Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Organická chemie

Studijní obor: Organická chemie

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Enzymová syntéza DNA modifikované v malém žlábku

Enzymatic synthesis of DNA modified in the minor groove

Disertační práce

Školitel: prof. Ing. Michal Hocek, PhD., DSc.

Praha, 2019

Disertační práce byla vypracována na Ústavu organické chemie a biochemie, Akademie věd České Republiky v Praze v období říjen 2015 –prosinec 2019.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce, ani její podstatná část, nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne

Podpis: Ing. Ján Matyašovský

Acknowledgement

First and foremost, I would like to thank my supervisor Prof. Michal Hocek for his guidance and trust. Without his support, none of this would be possible. I am thankful to Dr. Radek Pohl for his help with measurement and interpretation of complex NMR spectra, to Ing. Kateřina Nováková for the measurement of MALDI-TOF spectra and Dr. Dmytro Dzuiba for teaching me about fluorescence.

Special thanks to all of my colleagues in the group for creating a creative working atmosphere, many of whom should be mentioned by name. First of all, Alessandro Panattoni, my great friend, with whom I went through a lot of the same challenges and obstacles, Nemanja Milisavljevič, the clumsy friend with a big heart that every group should have, Vojtěch Havlíček, whose sense of humour never ceases to amaze me, Dr. Michal Tichý, who brought a bit of order to the chaos, Zuzana Vaníková, who kindly introduced me to the world of biochemistry, Dr. Agata Olszewska, whose drive and self-confidence I admired, Tereza Schröpferová, who made sure we were always properly stocked, Dr. Catherine Mullholland, who brought a bit of culture to the group and Dr. Veronika Sýkorová, whose shining smile always made everybody's day a lot better. Lastly, my big thanks go to Lucia Veselovská, my close friend, who made the good days great and the bad one bearable.

A special mention goes to my friends outside work. Dr. Pavol Lopatka, who remained a friend even though a great distance separated us and to whom I could always compare myself to. I thank Martin Chreno, Andrej Bielik and Ján Belan, my oldest friends, with whom I am able to talk nonsense, for being the people that I could always depend on.

Last but not least, I am grateful to my family. My aunt Andrea, whose life resembles mine in some ways and whose advice was invaluable. My parents, who supported me in everything I put my mind to and who raised me to be the person I am today. My father, the last alchemist, who showed me the beauty of chemistry at a very young age and from whom I inherited the patience required for great discoveries. My mother, who taught me to be responsible and from whom I got the organisation skill to also properly note the successful experiments leading to those discoveries. And also my brother, the young doctor, who, despite being the younger one, is a role model to me in many ways.

My Ph.D. work was a part of a multidisciplinary project performed by Prof. Hoceks research group at the Institute of Organic Chemistry and Biochemistry AS CR. All of the experiments described in the thesis were performed by me in person. Measurement and interpretation of complex NMR spectra were done by Dr. Radek Pohl. Ing. Kateřina Nováková measured the MALDI-TOF mass spectra of prepared oligonucleotides and the mass spectra of all of the synthesised compounds were measured by the mass spectrometry team at IOCB Prague.

This work was supported by the Czech Science Foundation (17-03419S), by Marie Sklodowska-Curie Innovative Training Network (ITN) Click Gene (H2020-MSCA-ITN-2014-642023) and by Gilead Sciences, Inc. (Forster City, CA, USA).

Summary

In the first part of the thesis, a series of six modified 2'-deoxyadenosine triphosphates, bearing small functional groups (chloro, amino, methyl, vinyl, ethynyl and phenyl) at position 2 of adenine, was designed and synthesised. They were then tested as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA. The 2-phenyl modified dATP was the only triphosphate unable to be incorporated, meaning that the phenyl group is already too big for minor-groove incorporations. All of the other tested nucleotides were good substrates for tested DNA polymerases [KOD XL, Vent(exo-) and Bst LF] affording minor-groove modified DNA bearing one or four modifications. The vinyl- and ethynyl-modified DNAs were then used for post-synthetic modification of DNA minor groove with fluorescent labels utilising click reactions. Ethynyl group reacted in copper-catalysed alkyne-azide cycloaddition (CuAAC), whereas the vinyl group participated in thiol-ene reaction. This procedure allowed for the attachment of big functional groups otherwise unable to be installed into the DNA minor groove using direct enzymatic incorporation.

The second part of the thesis was devoted to the study of 2-alkylamino-2'-deoxyadenosine triphosphates and their use in enzymatic synthesis of base-modified ONs and DNA. These N^2-alkyl diaminopurine nucleotides were synthesised and tested as substrates for DNA polymerases. The methylamino modified derivative was the only nucleotide which KOD XL DNA polymerase efficiently incorporated into DNA. All of the other nucleotides were poor for all tested DNA polymerases in those experiments. Therminator DNA polymerase was however able to extend the primer by incorporation of one modified adenine bases, when no other 2'-deoxynucleoside triphosphates (dNTPs) were present in the reaction mixture. Optimisation of reaction conditions for the experiment with template encoding for two consecutive adenine bases allowed for incorporation of either one or two modified triphosphates. When an excess of natural dNTPs was added to the reaction mixtures, PEX using these nucleotides resulted in site-specific minor-groove modified DNA. The allyl- and propargylamino- modified DNAs were then used for post-synthetic transformations of DNA minor-groove using click reactions with fluorescent labels. Ultimately this approach was used for the construction of DNA FRET probes, useful for the monitoring of DNA duplex denaturation/reannealing process or for the detection of specific ON sequences.

Finally, we also extended the portfolio of modified nucleotides useful for construction of minor-groove modified DNA to dGTP analogues. We prepared a series of five 2-substituted 2'-deoxyinosine triphosphate derivatives, bearing small substituents (chloro, methyl, vinyl, ethynyl and phenyl), which acted as surrogates of guanine nucleobase and base-paired with cytosine. Therminator DNA polymerase was able to produce full-length products with the 2-methyl and 2-vinyl modified nucleotides, whereas the other derivatives were not incorporated by any of the tested DNA polymerases. The prepared vinyl-modified DNA participated in thiol-ene reactions with thiols and also with a cysteine-containing minor-groove binding peptide providing a successful product of crosslinking thanks to the proximity effect of the functional groups.

All of the prepared 2-substituted purine nucleotides were ultimately used to study the influence of minor-groove modifications on cleavage of dsDNA by tape II restriction endonucleases. No general trend for the effect of these modifications was observed. Both enzymes cleaving the minor-groove modified DNA and enzymes whose activity was inhibited by these modifications were identified.

Souhrn

V první části práce byla navržena a syntetizována řada šesti modifikovaných 2'-deoxyadenosintrifosfátů, nesoucí malé funkční skupiny (chlor, amino, methyl, vinyl, ethynyl a fenyl) na adeninu v poloze 2. Připravené nukleotidy byly testovány jako substráty pro DNA polymerázy v enzymatické syntéze DNA modifikované v malém žlábku. 2-Fenyl modifikovaný dATP byl jediným trifosfátem, který nebyl inkorporovaný, což znamená, že fenylová skupina je již příliš velká pro inkorporaci do malého žlábku. Všechny ostatní testované nukleotidy byly dobrými substráty pro testované DNA polymerázy [KOD XL, Vent(exo-) a Bst LF], což poskytlo modifikovanou DNA s malým žlábkem nesoucím jednu nebo čtyři modifikace. Modifikované DNA s vinylovou nebo ethynylovou skupinou byly poté použity pro modifikaci malého žlábku DNA fluorescenčními značkami za použití click reakcí. Ethynylová skupina reagovala v mědí katalyzované cykloadici alkynu s azidem (CuAAC), zatímco vinylová skupina se účastnila thiol-en-ové reakce. Tento postup umožnil připojení velkých funkčních skupin, které by jinak nemohly být instalovány do malého žlábku DNA pomocí přímé enzymatické inkorporace.

Druhá část práce byla věnována studiu 2-alkylamino-2'-deoxyadenosin trifosfátů a jejich použití v enzymatické syntéze modifikovaných oligonukleotidů a DNA. N^2-alkyl diaminopurinové nukleotidy byly připraveny a testovány jako substráty pro DNA polymerázy. Methylamino modifikovaný derivát byl jediným nukleotidem, který KOD XL DNA polymeráza účinně inkorporovala do DNA. Všechny ostatní nukleotidy byly špatnými substráty pro všechny testované DNA polymerázy. Therminator DNA polymeráza však byla schopna prodloužit primer inkorporací jedné modifikované adeninové báze, v případě, že v reakční směsi nebyly přítomny žádné jiné 2'-deoxynukleosid trifosfáty (dNTP). Optimalizace reakčních podmínek pro experiment s templátem kódujícím dvě po sobě jdoucí adeninové báze v připravené DNA umožnila inkorporaci jednoho nebo dvou modifikovaných trifosfátů. Když byl do reakčních směsí přidán nadbytek přírodních dNTP, metoda prodlužování primeru (PEX) s použitím těchto nukleotidů vedla k DNA modifikované v malém žlábku na konkrétním míste. Allyl- a propargylaminem modifikované DNA byly poté použity pro transformaci malého žlábku DNA pomocí click reakcí s fluorescenčními značkami. Tento přístup byl nakonec použit pro konstrukci FRET sond, užitečných pro monitorování procesu

denaturace a opětovné hybridizace DNA duplexu nebo pro detekci specifických oligonukleotidových sekvencí.

Nakonec jsme také rozšířili portfolio modifikovaných nukleotidů užitečných pro konstrukci DNA s modifikací v malém žlábku na analogy dGTP. Připravili jsme sérii pěti 2-substituovaných 2'-deoxyinosintrifosfátů, obsahujících malé substituenty (chlor, methyl, vinyl, ethynyl a fenyl), které fungovaly jako náhražky guaninové nukleobáze a párovala se v DNA s cytosinem. Therminator DNA polymeráza byla schopna připravit produkty plné délky s 2-methyl a 2-vinyl modifikovanými nukleotidy, zatímco ostatní deriváty nebyly inkorporovány žádnou z testovaných DNA polymeráz. Připravená vinylem modifikovaná DNA se účastnila thiol-en-ových reakcí s thioly a také s DNA vazebným peptidem, který se vázal v malém žlábku a obsahoval cystein, což poskytlo produkt reakce mezi DNA a peptidem tvorbou kovalentní vazby díky jevu blízkosti funkčních skupin.

Všechny připravené 2-substituované purinové nukleotidy byly nakonec použity ke studiu vlivu modifikací v malém žlábku na štěpení dvouvláknové DNA restrikčními endonukleázami typu II. Nebyl pozorován žádný obecný trend účinku těchto modifikací. Identifikovány byly jak enzymy štěpící modifikovanou DNA s modifikací v malém žlábku, tak i enzymy, jejichž aktivita byla těmito modifikacemi inhibována.

List of abbreviations

Ac	acetyl
bp	base pair
Bu	butyl
COSY	correlation spectroscopy
CUAAC	copper-catalyse alkyne-azide cycloaddition
Da	Dalton
DABCO	1,4-diazabicyclo[2.2.2]octane
DCM	dichloromethane
DIPEA	N,N-diisopropyl(ethyl)amine
DMA	N,N-dimethylacetamide
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DMT	4,4′-dimethoxytrityl
dNMP	2'-deoxynucleoside-5'-*O*-monophosphate
dNTP	2'-deoxynucleoside-5'-*O*-triphosphate
DNA	deoxzribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
Et	ethyl
equiv.	equivalent
FAM	fluorescein
FG	functional group
FRET	Förster resonance energy transfer
HOMO	highest occupied molecular orbital
HPA	3-hydroxypicolinic acid
HPLC	high performance liquid chromatography
HMBC	heteronuclear multiple bond correlation
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence spectroscopy

iEDDA	inverse-electron-demand Diels Alder
i-Pr	isopropyl
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
Me	methyl
NEAR	nicking enzyme amplification reaction
NMR	nuclear magnetic resonance
nt	nucleotide
ON	oligonucleotide
PA	picolinic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDB	protein data bank
PEX	primer extension
Ph	phenyl
RE	restriction endonuclease
R_f	retention factor
RNA	ribonucleic acid
RP-HPFC	reverse-phase high-performance flash chromatography
rpm	rounds per minute
rt	room temperature
SPAAC	strain-promoted alkyne-azide cycloaddition
ss	single stranded
TBE	TRIS-borate-EDTA
TBTA	tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
t-Bu	*tert*-butyl
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetyl
THF	tetrahydrofurane
TINA	twisted intercalating nucleic acid
TLC	thin layer chromatography
T_m	denaturing (melting) temperature
Tol	toluoyl, 4-methylbenzoyl
TPPTS	3,3′,3″-phosphanetriyltris trisodium salt

Contents

1 Introduction

1.1 DNA structure

Nucleic acids are considered to be some of the most important biomacromolecules presented in nature. DNA (deoxyribonucleic acid) was first isolated by Friedrich Miescher in 1869, who correctly distinguished the isolated substance from proteins thanks to different properties and the presence of phosphorus in the structure.[1,2] He was aware of the importance of his discovery; however the assignment of the correct structure eluded scientists for a long time. Almost a century later, James Watson and Francis Crick described the structure of DNA using x-ray diffraction in 1953 (with a footnote acknowledgment of the contribution of Rosalind Franklin and Maurice Wilkins).[3]

Double helical DNA can be found in three major forms: A-DNA, B-DNA and Z-DNA (Figure 1).

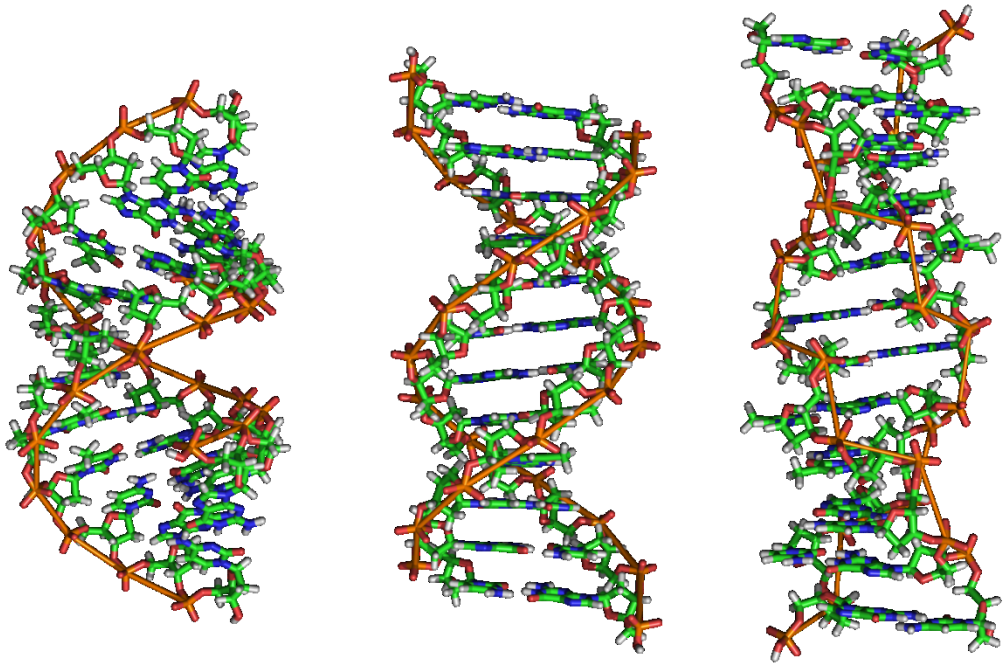


Figure 1. Structures of A-DNA, B-DNA and Z-DNA (left to right). (Figure taken from Wikimedia commons)

B-DNA structure, in which the helix twists in a right-hand direction, is the most abundant structure under the conditions found in cells.[4] It contains a wide major groove and a narrow minor groove which are important as sites for interactions of

proteins with DNA molecule.[5] A-DNA is also a right-handed double helix which exists mostly in partly dehydrated DNA samples; its major groove is quite deep and the minor groove is shallow.[6] A left-handed double helix appears in the structure of Z-DNA which forms upon methylation of nucleobases[7] and is mostly present in sequences of altering purine-pyrimidine bases.[8,9] This unusual structure can also be found *in vivo* and is thought to be involved in the regulation of transcription.[10]

Double helix of DNA is mostly held together by hydrogen bonds between complementary nucleobases of the antiparallel DNA strands; adenine (**A**) pairs with thymine (**T**) and guanine (**G**) pairs with cytosine (**C**) (Figure 2a).[11,12] There are also some other weaker bonding interactions that contribute to the overall structural stability of the molecule (such as π-π stacking of the nucleobases, etc.).[13] Apart from nucleobases, which are positioned perpendicularly to the axis of the helix, DNA is made up of sugar-phosphate backbone (Figure 2b).

