excluded from further study as insufficiently stable and insufficiently bulky to prevent the RE cleavage. When the (triisopropylsilyl)ethynyl-modified DNA was treated with TBAF and the product isolated by precipitation with EtOH, the resulting DNA was fully cleaved by all three REs which clearly indicated that the TIPS group was cleaved (see Figure S3 in the Supporting Information). The TMSE- and TESE-modified oligonucleotides (ONs) were deprotected by simple treatment with aqueous ammonia and the resulting ethynyl-DNA was cleanly cleaved by all three REs. This confirms the proof-of-principle of transient silyl protection against the restriction cleavage (Figure 1).

To investigate the potential for applications in molecular biology, we tested the protection and cleavage on real (larger) DNA. However, modifications are typically introduced into large DNA by polymerase chain reaction (PCR) rather than by PEX. Therefore, we tested **dA^{TESE}TP**s and **dA^{TIPSE}TP**s in PCR using a 98-mer template and two polymerases (Vent(*exo*$^-$) and KOD XL). The incorporation of **dA^{TESE}TP** worked reasonably well with Vent(*exo*$^-$) and very well with KOD XL polymerase, to give the full-length products (see Figure S4 in the Supporting Information), whereas the more bulky **dA^{TIPSE}TP** did not work with any of the polymerases. Therefore, we selected **dA^{TESE}TP** for the PCR synthesis of a 287-mer DNA (coding DNA for HIV protease) containing a recognition sequence for RsaI RE. Again, the PCR with KOD XL polymerase worked very well (Figure 2). When the PCR products, unmodified positive-control DNA, and fully TESE-modified DNA (every deoxyadenosine (dA) replaced by **dA^{TESE}** except for the primers), were treated with the RsaI enzyme, the unmodified DNA was cleaved, whereas the fully protected modified DNA completely resisted the cleavage. However, the treatment of the TESE-modified DNA with aqueous ammonia (desilylation of the TESE modifications) followed by the treatment with RsaI gave full cleavage of the DNA.

In summary, three (trialkylsilyl)ethynyl groups attached at the C7 position of 7-deazaadenine were tested as potential protecting groups against RE cleavage of DNA. All three modified **dA^{XE}TP**s were prepared and tested for PEX and PCR. In PEX, all of them worked well with several polymerases. On the other hand, in PCR, the more bulky **dA^{TIPSE}TP** was an unsuitable substrate, whereas the **dA^{TESE}TP** worked very well even for larger templates. The protection was first tested on shorter (30-mer) DNAs containing one **dA^{XE}** modification within a recognition sequence for an RE (KpnI, RsaI, or SacI). The RE cleavage of the silylethynyl-modified DNA was fully (TESE and TIPSE) or partly (TMSE) prevented. Only after desilylation

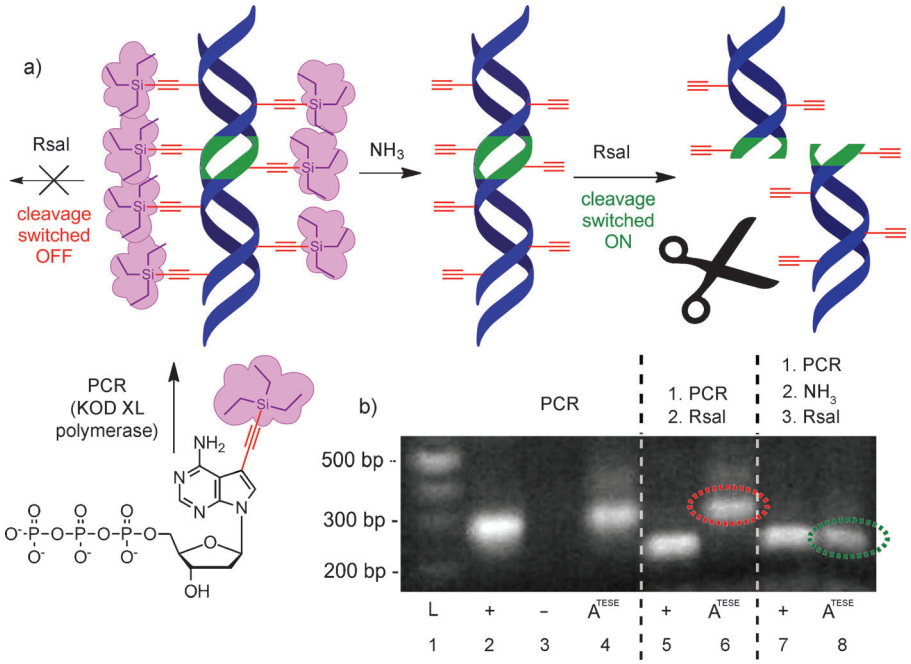

Figure 2. a) Scheme of the PCR incorporation of **dA^{TESE}TP**, and deprotection and cleavage of the DNA (blue) by RsaI (recognition site for RsaI in green). b) Agarose gel analysis of PCR, deprotection, and cleavage experiments with 297-mer template. Lane 1: DNA ladder; lane 2: product of PCR with natural dNTPs; lane 3: product of PCR with dTTP, dCTP, dGTP; lane 4: product of PCR with dTTP, dCTP, dGTP, **dA^{TESE}TP**; lane 5: cleavage of the unmodified PCR product with RsaI; lane 6: treatment of the TESE-modified PCR product with RsaI (no cleavage, circled in red); lane 7: treatment of the unmodified PCR product with NH_3 followed by cleavage with RsaI; lane 8: treatment of the TESE-modified PCR product with NH_3 followed by cleavage with RsaI (full cleavage, circled in green). bp = base pair.

by treatment with NH_3 (TESE and TMSE) or TBAF (TIPSE), was the resulting ethynyl-modified DNA fully cleaved by all three REs. Apparently, the TESE protecting group is the most useful one in terms of good substrate activity toward the polymerase and ease of deprotection. This approach was also successfully tested on a larger DNA. A 287-mer dsDNA that was fully modified with **dA^{TESE}** was prepared by PCR and was found to fully resist the cleavage by RsaI. Only after its treatment with NH_3 (deprotection), could the resulting DNA be fully cleaved by this RE.

We have shown that the TESE group attached to 7-deazaadenine can be efficiently used for the protection of DNA against RE cleavage. So far, we have identified only three REs that are compatible with this protocol. However, we believe that other suitable REs may be found by further screening, and that other transient protecting groups may be found for REs that do not tolerate 7-ethynyl-dA. This approach may find applications in the manipulations of larger DNA sequences (i.e. genomic DNA), where target sequences for some REs may appear in several copies that must be distinguished and cleaved specifically (only one at a time). As the deprotection releases free ethynyl group(s), the deprotected DNA can also be used for further modifications by click chemistry with azides.[8,14] Further interesting questions for future research on this topic might concern the interactions of the modified DNA with transcription factors, the possible regulation of gene expression by transient protection, and the possibility of using RE-resistant protected DNA to combat bacteria. Studies along these lines will continue in our laboratory.

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