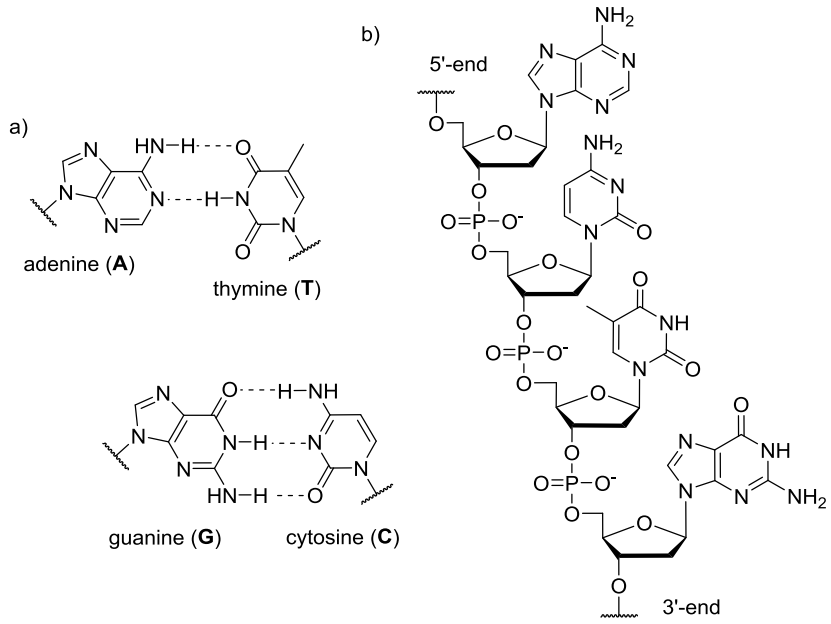
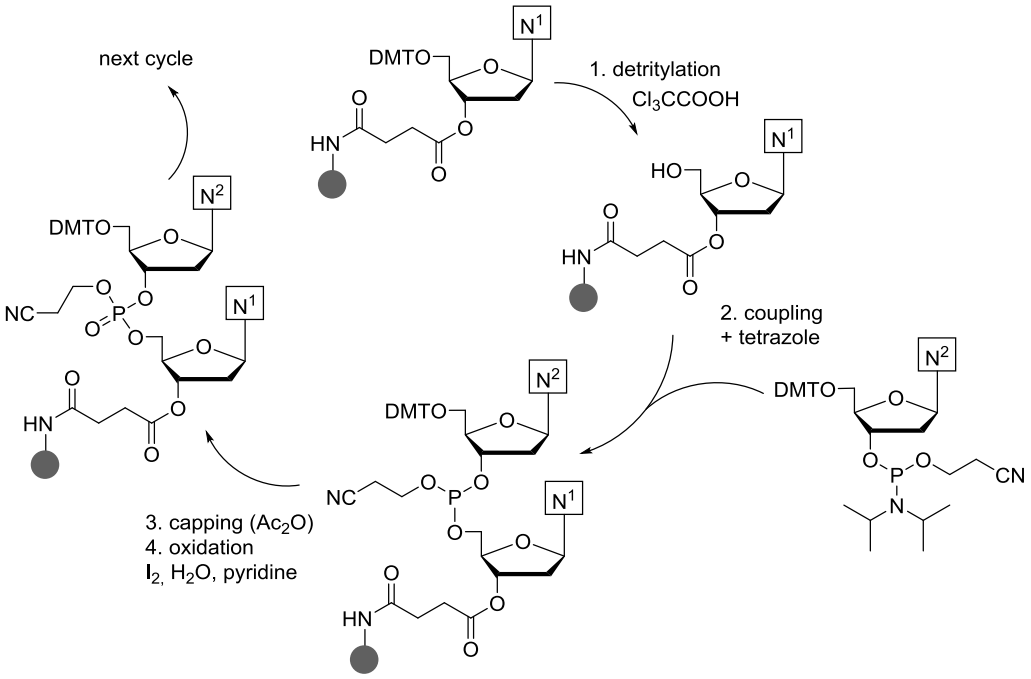


Figure 2. a) Watson-Crick base pairing of nucleobases. b) Part of a single stranded 2'-deoxyoligonucleotide with ACTG sequence.

1.2 Synthesis of nucleic acids

1.2.1 Chemical synthesis of oligonucleotides on solid support

Chemical synthesis of DNA (and RNA) is now an established method for preparation of up to 100-mer oligonucleotides (ONs).[14] Multiple methodologies have been developed for the synthesis of ONs, such as phosphodiester,[15] phosphotriester,[16,17] H-phosphonate,[18,19] phosphite triester.[20] Phosphoramidite synthesis is the most commonly used method which was developed by the Caruthers lab and was fully automated in the late 1980s (Scheme 1).[21]



Scheme 1. Standard solid-phase phosphoramidite-based oligonucleotide synthesis.

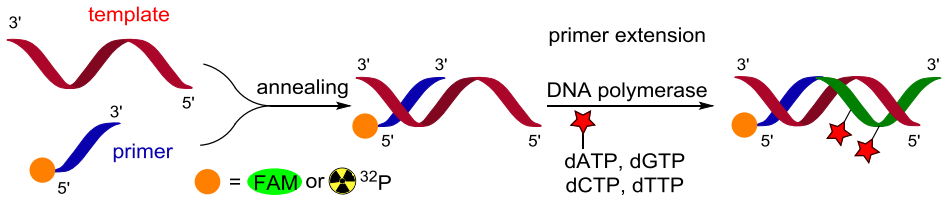
The synthetic strategy is to build the oligonucleotide from the 3'-end. The synthesis starts with detritylation by strong acid, upon which a primary hydroxyl group at 5'-position is released. Next step is a coupling reaction with nucleoside 3'-phosphoramidite, where tetrazole is used to promote the reaction and a phosphite triester is formed. After reaction of unreacted 5'-hydroxyl groups with acetic anhydride (to minimise the formation of undesired shorter products) the phosphite triester is oxidised by iodine in water/pyridine mixture to phosphate triester. The cycle is repeated in order to achieve the ON of desired length and sequence. At the end of the synthesis the formed ON is cleaved from the solid support usually by aqueous ammonia solution and finally purified. Polyacrylamide gel electrophoresis (PAGE) or high-performance

liquid chromatography (HPLC) are the methods of choice for purification of ONs prepared by chemical synthesis. This methodology has many advantages and is regularly used for the preparation of shorter ONs in high amount. For the preparation of longer ONs (>100-mer) this approach becomes ineffective due to low overall yield of the synthesis. Moreover, if the desired product of the synthesis is a modified ON, problem with compatibility of some functional groups with the synthesis protocol (strong acid, oxidation) might also arise.

1.2.2 Enzymatic synthesis of oligonucleotides

Enzymatic synthesis is an alternative to synthesis of ONs on solid support.[22] It is advantageously used especially when the chemical synthesis would be difficult or impossible. Preparation of longer ONs (>100-mer) is easily achievable using enzymatic synthesis. It is also possible to prepare modified ONs in cases when modifications are not compatible with the conditions used during the prosphoramidite synthesis. Compared to chemical synthesis, enzymatic synthesis proceeds from 5'- to 3'- terminus. There are two main ways of enzymatic preparation of DNA: primer extension (PEX) and polymerase chain reaction (PCR).

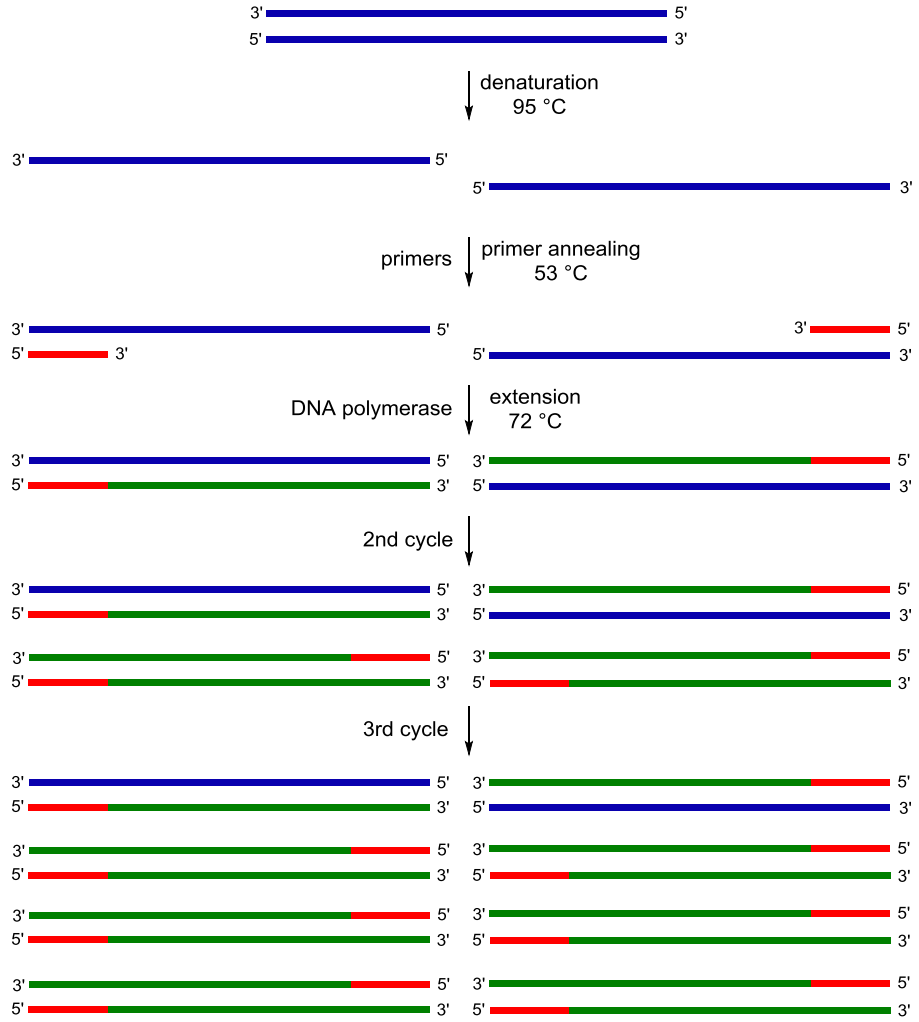
PEX is a method that is usually used to prepare DNA of <80 base pairs. The reaction itself requires a DNA polymerase, template, natural 2'-deoxynucleoside triphosphates (or modified triphosphates used instead of natural ones) and a primer that is being extended during the reaction. The primer can be labelled at the 5'- end by a fluorescent label or by ^{32}P-phosphate in order to have a way of visualization of the products of the reaction (Scheme 2). In the case when modified nucleotides are used in the reaction, modifications are present only in the synthesised strand.



Scheme 2. Primer extension using a labelled primer and modified 2'-deoxyadenosine triphosphate with 2 modifications incorporated into the DNA product.

PCR, developed in the 1980s,[23] is a method used for amplification of longer DNA (Scheme 3). The process is made up of repeating cycles where each cycles contains three steps: 1. denaturation of dsDNA, 2. annealing of complementary primers

and 3. extension of primers by DNA polymerase based on complementarity. The denaturation is usually carried out at 95 °C; therefore thermostable enzymes have to be used in the reaction. For successful annealing to take place, correct temperature has to be chosen. This temperature depends on the sequences of primer and template and therefore on the stability of the duplex formed (usually 50-55 °C). Finally, the extension is performed at (usually) 72 °C, depending on the DNA polymerase and triphosphates used. The amount of DNA increases exponentially during PCR (Scheme 3) and can provide dsDNA modified in both of the strands, if modified nucleotides are used. On the other hand, compared to PEX, PCR with modified nucleotides is much more challenging, as it not only requires that the modified 2'-deoxynucleoside triphosphates (dNTPs) are substrates for DNA polymerase and are incorporated, but the enzyme needs to be able to read through the modified DNA template in the next cycle of the reaction.



Scheme 3. Polymerase chain reaction.

1.3 Enzymatic synthesis of base-modified DNA

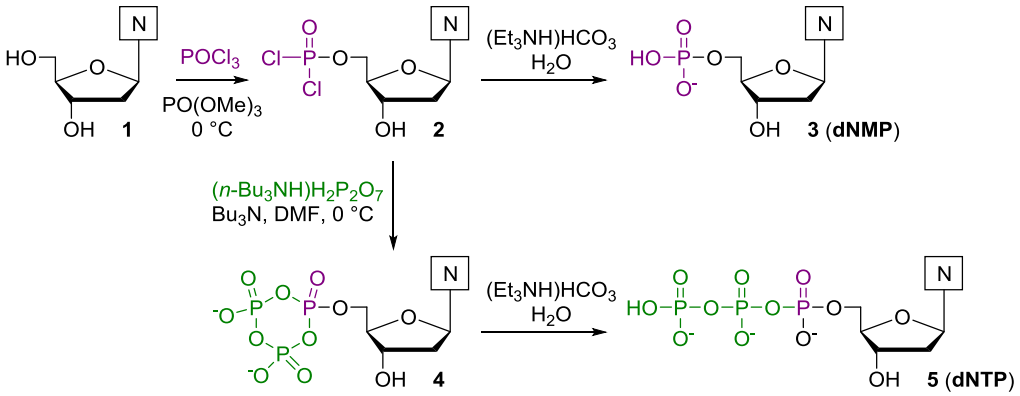
As was already mentioned, functionalised DNA can be prepared enzymatically, when modified dNTPs are used instead of their natural counterparts in PEX or PCR. The first example of this approach[24] had shown that (2'-deoxy)uridine triphosphate derivatives modified by biotin served as substrates for both DNA and RNA polymerases.

Modified nucleic acids became important tools in chemical biology, material science or diagnostics since then.[25] Diverse modifications including fluorophores,[26–29] redox[30,31] or spin labels,[32] reactive groups for bioconjugations[33–36] and even biomolecules (oligonucleotides,[37] proteins,[38] antibodies,[39] etc.) have been attached to ONs and used for different applications. Preparation of short base-modified ONs, useful as modified primers, using isothermal nicking-enzyme amplification reaction (NEAR) is also possible.[40,41] The building blocks for the preparation of these modified ONs and DNAs are the corresponding modified dNTPs. They are prepared by the means of organic synthesis and position at which the modification is attached plays a crucial role in whether they act as substrates for DNA polymerases or not.

1.3.1 Synthesis of modified nucleosides and nucleotides

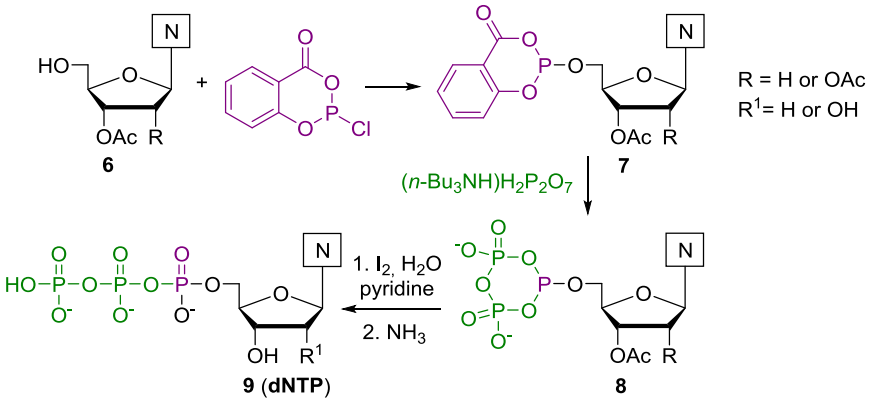
A general and high-yielding method for the preparation of dNTPs from corresponding 2'-deoxynucleosides has not been described up to date. However, multiple synthetic methods are available,[42] where optimisation of the reaction conditions is usually required depending on the functionality of the nucleosides and careful purification has to be performed to isolate the desired triphosphates.

Phosphorylation developed by Yoshikawa is one of the most commonly used reactions for the preparation of (modified) dNTPs (Scheme 4).[43,44] $POCl_3$ is used to selectively react with the 5'-hydroxyl group of 2'-deoxynucleoside **1**, which results in the formation of highly reactive phosphorodichlorate nucleoside **2**. This intermediate can then by hydrolysed to form the 2'-deoxynucleoside monophosphate (dNMP, **3**). Alternatively, upon *in situ* treatment of intermediate **2** with (tributylammonium) pyrophosphate, cyclic triphosphate **4** is formed, which can then by hydrolysed to obtain the desired triphosphate **5** (dNTP).



Scheme 4. Yoshikawa's synthesis of 2'-deoxynucleoside 5'-*O*-mono- and triphosphates.

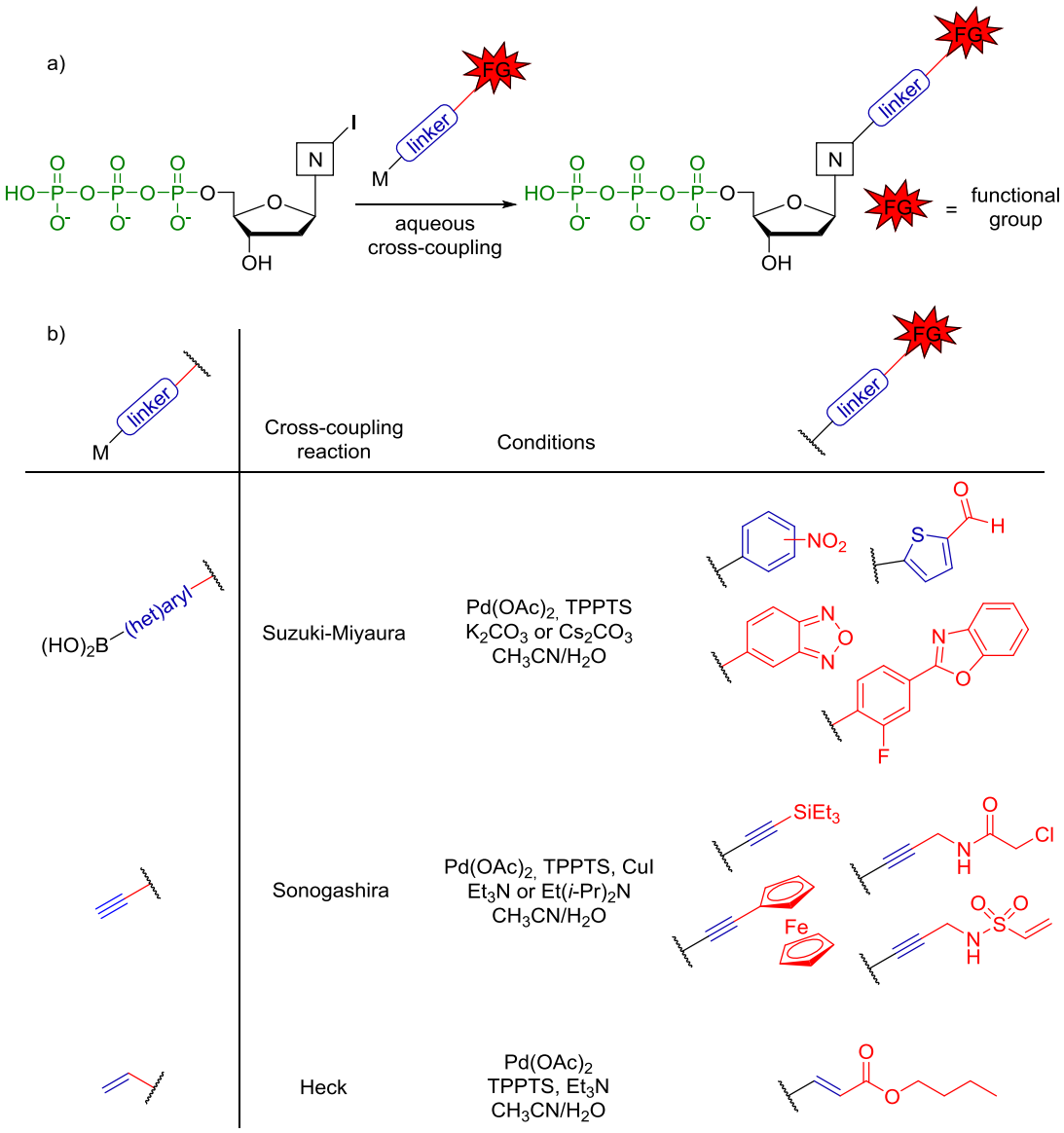
Another popular methodology, developed by Ludwig and Eckstein,[45] uses a more reactive P(III) species (Scheme 5). It utilizes 3'-*O*-protected nucleosides (such as **6**), which reacts with salicyl chlorphosphite to form the phosphite derivative **7**. Reaction of **7** with pyrophosphate leads to the cyclic intermediate **8**, which is then transformed by hydrolysis and oxidation to the corresponding nucleotide **9**.



Scheme 5. Preparation of nucleoside triphosphates developed by Ludwig and Eckstein.

The use of palladium-catalysed reactions is universally a method of choice for C-C bond formation. It can also be applied to functionalisation of heterocyclic nucleobases of nucleosides, but also nucleoside mono- and triphosphates, when water soluble catalytic system is used.[46] The very first example of alkylation of pyrimidine nucleotides taking place in aqueous media utilizing palladium catalysis was reported in 1990,[47] where Sonogashira reaction proceeded in acetonitrile/water mixture with propargylamine in good yields. Later on, modification of 5-iodouridine nucleotides with fluorescent labels by Sonogashira cross-coupling reaction was reported, where $Pd(OAc)_2$/TPPTS [tris(3-sulfonatophenyl)phosphine trisodium salt] catalytic system was described for the first time.[48] Suzuki-Miyaura reaction can also be performed in

aqueous media as reported by Davis for modification of short iodinated oligonucleotides.[49] The scope of different acetylenes and boronic acids was broadened by the work of Hocek group, where this straightforward way of synthesis of modified nucleotides was studied extensively.[50,51] In general the reactions are performed in the presence of $Pd(OAc)_2$/TPPTS and base in aqueous acetonitrile at elevated temperatures, where short reaction time (≥1 hour) is crucial to minimise the degree of hydrolysis of the triphosphate moiety. The possibility of using Heck reaction, where the modification would be attached by a double bond, was also explored; however the use of this approach is limited to reactions of purine nucleotides (Scheme 6).[52]



Scheme 6. a) General depiction of aqueous cross-coupling reaction of iodinated nucleotide. b) Selected examples of functional groups attached to nucleobases by Suzuki-Miyaura, Sonogashira or Heck aqueous coupling performed in Hocek group.

1.3.2 Modified nucleoside triphosphates as substrates for DNA polymerases

Similarly to ON synthesis on solid support, also enzymatic synthesis has its limitations. Although it proceeds under mild conditions and tolerates a large number of functional groups without the need for protection, the location of the attachment of the modification is crucial for successful incorporation of modified nucleotides. DNA polymerases generally do not recognise nucleotides bearing modification on the sugar moiety or the phosphate as substrates.[53] The modification therefore has to be located at the nucleobase. Position 5 of pyrimidines and position of 8 of purines seems like the obvious choice at first glance (these positions are not essential for successful Watson-Crick). However, position 8 of purines was deemed unsuitable for modification, since it has been shown that that bulky modifications (methyl and bromo derivatives still work in the enzymatic synthesis) prevent the typical anti conformation between sugar hydrogens and nucleobase which in turn prevents these nucleotides to be recognised as substrates by DNA polymerases (Figure 3a).[54] Therefore the modification is usually attached at the position 7 of 7-deazapurines (nitrogen at position 7 of purines is replaced by carbon).[50,51] It has also been reported that some N^4-modified cytosine dNTPs worked reasonably well as substrates of some DNA polymerases (Figure 3b).[55–57] The choice of DNA polymerase also has to be taken into consideration, since DNA polymerases from sequence family B process modified nucleotides more efficiently than enzymes from family A.[58,59]

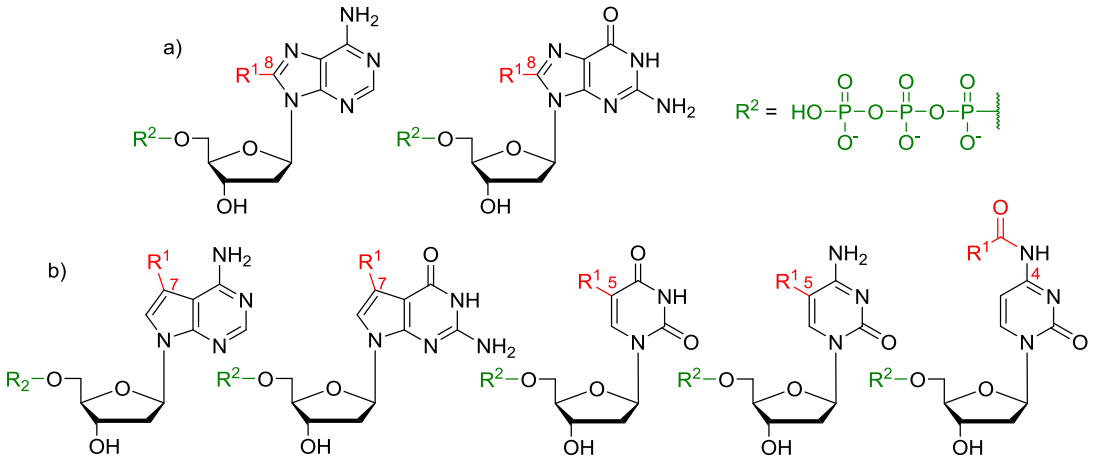


Figure 3. Structures of modified nucleotides generally a) not recognised and b) recognised as substrates for DNA polymerases in enzymatic synthesis of modified DNA.

Placement of modifications at position 5 of pyrimidines or position 7 of purines ensures that this modification is pointing out to the DNA major groove and these

nucleotides are generally good substrates for DNA polymerases. Extensive structural studies have shown the ability of DNA polymerases to accommodate different modification inside their active sites.[60] Some modified nucleotides possessing π-electron rich modifications have been described as even better substrates as their natural counterparts due to increased π-π stacking.[61,62] In general, even bulky modifications, such as fluorescent dyes or biotin, are well accepted, when a long flexible linker is used for the attachment to the nucleobase[63] (this approach is used for the design of fluorescently labelled reversible terminator nucleotides used in next-generation sequencing).[64–66]

On the other hand, information about modification of DNA minor groove is scarce in the literature. Since minor groove in B-DNA structure is quite narrow, presumed attractiveness of modifying of this DNA region is limited. Moreover, base-modification, which works well in case of major groove modified DNA, could interfere with Watson-Crick base-pairing. Therefore this type of DNA modification is not described well and only a handful of examples can be found. Richert et al. published a study showing that, when thymidine was replaced by 2-ethynylpyridone-C-nucleoside, the overall stability of the DNA duplex was increased thanks to additional Van der Waals interaction with adenine.[67] Another study showed that DNA modified by imidazolylpropyl group attached at the position 2 of purines also stabilised the DNA.[68] Chemical synthesis of ONs bearing a 2'-*O*-propargyl ribonusleoside was used for post-synthetic modification of DNA minor groove with fluorescent labels in CuAAC.[69,70] N^2-furfuryl-2'-deoxyguanosine was used to study the possible structural changes of B-DNA structure caused by minor-groove modifications (Figure 4).[71]

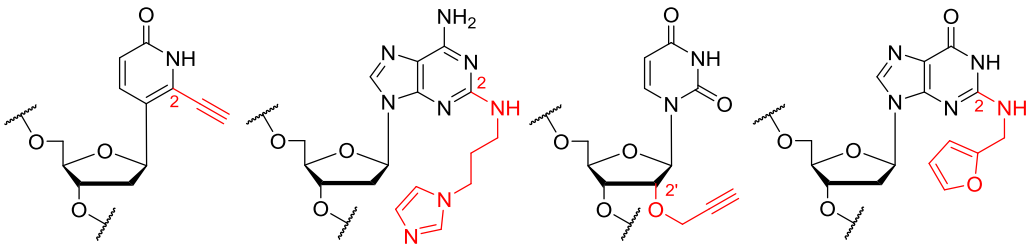


Figure 4. Structures successfully incorporated into DNA in chemical synthesis resulting in minor-groove modified DNA.

Minor-groove modified DNA was prepared enzymatically mostly in case sugar modified nucleotides were used for the enzymatic synthesis. Wagenknecht et al. reported that 2'-*O*-propargyl modified ribonucleotides were successfully incorporated into DNA by Therminator DNA polymerases and the propargyl group was utilised for

post-synthetic fluorescent labelling.[72] Ribonucleotides modified at the 2' hydroxyl were successfully used for enzymatic synthesis of DNA also by the group of Veedu.[73] Recently Floyd Romesberg reported evolution of a thermostable DNA polymerase able to incorporate ribonucleotides and 2'-*O*-modified ribonucleotides into DNA with great efficiency.[74] 4'-Modified nucleotides were also used for enzymatic synthesis of DNA,[75] although these served as chain-terminating nucleotides. This ultimately led to studies of incorporation of 2',4'-dimodified nucleotides[76] and 2',4'-bridged nucleoside triphosphates (Figure 5a).[77]

The only examples of minor-groove base-modified modified DNA involved the 2-chloro-[78] (**10**) and 2-amino-2'-deoxyadenosine triphosphate[79,80] (**11**), which were substrates for DNA polymerases in enzymatic synthesis of DNA. On the other hand, 2-arylaminoadenosine triphosphates (e.g. **12**) acted as inhibitors of these enzymes (Figure 5b).[81,82]

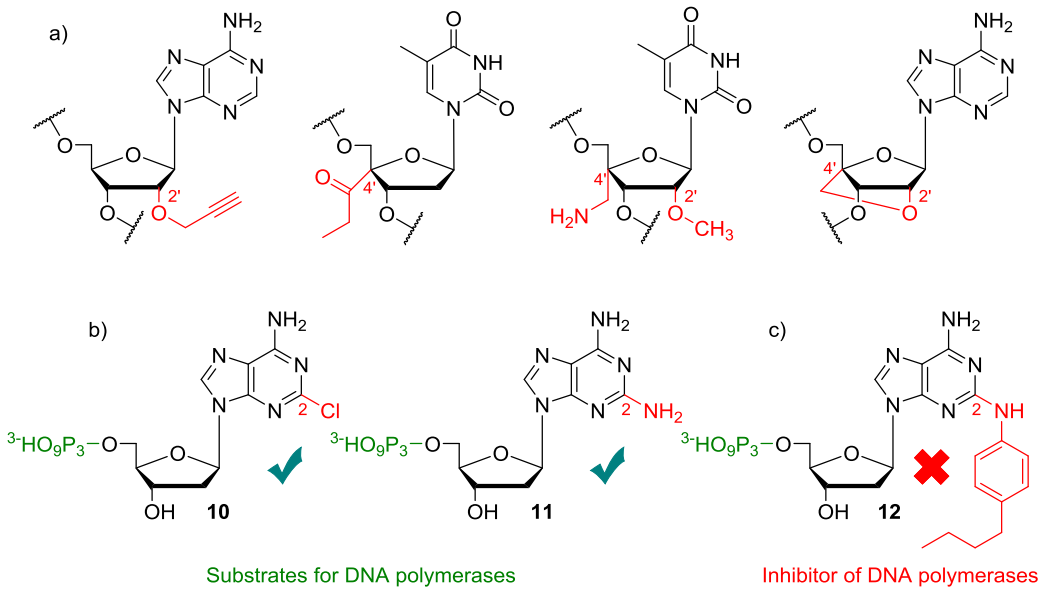
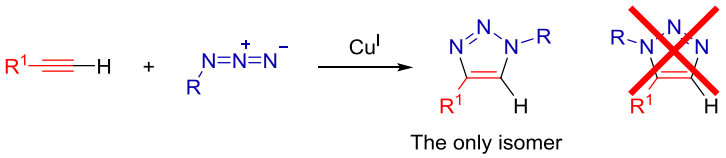


Figure 5. a) Sugar-modified structures successfully incorporated into DNA by DNA polymerases. b) Modified nucleotide triphosphates **10** and **11** successfully incorporated into DNA. c) Structure of 2-(*p*-butylphenylamino)-2'-deoxyadenosine triphosphate **12** which is an inhibitor of DNA polymerases.

1.4 Post-synthetic modification of DNA

In case base-modified dNTPs do not serve as substrates for DNA polymerases in enzymatic synthesis of DNA and the modifications are not compatible with the conditions of chemical synthesis, post-synthetic modification of DNA might be an advantageous approach for the introduction of additional functionalities into DNA.[83] In that case, DNA should be synthesised (either chemically or enzymatically) bearing small reactive modifications and the attachment of the desired functional group is done subsequently. The reactions have to comply with certain specifications for this approach to be possible. The small reactive handles have to react chemoselectively with the reactive group of the desired label and they have to be inert towards other functionalities they might come in contact with (found in DNA or even in other biomolecules). The reactions should ideally proceed in water with fast reaction rates and they should run under mild conditions with low concentration of reagents and give the desired products in high yields.[84]

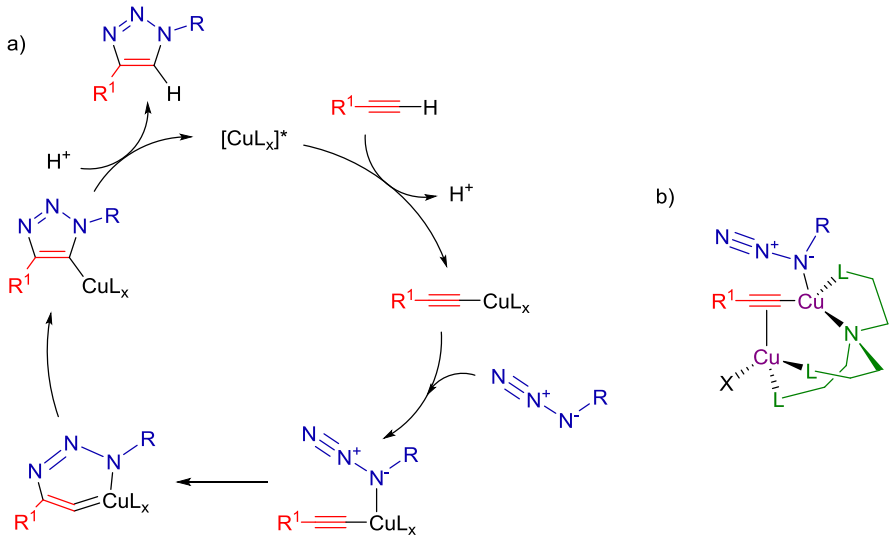
Over the past years, the CuAAC (copper-catalysed alkyne-azide cycloaddition) has become the method of choice for the post-synthetic modification of ONs. Huisgen originally described the uncatalysed variant of this triazole yielding reaction,[85] however the reaction became extremely popular only after the catalysed version of the reaction was introduced by Meldal[86] and Sharpless.[87] The Cu^{I} catalysis promotes regioselectivity of the reaction and increases the reaction rate (Scheme 7). Sharpless also described the reaction to work in aqueous media, which is necessary when thinking about performing reactions on nucleic acids.



Scheme 7. Copper-catalysed alkyne-azide cycloaddition with the 1,4-disubstituted triazole being the sole product of the reaction.

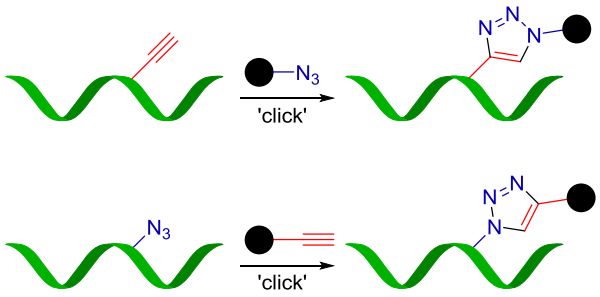
The drawback of the presence of Cu^{I} ions, which might cause formation of oxidised side products, was solved by development of Cu^{I} chelating ligands like tris(benzyltriazolylmethyl)amine (TBTA)[88] and its water-soluble derivatives.[89] Single[90] and bimetallic[91] mechanisms have been proposed based on computational studies and the reaction in the presence of tris(triazolylmethyl)amine ligands is also

thought to proceed through an intermediate with two copper ions,[92] however the definitive mechanism has still not been agreed upon (Scheme 8).



Scheme 8. a) Proposed single metallic mechanism of CuAAC reaction. b) Bimetallic intermediate formed in the presence of copper binding ligands.

CuAAC, fittingly deemed "click" reaction,[93] has been used extensively for post-synthetic modification of nucleic acids thanks to its versatility (Scheme 9).[94,95] Variety of building blocks bearing either azido or ethynyl group used in chemical or enzymatic synthesis of modified ONs has been described up to date (Figure 6). Ethynyl group attached directly to a nucleobase[96,97] or the 1,7-octadiynyl group[98] as a source of the triple bond seem to be the most popular choices for modified DNA. Recently Hocek group reported that the reaction proceeds faster when longer linkers are used to attach the ethynyl moiety to a nucleobase.[99] Modified RNA has been prepared having a propargyloxo[69,100] or an azido[101,102] group at the 2' position. Azido group, long overlooked due to presumed incompatibility with conditions of chemical synthesis of ONs (P^{III} oxidation step), has been installed into DNA both by direct attachment to a nucleobase (after nucleophilic aromatic substitution)[103] or *via* a longer linker.[104] Notably, 5-ethynyl-2'-deoxyuridine[105] (and later other nucleosides)[106–108] have been used for genomic labelling of DNA after successful phosphorylation by cellular kinases, incorporation into DNA and subsequent click reaction with a fluorescent label.



Scheme 9. CuAAC of either ethynyl or azido modified nucleic acid with the corresponding reagent.

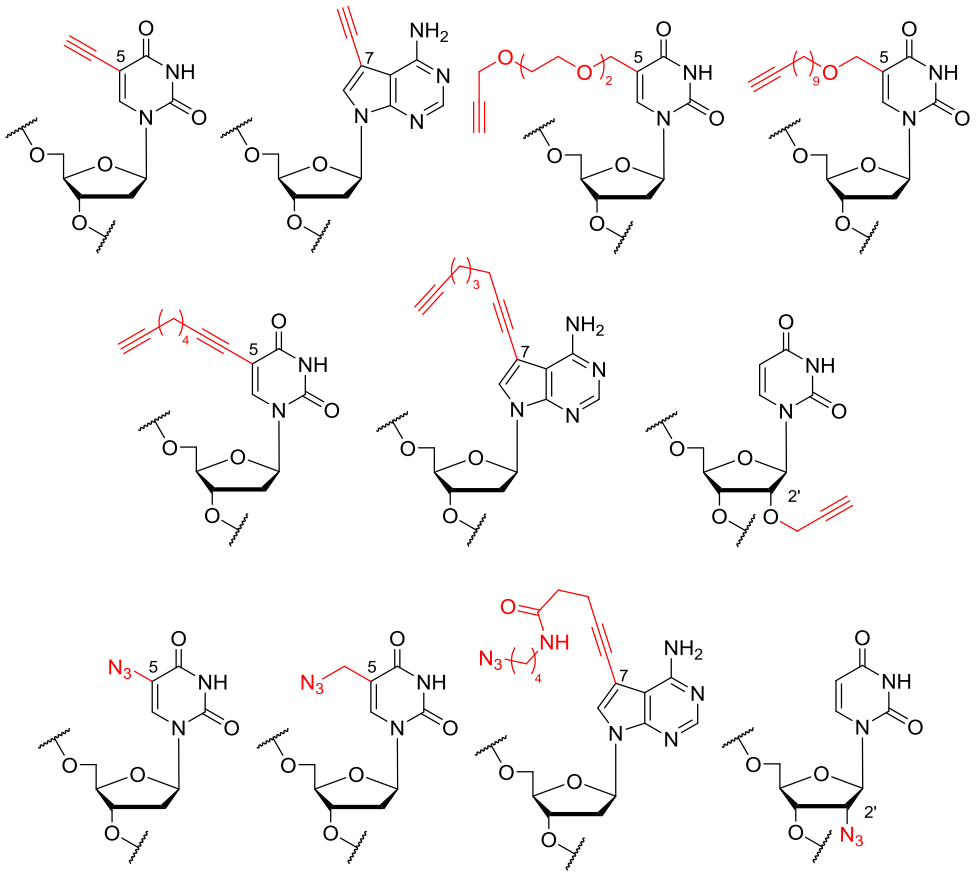


Figure 6. Selected examples of ethynyl or azido modified nucleosides that were incorporated into nucleic acids and used for post-synthetic modification.

Although CuAAC definitely has its advantages, there is always a need for alternative reactions (in the case of CuAAC especially, since any application of this reaction *in vivo* is problematic due to toxicity of copper ions).[109] The copper-free variant of this reaction (strain-promoted azide-alkyne cycloaddition; SPAAC) utilises the reactivity of strained cyclooctynes[110] and was developed as a bioorthogonal reaction by Bertozzi.[111] Tuning of the reactivity of cyclooctynes allowed the reaction to proceed efficiently not only with azides, but also with nitrile-oxides and nitrones. To

this end, cyclooctynes were modified by the addition of electron-withdrawing groups (**13**),[112] dibenzoannulation (**14**),[113] fusion with cyclopropane ring (**15**)[114] and by monobenzoannulation (**16**)[115] in order to battle the lipophilicity of the compounds (Figure 7a). Multiple examples of this methodology being used for post-synthetic nucleic acid modification have emerged with the cyclooctyne-modified DNA and RNA being prepared mostly by phosphoramidite chemistry.[116–118] SPAAC has also been described for modification of azido-modified nucleic acids with cyclooctyne-based reagents (Figure 7b,c).[108,119]

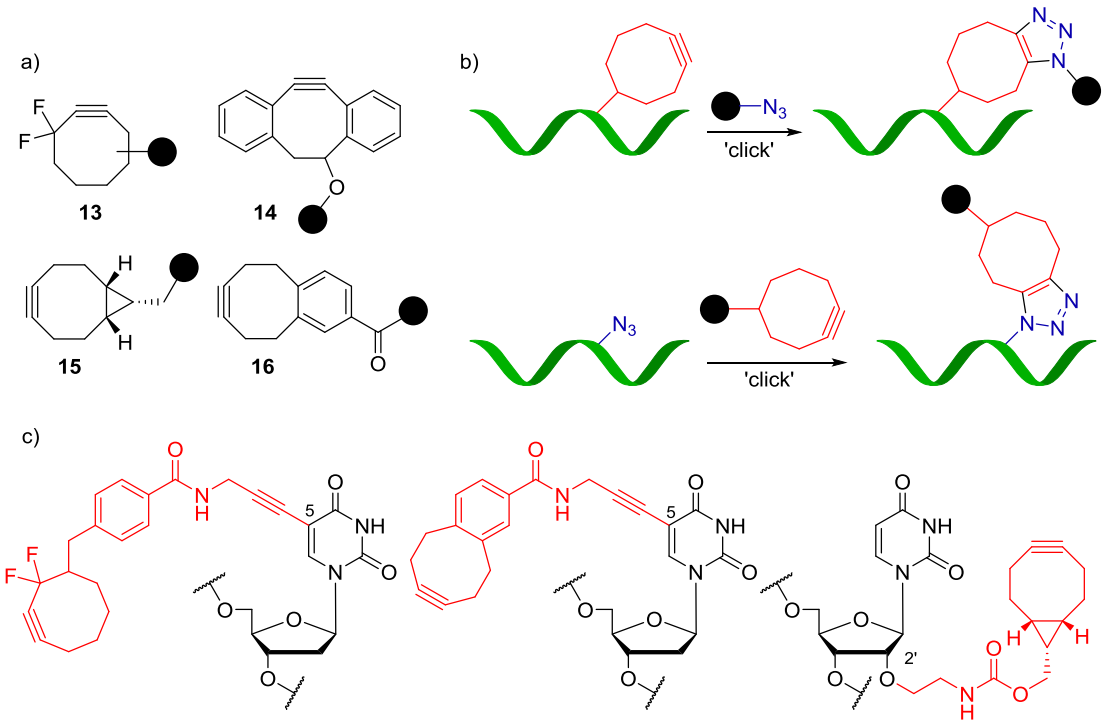


Figure 7. a) Selected cyclooctynes with increased reactivity in SPAAC. a) SPAAC of either cyclooctynyl or azido modified nucleic acid with the corresponding reagent. b) Selected examples of cyclooctynyl modified nucleosides that were incorporated into nucleic acids and used for post-synthetic modification.

Other cycloaddition reactions, not only between alkynes and azides, have been developed and reported for post-synthetic modification of nucleic acids. Wagenknecht described chemical synthesis of ON bearing a diaryltetrazole moiety, which reacted with dipolarophiles upon irradiation with 365 nm in a "photoclick" reaction (Scheme 10a),[120] which was later used for fluorescence DNA labelling.[121] This approach was lately used for *in vivo* fluorescence imaging of DNA.[122] Rentmeister et al. used the same approach for the attachment of a 5'-cap for RNA strands.[123] Crosslinking of aziridine-containing DNA upon irradiation, used to study DNA repair,[124]

methyltransferases[125] or human DNA polymerase β,[126] was also reported. Diels-Alder reaction was reported in the work of Howorka et al., who reacted buta-1,3-dienyl modified DNA with maleimides.[127] Most Diels-Alder reactions reported on nucleic acids are, however, of inverse-electron-demand nature (iEDDA, Scheme 10b).[128] The reaction proceeds the best with heteroatom containing dienes (such as tetrazines) and dienophiles of high HOMO energy, such as ring-strained molecules (norborenes, cyclopropenes, etc.).[129] iEDDA reaction of nucleic acids has been reported mostly for dienophile-labelled nucleic acids[130–133] due to incompatibility of tetrazines with the phosphoramidite protocol, although lately this has been accomplished as well.[134–136]

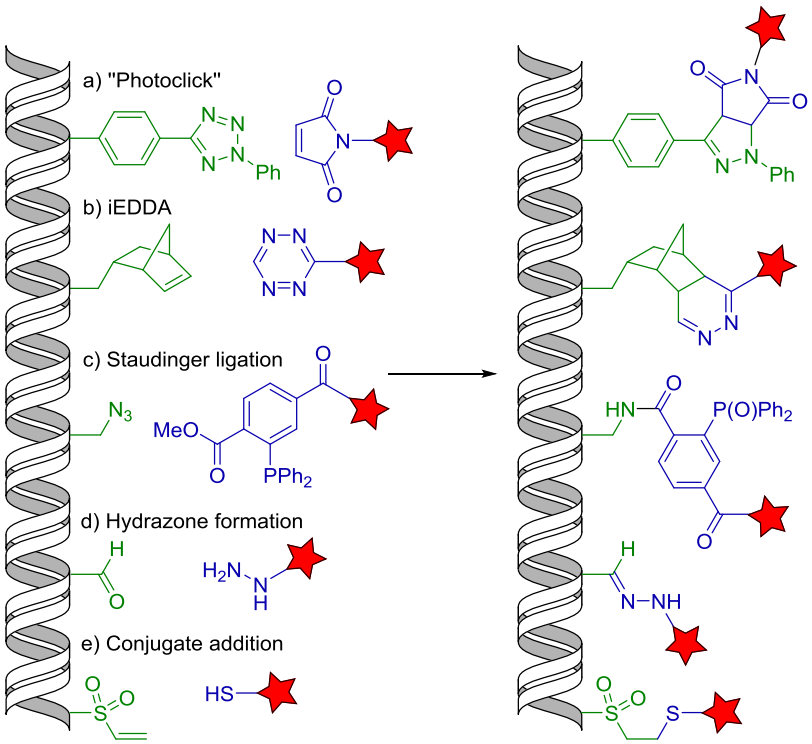
All of the reactions mentioned above are cycloaddition-based reactions. However, other important reactions used for post-synthetic nucleic acid modification are worth mentioning. The Staudinger ligation requires an azide and a triphenylphosphine as reaction partners (Scheme 10c), both of which are quite problematic with regards to chemical synthesis of ONs. This problem is circumvented in the literature by preparation of modified nucleotides bearing these moieties, which are then used in enzymatic synthesis.[137]

Transformation of aldehyde functions is used frequently for DNA modification, although aldehydes again suffer from reactivity issues in chemical ON synthesis. The early example reported by Sheppard described the attachment of a more stable ketone, which was used for further oxime formation.[138] 5-Formylcytosine has since been recognised as an epigenetic base[139] and has been shown to react with hydroxylamines,[140] even forming covalent cross-links between DNA strands.[141] Transformation of formyl-linked DNA has been studied also in Hocek group. The aldehyde function was incorporated into DNA by enzymatic synthesis using corresponding modified nucleotides. The formyl group was attached either by a thiophene linker, by a saturated carbon chain or even prepared *in vitro* by oxidative cleavage of a vicinal diol. Aldehyde-modified DNA was then used to form hydrazones (Scheme 10d) of redox or fluorescent labels, or bioconjugates with amino acids and peptides via reductive amination.[35,142,143]

Conjugate addition of thiol to vinylsulfoneamide has been used to trap DNA-binding protein through its cysteine residue (Scheme 10e)[33] and enzymatic incorporation of chloroacetamide moiety into DNA resulted in a covalent DNA-protein conjugate through either cysteine or histidine.[36] Squaramate-linked DNA has been used to react with lysine and amino-group-rich histone.[34] On the other hand, cysteine

modified DNA was used to react with a short peptide with maleimide functionality at the N terminus.[144] Preparation of N-hydroxysuccinimide ester modified DNA allowed for the attachment of carbohydrates onto the DNA duplex.[145]

Aqueous cross-couplings are used not only for modification of nucleosides and nucleotides,[51] but also for post-synthetic modification of DNA containing halogeno-substitutions on the nucleobases. Iodo-functionalized DNA was reported to react in Suzuki,[49,146] Stille[147] and Buchwald-Hartwig-Migata[148] coupling reactions.



Scheme 10. Post-synthetic modification of DNA via a) photoclick reaction, b) inverse-electron-demand Diels Alder reaction, c) Staudinger ligation, d) hydrazine formation and e) Michael conjugate addition.

1.5 DNA-protein interactions

DNA-binding proteins are an important part of many cellular mechanisms. Transcription binding factors (responsible for transcription of gene into RNA), polymerases (synthesis of nucleic acid chains), nucleases (cleavage of nucleic acids) or histones (packing of chromosomes inside cellular nucleus) are all examples of DNA-binding proteins. The proteins bind to DNA thanks to its unique structure, which offers DNA functional groups to the outside environment via major and minor groove. Sequence-specific DNA-binding proteins, such as transcription factors or nucleases, require a specific sequence to be recognised as a binding site for the enzymes. Most of the sequence-specific enzymes bind to DNA through interactions in the DNA major groove,[5] however there are a few examples of minor-groove binding proteins[149] and ligands.[150] Upon closer look at the DNA-protein binding motifs, such as helix-loop-helix,[151] leucine zipper,[152,153] zinc finger[154,155] or helix-turn-helix (Figure 8),[156,157] it can be seen that the binding is secured mostly by hydrogen bonding interactions, but also by local conformation and configuration of the DNA molecule.[158]

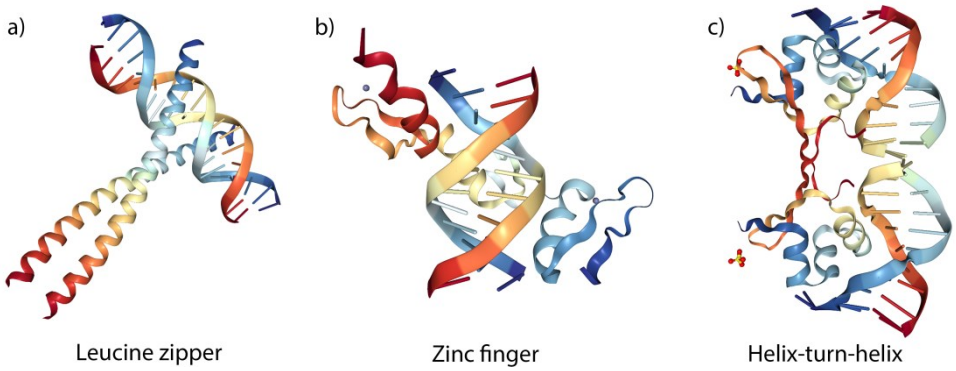
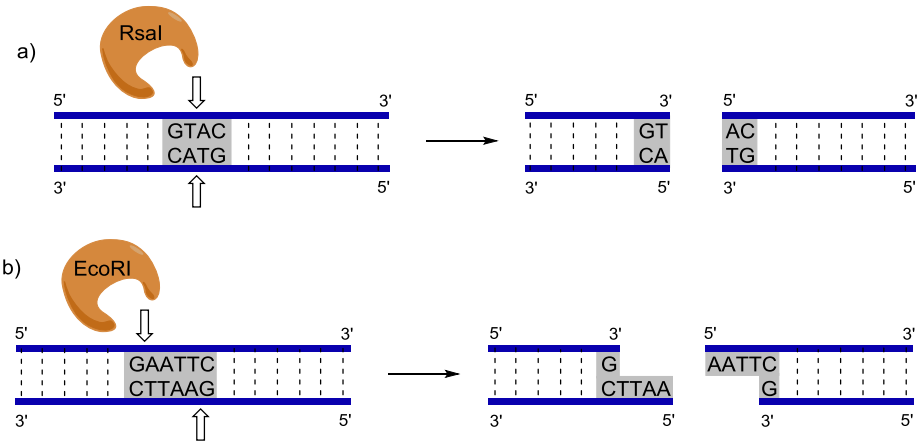


Figure 8. Different types of DNA-protein binding motifs. a) Leucine zipper, b) zinc finger, c) helix-turn-helix. Images taken from the available PDB data.[153,155,157]

1.5.1 Restriction endonucleases

Restriction endonucleases (REs) are sequence-specific DNA-binding enzymes that seek out and bind to short palindromic sequences of DNA (usually 4-8 bp), resulting in DNA cleavage leaving single-stranded DNA blunt ends or overhangs (Scheme 11). They represent a protection mechanism of bacteria against foreign (bacteriophage) DNA.



Scheme 11. Cleavage of DNA by type II restriction endonucleases leaving a) blunt ends or b) overhangs.

REs have been studied extensively throughout 1960s and 70s, which ultimately led to a Nobel Prize in Physiology or Medicine awarded to Werner Arber, Hamilton Smith and Daniel Nathans "for the discovery of restriction enzymes and their application to problems of molecular genetics" in 1978. Nowadays, more than 5000 REs have been described according to REBASE.[159] They are divided into four main groups based on their properties (mode of action, site specificity, subunit composition, etc., Table 1).[160]

Table 1. Description of four types of restriction enzymes.

Type	Type I	Type II	Type III	Type IV
Characteristic	Multi subunit complex, ATP required for cleavage	Separate RE	Bifunctional complex of 2 subunits	Methylation-dependant REs
Cleavage site	Variable, often far from recognition site	Specific within the recognition site	Random 24-26 bp from the recognition site	Cleave randomly outside the recognition site
Typical examples	EcoAI, CfrAI	EcoRI, RsaI	EcoPI15I, HinfIII	EcoKMcrBC

REs soon became indispensable tools for DNA cutting in gene analysis and cloning.[161] Type II REs are the most deeply studied, with >200 of them

commercialised. Since most DNA-binding enzymes bind to DNA through interactions in the major groove, the effect of major-groove modification on cleavage of DNA by REs was studied, although not consistently.[162–165] Lately however, an extensive studies by Hocek group evaluated the effect of major-groove modification of RE cleavage.[166–169] REs tolerated some small modifications attached to uracil and adenine bases, while bulky modifications inhibited the cleavage. On the other hand, no modifications were allowed to be attached to either cytosine or guanine base, suggesting that G:C pairs seem to be more important than A:T pairs for the recognition and cleavage of DNA (Figure 9).

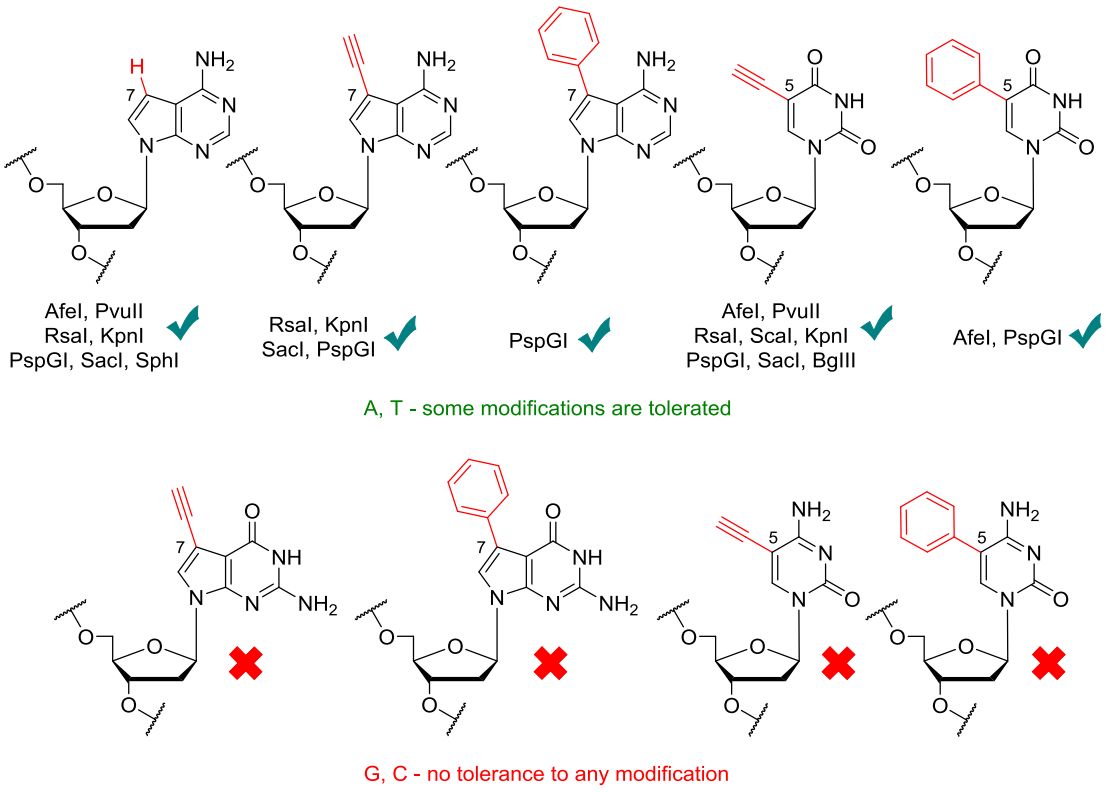
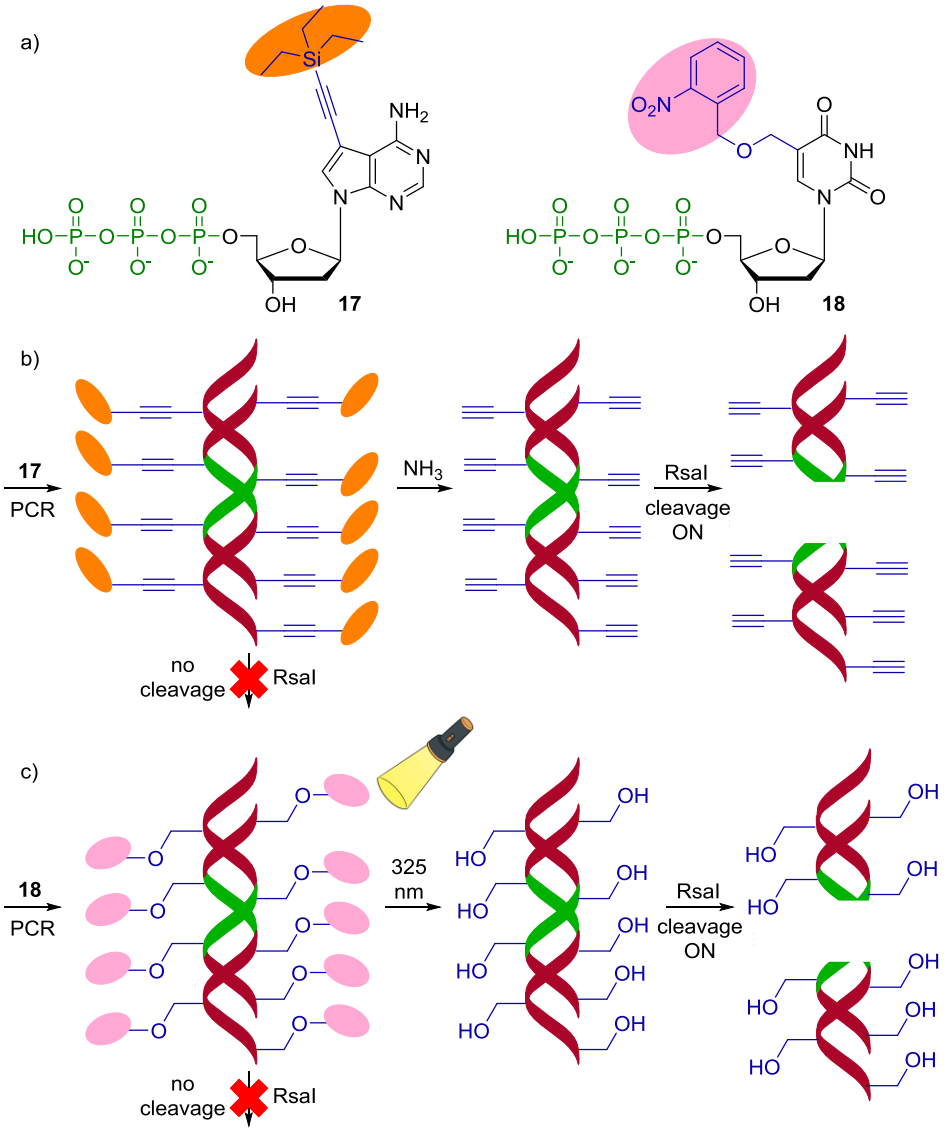


Figure 9. Modified adenosines and thymines that allowed cleavage of DNA by REs and structures of modified guanosines and cytidines that inhibited the cleavage.

Soon thereafter, a transient chemical protection of from RE cleavage was developed, where 7-(triethylsilylethynyl)-7-deaza-2'-deoxyadenossine triphosphate **17** was efficiently incorporated into DNA (Scheme 12a). The bulky group completely inhibited the cleavage of the DNA by RsaI, whereas upon cleavage of the silyl groups by aqueous ammonia, the ethynyl-modified DNA was cleaved efficiently (Scheme 12b).[170] Later, a more biocompatible approach was described, where modified DNA was prepared by incorporation of 5-nitrobenzyloxymethyl-2'-deoxyuridine triphosphate

18 (Scheme 12a). While no cleavage was observed for this photocaged DNA, after irradiation of the DNA by 365 nm light and release of hydroxymethyl-modified DNA, several REs readily cleaved the DNA (Scheme 12c).[171] In an effort to make this approach viable for *in cellulo* or *in vivo* applications, DNA photocaged by groups cleavable by light of higher wavelengths was also prepared and successfully used to demonstrate a way of turning on of the RE cleavage.[172,173] Notably, the same approach was later used to make an artificial switch for bacterial transcription.[174] Modification of DNA by acyl groups has also been used to protect the DNA against RE cleavage.[57]



Scheme 12. a) Structures of modified nucleotides bearing cleavable groups. Protection of DNA against cleavage by REs by b) bulky triethylsilyl group or c) photocleavable nitrobenzyl group and subsequent turn on of the cleavage upon deprotection.

Compared to the extensive studies on the effect of major-groove modifications on the cleavage by REs, the effect of minor-groove modifications has been studied more scarcely. It has been shown for a number of REs that DNA containing 2,6-diaminopurine or hypoxanthine is cleaved at a slower rate compared to non-modified DNA.[79,175–177] Cosstick et al. showed that the 3-deazaadenosine derivative also had an inhibiting effect on *EcoRV*.[178] The alkylation of the minor groove sites of nucleobases also caused inefficient cleavage, most probably due to changes in the binding mode of the RE.[179,180] Williams et al. studied the effect of 2'-fluorothymidine on the interaction with *EcoRV* and concluded that the local conformational changes induced by the introduction of the fluorine atom are the cause of the partial inhibition of the cleavage.[181] On the other hand, presence of riboadenosine inside the DNA strand almost completely inhibited the cleavage by *EcoRI*.[182]

2 Specific aims of the thesis

1. Design and synthesis of 2-substituted 2'-deoxyadenosine triphosphates and evaluation of their ability to act as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA.

2. Synthesis of 2-alkylamino-2'-deoxyadenosine triphosphates and their use in enzymatic synthesis of site-specifically minor-groove modified DNA.

3. Synthesis of 2-modified 2'-deoxyinosine triphosphates as guanosine nucleotide surrogates and their use in enzymatic synthesis of minor-groove modified DNA.

4. Study of post-synthetic transformations of variously minor-groove modified DNA using click reactions.

5. Evaluation of the influence of minor-groove modifications on the ability of type II restriction endonucleases to cleave the modified DNA sequences.

2.1 Rationale of the specific aims

Enzymatic synthesis of (base-)modified DNA is now an established alternative of chemical synthesis of nucleic acids.[25] Typically the modification is attached at the position 5 of pyrimidine or position 7 of 7-deazapurine nucleotides.[50,51] This ensures that the modification is pointing out into the DNA major groove and the corresponding nucleotides are generally good substrates for DNA polymerases. Enzymatic synthesis of minor-groove modified DNA has not been studied extensively, only being reported for sugar-modified nucleotides.[72–77] My task was therefore to synthesise a series of 2-modified 2'-deoxyadenosine triphosphates and test them as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA.

Installation of minor-groove modification into the DNA strand would inherently cause destabilisation of the DNA duplex, since the modification is likely to affect or interfere with Watson-Crick base-pairing. 2-Alkylamino modified dATPs, having a flexible alkylamino chain attached to the nucleobase, could in principle compensate for the possible steric effect of the modification by an additional hydrogen bond with thymine nucleobase. These compounds on the other hand could interfere with minor-

groove interactions of DNA and DNA polymerase, essential during DNA synthesis.[183] One of the aims of the thesis was to test them as potential substrates for enzymatic synthesis with a potential for site-specific DNA minor-groove labelling.

2-Substituted dATP derivatives are the obvious choice for the testing of the enzymatic synthesis of minor-groove modified DNA, since position 2 of adenine does not directly take part in Watson-Crick base-pairing. The question was whether also some analogues of guanine base can be used. Therefore my next task was to design and synthesise 2-substituted 2'-deoxyinosine triphosphates in order to be tested as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA.

Post-synthetic DNA modification is a viable option, when other means for modifying of DNA are unavailable.[83] With the expected size limitations of the substituents able to be directly installed into the DNA minor groove via enzymatic synthesis in mind, we envisaged click reactions as means of subsequent post-synthetic transformations of DNA.

Type II restriction endonucleases (REs) are widely used in biochemistry and molecular biology for site-specific cleavage of dsDNA.[161] It is known that REs recognise and bind to DNA through interaction in the major groove and the effect of the major-groove modifications on the RE cleavage has been studied extensively.[166–169] On the other hand, it remains unclear whether minor-groove modifications could also affect and modulate the cleavage of DNA by REs. My task was therefore to synthesise DNA modified in the recognition site of REs by minor-groove modifications and evaluate the effect these modifications have on the RE cleavage.

3 Results and discussion

3.1 Preparation of 2-substituted 2'-deoxyadenosine triphosphates and testing of their viability as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA

Base-modified nucleic acids are important tools in chemical biology, material science or diagnostics.[25] ONs or DNA bearing diverse substituents has been prepared and used for different applications. Chemical synthesis on solid support or enzymatic synthesis using DNA polymerases can be used for the construction of these modified nucleic acids. In the case of enzymatic synthesis, the corresponding building blocks [(modified nucleoside triphosphate (dNTPs)] have to be modified accordingly. The modification is usually attached at the position 5 of pyrimidines and position 7 of 7-deazapurines. This ensures that the modified nucleotides are generally good substrates for DNA polymerases and that the modification is pointing out into the major groove of DNA and thus not interfering with the crucial Watson-Crick hydrogen bonding. This approach therefore leads to the construction of major-groove modified DNA and its viability has been proven both empirically and by structural studies of DNA-DNA polymerase complexes.[50,51,60]

On the other hand, information about modification of DNA minor groove is quite scarce in the literature. Minor-groove modified DNA has been prepared mostly chemically[67–71] and enzymatic synthesis is described mostly using sugar-modified nucleotides.[72,73,75–77] The only base-modified triphosphates used for the synthesis of minor-groove modified DNA were 2-chloro- (**10**)[78] and 2-amino-2'-deoxyadenosine triphosphate (**11**).[79,80] It seems that position 2 of adenine seems to be an ideal choice for modification, since this position is not directly taking part in Watson-Crick base-pairing, although it was not entirely clear, whether it might still not interfere with the base-pairing itself (Figure 10). In addition to the already known chloro- and amino-modified nucleotides, we chose to prepare four more compounds bearing substituents of increasing bulkiness (methyl, vinyl, ethynyl and phenyl) in order to determine the effect of the size of the modification on the ability of DNA polymerases to recognise these compounds as substrates in enzymatic synthesis of minor-groove modified DNA.

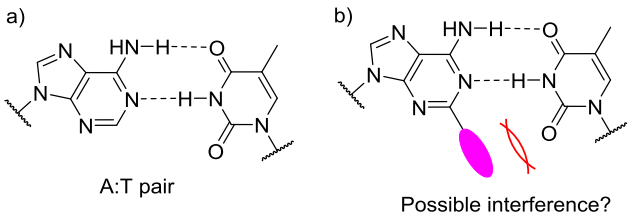
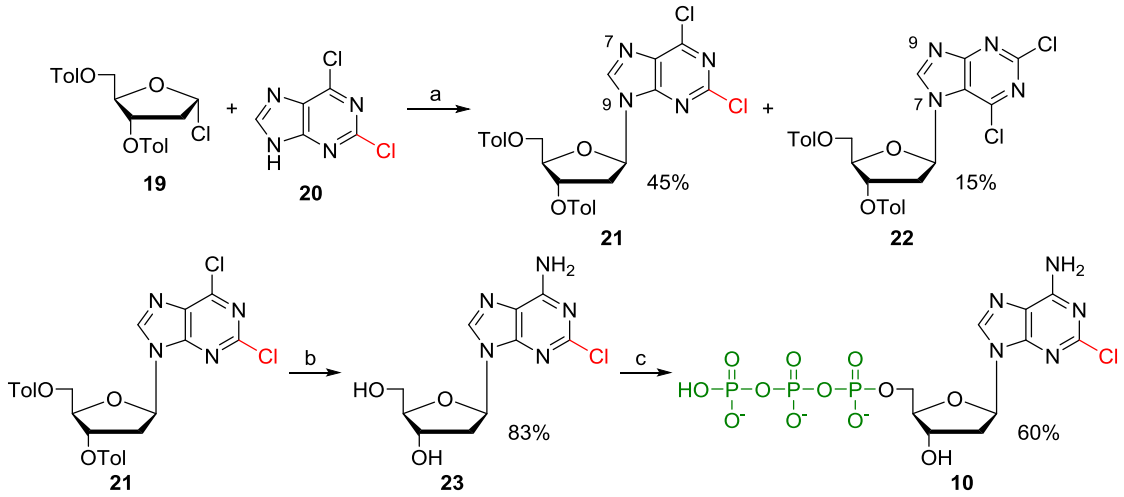


Figure 10: Watson-Crick base-pairing of A:T pair a) normally present in DNA or b) being possibly interfered with in the presence of 2-modified adenine.

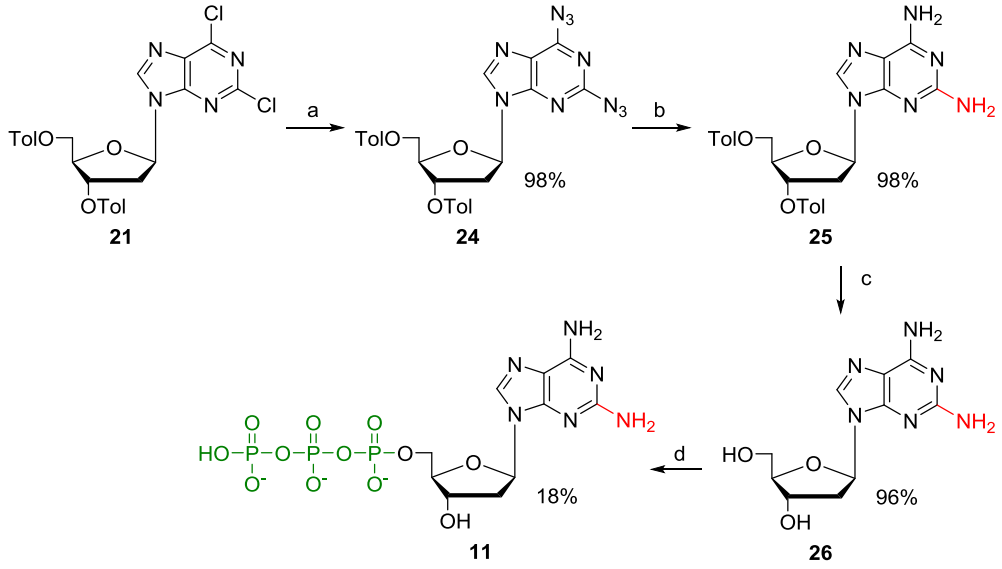
3.1.1 Synthesis of 2-modified 2'-deoxyadenosine triphosphates ($d^{R}ATPs$)

In order to prepare the required nucleotides, Yoshikawa's phosphorylation method can be used (Chapter 1.3.1).[43,44] For this, the parent 2'-deoxynucleosides need to be synthesised. The synthesis of 2-chloro modified nucleoside **23** started by following a known glycosylation procedure[184] using toluoylated halogenose **19**[185] and commercial 2,6-dichloropurine **20** in acetonitrile and NaH as a base. The desired protected nucleoside **21** was isolated in 45% yield together with N-7 glycosylated regioisomer **22** (15%). Nucleoside **21** was then transformed into 2-chloro modified adenosine **23** using methanolic ammonia in 83% yield.[185] Phosphorylation of **23** using $POCl_3$ in trimethyl phosphate followed by the addition of tributylammonium pyrophosphate and Bu_3N in DMF and subsequent quenching of the reaction using TEAB solution (Yoshikawa's method) afforded the desired triphosphate **10** ($d^{Cl}ATP$) in 60% yield (Scheme 13).



Scheme 13. Synthesis of $d^{Cl}ATP$. Reaction conditions: a) **20**, NaH, CH_3CN, rt, 30 min; then **19**, rt, overnight; b) NH3 in MeOH, 100 °C, 5 h; c) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min.

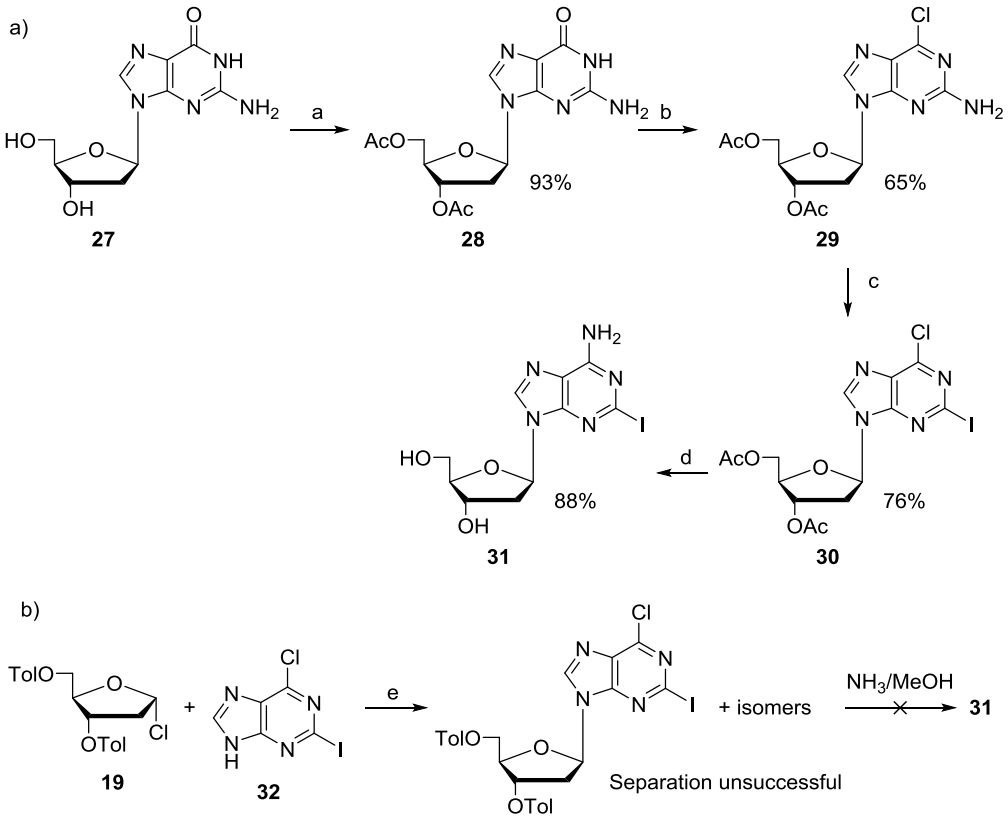
Protected nucleoside **21** was then also used in the preparation of the 2-amino modified adenosine **26**. Thus, aromatic nucleophilic substitution of chlorine atoms by azido groups proceeded with NaN_3 in $EtOH/H_2O$ mixture and the resulting nucleoside **24** was prepared in almost quantitative yield (98%). Diaminopurine bearing nucleoside **25** was prepared by reduction of the azido groups of **24** by gaseous H_2 using 10% palladium on charcoal in EtOH/DMA mixture in 96% yield. Final cleavage of the ester protecting groups by K_2CO_3 in MeOH afforded the desired (known)[186] nucleoside **26** in 96% yield. 2-Amino modified triphosphate **11** (**$d^{NH_2}ATP$**) was prepared following the same procedure as described above (Yoshikawa's phosphorylation) starting from nucleoside **26** in satisfactory 18% yield (Scheme 14).



Scheme 14. Synthesis of **$d^{NH_2}ATP$**. Reaction conditions: a) NaN_3, $EtOH/H_2O$, reflux, 30 min; b) H_2 (g), Pd/C 10%, EtOH/DMA, rt, 24 h; c) K_2CO_3, MeOH, rt, 1 h; d) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min.

The synthetic strategy for the preparation of the remaining nucleosides consisted of performing a series of cross-coupling reactions starting from a common intermediate. Thus, commercially available 2'-deoxyguanosine **27** was first acetylated using Ac_2O, Et_3N and DMAP in acetonitrile in 93% yield and then chlorination of protected guanosine **28** using $POCl_3$, dimethylaniline and benzyl(triethyl)ammonium chloride in acetonitrile afforded the purine nucleoside **29** in 65% yield. These reaction steps were performed following procedures described in the literature.[187] Next, the iodo-deamination using CH_2I_2, I_2, CuI and isopentyl nitrite in THF gave the 2-iodo nucleoside **30** in the yield of 76%. Finally, treatment of **30** with methanolic ammonia at elevated temperature led to the common intermediate 2-iodo adenosine **31** in 88% yield

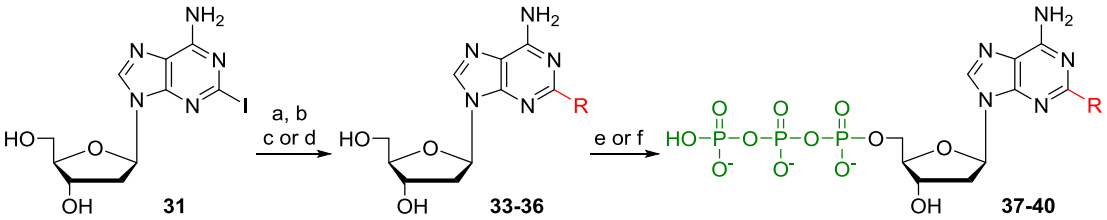
(Scheme 15a). Alternatively, iodinated nucleoside **31** can be prepared following a literature procedure of glycosylation using 6-chloro-2-iodopurine **32** and halogenose **19** and subsequent deprotection and amination reaction.[187] However, in our hands this approach led to a mixture of isomers (regioisomers or even diastereomers) where these isomers inseparable using chromatography (Scheme 15b). Additionally, since the preparation of purine **32** requires three steps starting from commercial 2-amino-6-chloropurine,[188] the procedure for the preparation of **31** starting from **27** is faster and easier.



Scheme 15. Syntheses of 2-iodoadenosine **31**. a) Successful synthesis starting from 2'-deoxyguanosine and b) unsuccessful attempt at the literature procedure. Reaction conditions: a) Ac_2O, Et_3N, DMAP, CH_3CN, rt, 3 h; b) $POCl_3$, dimethylaniline, benzyl(triethyl)ammonium chloride, acetonitrile, reflux, 10 min; c) CH_2I_2, I_2, CuI, isopentyl nitrite, THF, reflux, 45 min; d) methanolic ammonia, 100 °C, 5 h; e) **32**, KOH, tris[2-(2-methoxyethoxy)ethyl]amine, acetonitrile, rt, 5 min; then **19**, rt, 1 h.

Iodinated nucleoside **31** was then used as a substrate in a series of cross-coupling reactions. Reaction of **31** with trimethylaluminium, $Pd(PPh_3)_4$ in THF afforded the methyl nucleoside **33** in 70% yield. Suzuki-Miyaura coupling of **31** with either phenylboronic acid or potassium vinyltrifluoroborate in the presence of $Pd(OAc)_2$, trisodium salt of tris(3-sulfonatophenyl)phosphine (TPPTS) and Cs_2CO_3 in aqueous

acetonitrile afforded the 2-phenyl (**34**) and 2-vinyl (**35**) derivatives (91% and 80% respectively). Lastly, Sonogashira coupling of **31** proceeded with trimethylsilyl acetylene, $PdCl_2(PPh_3)_2$, CuI and Et_3N in DMF followed by treatment with methanolic ammonia[187] to give the desired product **36** in the yield of 79%. All of the prepared modified nucleosides were then subjected to phosphorylation reaction. The reaction was performed as described above for nucleosides **33** and **34** yielding the desired nucleotides **37** (**d^{Me}ATP**) and **38** (**d^{Ph}ATP**) (23% and 24%), while the conditions were modified for the preparation of 2-vinyl (**39**, **d^{V}ATP**) and 2-ethynyl (**40**, **d^{E}ATP**) modified nucleotides. Thus the reaction was performed with the addition of 1,8-bis(dimethylamino)naphthalene (proton sponge) in the first step of the phosphorylation reaction, upon which the 2-modified nucleotides **39** and **40** were obtained in respectable yields (22% and 31%) (Scheme 16). The reaction conditions and yields are summarised in Table 2. (Nucleosides **33**,[189] **35**[190] and **36**[187] have been previously synthesised).



Scheme 16. Synthesis of 2-modified adenosine nucleotides. Reaction conditions: a) Me_3Al, $Pd(PPh_3)_4$, THF, 60 °C, 3 h; b) $PhB(OH)_2$, Cs_2CO_3, TPPTS, $Pd(OAc)_2$, H_2O/MeCN, 80 °C, 1.5 h; c) potassium vinyltrifluoroborate, Cs_2CO_3, TPPTS, $Pd(OAc)_2$, H_2O/MeCN, 80 °C , 2.5 h; d) 1. trimethylsilyl acetylene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, DMF, rt, 3 h; 2. methanolic ammonia, rt, 1.5 h; e) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min; f) 1. proton sponge, $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min.

Table 2. Summarised results of the synthesis of remaining **d^{R}ATP**s.

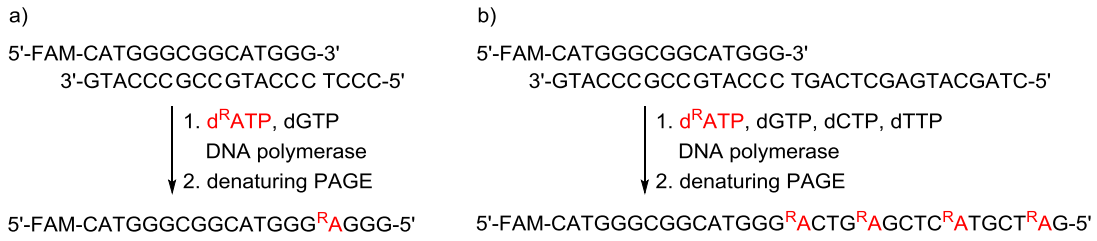
Entry	Product	R substituent	Conditions	Yield [%]
1	**33**	methyl	a	70
2	**34**	phenyl	b	91
3	**35**	vinyl	c	80
4	**36**	ethynyl	d	79
5	**37**	methyl	e	23
6	**38**	phenyl	e	24
7	**39**	vinyl	f	22
8	**40**	ethynyl	f	31

3.1.2 $d^{R}ATP$s as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA

With all of the desired **$d^{R}ATP$s** in hand, we decided to test whether they will serve as substrates for DNA polymerases in enzymatic synthesis of DNA modified in the minor groove. First we wanted to perform primer extension (PEX) experiment with primer Prim15-FAM and templates encoding for either one (Temp19A-FAM) or four (Temp31) modifications in the synthesised DNA strand [for sequences of all oligonucleotides (ONs) used in this study, see Table 3]. The reaction products would then be visualised by fluorescent scanning after denaturing polyacrylamide gel electrophoresis (PAGE) (Scheme 17). PEX in the presence of KOD XL DNA polymerase showed formation of full-length products (**DNA1^{R}A**) for all tested **$d^{R}ATP$s** with the exception of 2-phenyl modified derivative **$d^{Ph}ATP$** which means that phenyl modification is therefore already too big to be incorporated (Figure 11).

Table 3. List of all oligonucleotides used in this study. [a] 6-Carboxyfluorescein (6-FAM) used for oligonucleotide labeling at 5'-end. [b] Primer sequences in template are underlined. [c] Template biotinylated at 5'-end. [d] Template labeled by *ortho*-TINA at 5'-end.

Oligonucleotide	Sequence 5'→ 3'	Length
Prim15	CATGGGCGGCATGGG	15-mer
Prim15-FAM [(a)]	CATGGGCGGCATGGG	15-mer
Temp19A [(b)]	CCCTCCCATGCCGCCCATG	19-mer
Temp19A-bio [(c)]	CCCTCCCATGCCGCCCATG	19-mer
Temp31	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp31-TINA [(d)]	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp31-bio	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp16A	TCCCATGCCGCCCATG	16-mer
Prim_FOR	GACATCATGAGAGACATCGC	20-mer
Prim_REV	CAAGGACAAAATACCTGTATTCCTT	25-mer
Temp98	GACATCATGAGAGACATCGCCTCTGGGCTAATAGGAC TACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGC AAGGAATACAGGTATTTTGTCCTTG	98-mer
ON1^{R}A	CATGGGCGGCATGGG^{R}AGGG	19-mer
ON4^{R}A	CATGGGCGGCATGGG^{R}ACTG^{R}AGCTC^{R}ATGCT^{R}AG	31-mer



Scheme 17. Depiction of PEX with DNA polymerase, Prim15-FAM, modified **d^{R}ATP** and remaining natural dNTPs and a) Temp19A or b) Temp31, giving products with either a) one or b) four modifications in the synthesised DNA strand, followed by denaturing PAGE analysis.

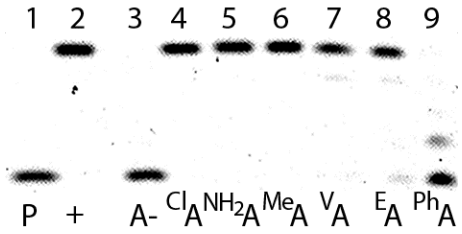


Figure 11. Denaturing PAGE of PEX experiment in presence of KOD XL DNA polymerase, Prim15-FAM and Temp19A. Lanes: 1, P: primer; 2, +: products of PEX with natural dNTPs; lane 3, A-: products of PEX with dTTP, dCTP, dGTP; lanes 4-9, ^{R}A: products of PEX with dTTP, dCTP, dGTP and functionalized **d^{R}ATP** used instead of natural dATP.

Next we investigated the possibility of preparation of DNA bearing four modifications (Scheme 17b). PEX was performed using the same primer and DNA polymerase, but in the presence of Temp31-TINA. The ON labelled by TINA (Figure 12a) is used in PEX reactions to minimise the non-templated extension of the synthesised strand.[191] KOD XL DNA polymerase was again able to synthesise full-length products (**DNA4^{R}A**) with the use of modified **d^{R}ATP**s. In the case of synthesis of ethynyl modified DNA (DNA4^{E}A) the synthesis was partially halted at the n-1 position. This means that all four 2-ethynyl modified adenine bases are present in the synthesised product and the DNA polymerase was not properly able to incorporate the last guanosine, most probably due to some spatial constrictions. On the other hand, synthesis of phenyl modified DNA was again not accomplished, therefore **d^{Ph}ATP** is not a substrate for this DNA polymerase (Figure 12b).

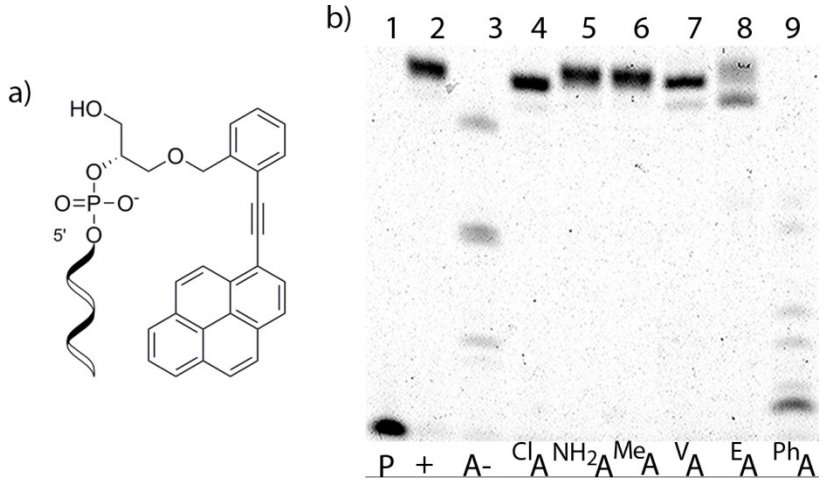


Figure 12. a) Structure of *ortho*-twisted intercalating nucleic acid (*o*-TINA). b) Denaturing PAGE of PEX experiment in presence of KOD XL DNA polymerase, Prim15-FAM and Temp31-TINA. Lanes: 1, P: primer; 2, +: products of PEX with natural dNTPs; lane 3, A-: products of PEX with dTTP, dCTP, dGTP; lanes 4-9, ^{R}A: products of PEX with dTTP, dCTP, dGTP and functionalized **d^{R}ATP** used instead of natural dATP.

We also studied the ability of other DNA polymerases to incorporate these modified adenosine triphosphates into DNA. PEX experiments with Vent(exo-) and Bst Large Fragment (LF) DNA polymerases were repeated with the same primer and templates and the PAGE analysis showed formation of full-length products in all cases (even in the case of experiment with Temp31-TINA and **d^{E}ATP**). On the other hand, none of the tested enzymes were able to incorporate the **d^{Ph}ATP**, so we can conclude that phenyl group is already too big to be incorporated (Figures 13, 14). It is important to note, that while KOD XL and Vent(exo-) DNA polymerases are enzymes from family B of DNA polymerases, Bst LF DNA polymerase is classified as a family A DNA polymerase. The ability of these enzymes to incorporate modified dNTPs is usually lower to that of enzymes from family B.[58,60]

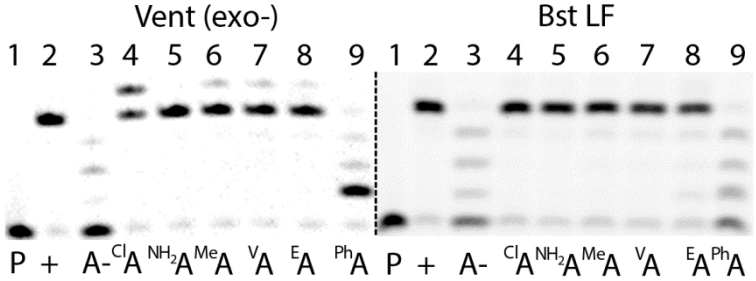


Figure 13. Denaturing PAGE of PEX experiment in presence of either Vent(exo-) or Bst Large Fragment DNA polymerases, Prim15-FAM and Temp19A. Lanes: 1, P: primer; 2, +: products of PEX with natural dNTPs; lane 3, A-: products of PEX with dTTP, dCTP, dGTP; lanes 4-9, ^{R}A: products of PEX with dTTP, dCTP, dGTP and functionalized **d^{R}ATP**.

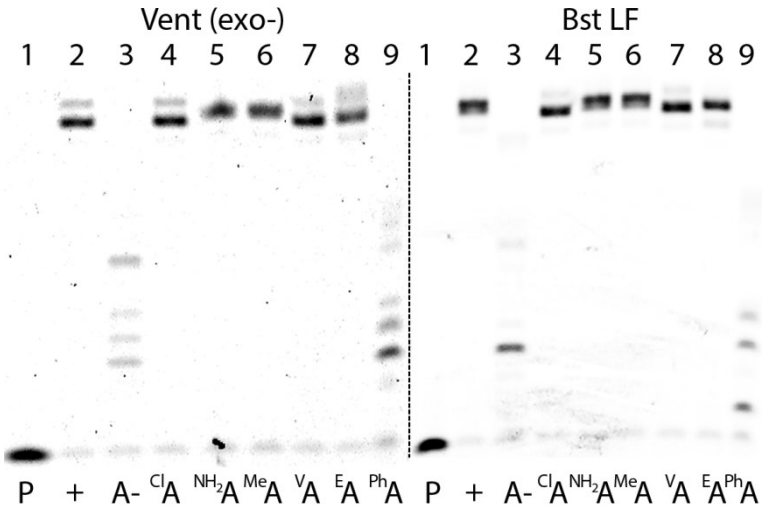


Figure 14. Denaturing PAGE of PEX experiment in presence of either Vent(exo-) or Bst Large Fragment DNA polymerases, Prim15-FAM and Temp31-TINA. Lanes: 1, P: primer; 2, +: products of PEX with natural dNTPs; lane 3, A-: products of PEX with dTTP, dCTP, dGTP; lanes 4-9, ^{R}A: products of PEX with dTTP, dCTP, dGTP and functionalized **d^{R}ATP** used instead of natural dATP.

We also performed a simple kinetic study (Figure 15), where we compared the rates of incorporation of **d^{R}ATP**s compared to natural dATP. PEX with KOD XL DNA polymerase, Prim15-FAM and Temp16A, which affords a 16-bp product having the modified nucleoside be the only one being incorporated. The reactions were run with dATP or modified **d^{R}ATP**s for the specified time followed by immediate denaturation by heating. PAGE analysis of the products showed that small modifications (chloro, amino and methyl) did not affect the incorporation and the synthesis was finished in time comparable to that of reaction with natural dATP (<1 minute). Reactions with **d^{V}ATP** and **d^{E}ATP** took a bit longer to reach completion (ca. 2 minutes).

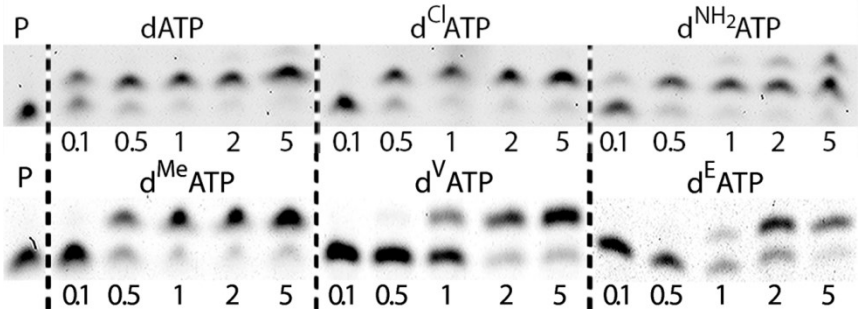
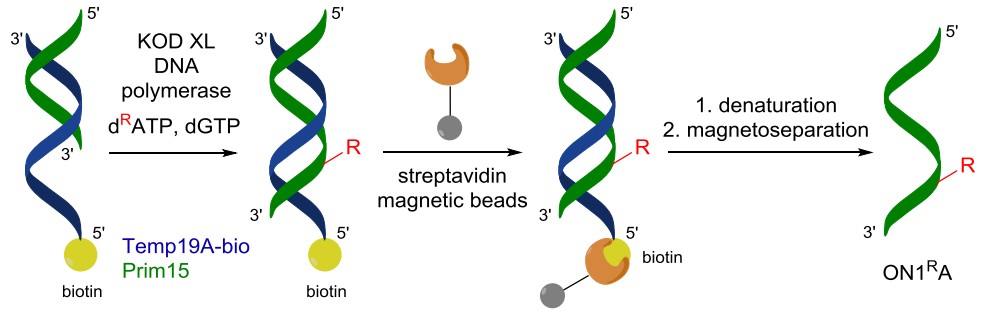


Figure 15. PAGE analyses of kinetic single nucleotide extension experiments with Temp16A using KOD XL DNA polymerase and **d^{R}ATP**s in comparison with natural dATP. Time intervals are given in minutes.

By performing PEX with KOD XL DNA polymerase, **d^{R}ATP**s, Prim15 and templates labelled by biotin at the 5'-end on a semi-preparative scale, we were able to isolate single stranded DNA (ssDNA) following a magnetoseparation procedure using streptavidin magnetic particles (for depiction of the procedure, see Scheme 18).[192] The isolated ONs (ON1^{R}A or ON4^{R}A, for sequences see Table 3) were then analysed by MALDI-TOF analysis, which confirmed formation of all the desired products (Table 4).



Scheme 18. PEX with biotinylated template Temp19A-bio, Prim15, KOD XL DNA polymerase, dGTP and modified **d^{R}ATP** followed by magnetoseparation using streptavidin magnetic particles resulting in single stranded DNA ON1^{R}A.

Table 4. MALDI data of modified ONs.

ON	Template used	Number of ^{R}A modifications	M (calculated) / Da	M (found) [M+H] / Da
ON1^{Cl}A	Temp19A-bio	1 × ^{Cl}A	6008.4	6009.0
ON1^{NH_2}A	Temp19A-bio	1 × ^{NH_2}A	5989.0	5989.8
ON1^{Me}A	Temp19A-bio	1 × ^{Me}A	5988.0	5988.9
ON1^{V}A	Temp19A-bio	1 × ^{V}A	6000.9	6001.0
ON1^{E}A	Temp19A-bio	1 × ^{E}A	5998.9	5999.8
ON1^{CM}A	Temp19A-bio	1 × ^{CM}A	6209.0	6209.2
ON1^{Cy3}A	Temp19A-bio	1 × ^{Cy3}A	6539.3	6540.2
ON4^{Cl}A	Temp31-bio	4 × ^{Cl}A	9755.0	9755.0
ON4^{NH_2}A	Temp31-bio	4 × ^{NH_2}A	9677.3	9678.1
ON4^{Me}A	Temp31-bio	4 × ^{Me}A	9672.4	9673.0
ON4^{V}A	Temp31-bio	4 × ^{V}A	9721.5	9723.0
ON4^{E}A	Temp31-bio	4 × ^{E}A	9713.4	9714.8
ON4^{CM}A	Temp31-bio	4 × ^{CM}A	10555.0	10558.4
ON4^{Cy3}A	Temp31-bio	4 × ^{Cy3}A	11868.0	11865.9

In order to study the effect of DNA minor groove modifications, we also measured the denaturing temperatures of these minor-groove modified DNA duplexes

(DNA1^{R}A or DNA4^{R}A) and compared them with the natural DNA of the same sequence. Most of the modifications destabilised the duplexes, while the 2,6-diaminopurine base stabilised the DNA by about 0.5 °C per modification. This is most probably thanks to additional hydrogen bond with the thymine base (Table 5).[193]

Table 5. Denaturing temperatures of minor-groove modified DNA duplexes compared to natural DNA of the same sequence. [a] $\Delta T_m = (T_{m\,mod} - T_{m+})/n_{mod}$

DNA	T_m / °C	ΔT_m / °C [(a)]	DNA	T_m / °C	ΔT_m / °C [(a)]
DNA1A	72.2 ± 0.1	–	DNA4A	79.4 ± 0.1	–
DNA1^{Cl}A	69.6 ± 0.1	– 2.6	DNA4^{Cl}A	71.0 ± 0.2	– 2.1
DNA1^{NH_2}A	72.5 ± 0.1	+ 0.3	DNA4^{NH_2}A	81.4 ± 0.2	+ 0.5
DNA1^{Me}A	70.5 ± 0.1	– 1.7	DNA4^{Me}A	72.3 ± 0.2	– 1.8
DNA1^{V}A	66.6 ± 0.2	– 5.6	DNA4^{V}A	72.6 ± 0.3	– 1.7
DNA1^{E}A	66.1 ± 0.3	– 6.1	DNA4^{E}A	71.1 ± 0.3	– 2.1
DNA1^{Cy3}A	69.0 ± 0.5	– 3.2			

We also tested the possibility of using **d^{R}ATP**s as substrates in PCR. Compared to PEX, PCR is more demanding, since the DNA polymerase has to not only be able to incorporate the modified nucleotide into the strand that is being extended, but also read through the modified template strand in the next cycle of the reaction. The result is a DNA modified in both strands. PCR was run in the presence of KOD XL DNA polymerase, Prim_FOR, Prim_REV and Temp98 (Table 3) for 30-35 cycles. The products of the reaction were analysed by agarose gel electrophoresis and GelRed fluorescence staining. Full-length products were observed only in the case of **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP**, whereas no product was detected for the vinyl- and ethynyl-modified nucleotides (Figure 16). PCR with the diaminopurine nucleotide was comparably efficient to PCR with natural dNTPs, while the reaction with the chloro and methyl modified triphosphates was less efficient.

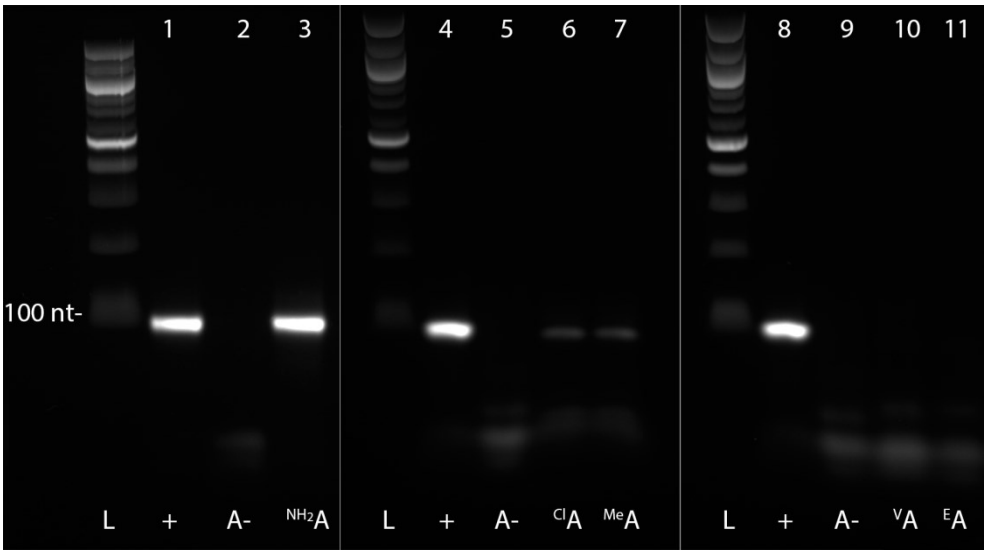


Figure 16. Agarose gel analysis of PCR products amplified by KOD XL DNA polymerase in the presence of **d^{R}ATP**s. Lanes: L: 100-nt ladder; 1,4,8, +: PCR in the presence of natural dNTPs; 2,5,9, A-: PCR in the presence of dCTP, dTTP and dGTP; 3,6,7,10,11, ^{R}A: PCR in the presence of corresponding **d^{R}ATP** instead of natural dATP.

3.1.3 Post-synthetic transformation of minor-groove modified DNA

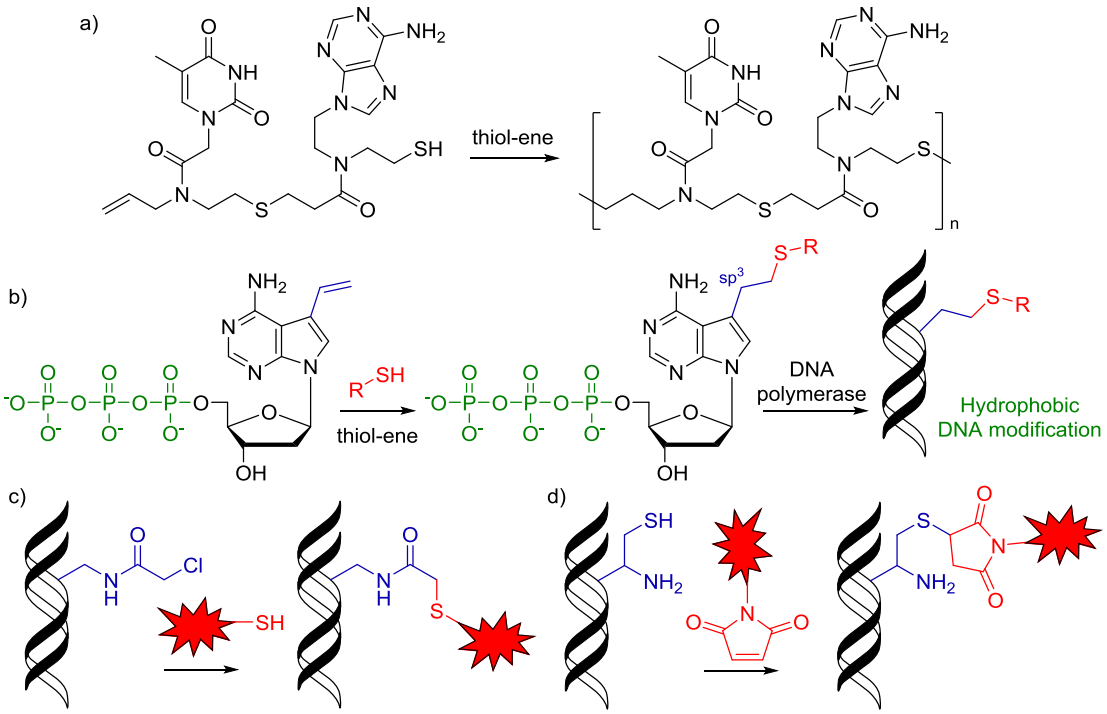
Since 2-phenyl modified dATP did not serve as a substrate for DNA polymerases in enzymatic synthesis of minor-groove modified DNA, one of the ways to prepare DNA modified by a larger functional group would be to utilise post-synthetic DNA modification reactions. Luckily, we were able to prepare vinyl- (DNA1^{V}A and DNA4^{V}A) and ethynyl-linked DNA (DNA1^{E}A and DNA4^{E}A) and therefore post-synthetic modification was an option in this case. We envisaged that ethynyl modified DNA could react in copper-catalysed alkyne-azide cycloaddition (CuAAC) which has been used for modification of nucleic acids quite extensively.[94,95] The vinyl group could in principle be considered for a number of reactions such as "photoclick" reaction or inverse-electron-demand Diels-Alder cycloaddition (iEDDA). Both of these reactions have their disadvantages in this particular case. Even though the reactions have both been described for post-synthetic labelling of DNA, the described examples all take place in the DNA major groove or at the terminal positions of ONs.[120,121,130–133] Therefore, since the "photoclick" reaction requires a diaryltetrazole as a reaction partner of the vinyl group, the tight spatial requirements of the minor-groove might disfavour this reaction. iEDDA requires the reaction to be performed with electron-deficient heteoaromatics (such as tetrazines, commonly again diaryltetrazines) and strained

alkene, which does not comply with the vinyl group. The reaction is described also with some non-strained alkenes; these are however almost exclusively terminal alkenes of longer saturated carbon chains.[194–196] The vinyl group modified nucleoside did react with a tetrazine in the work of Luedtke et al., who then used the nucleoside for introduction of the vinyl group into cellular DNA.[197] However this reaction again proceeded in the major groove of DNA.

Another option for post-synthetic DNA modification utilising the vinyl group would be thiol-ene reaction. These reactions possess many attributes typical for click reactions, whether the reaction proceeds via a radical mechanism (thiol-ene) or via a Michael type conjugate addition. Thiol-ene radical chemistry is often used for an efficient construction of polymers thanks to its exceptional properties (high yields, high reaction rates, etc.). It is typical that a thiol-ene reaction is photoinitiated (light-mediated thiol-ene radical reaction) which increases the usefulness of this reaction even more for polymerisation purposes.[198,199] Interestingly, nucleobase-containing polymers have been constructed using either thiol-ene reaction of a thiol and alkene[200] or a combination of Michael and thiol-ene radical reaction (Scheme 19a).[201]

Thiol-ene reaction has been used before for modification of vinyl-modified nucleosides and nucleotides, the result of which is a modification attached to the nucleobase via sp^3 hybridised carbon, quite uncommon in the field (the modification are usually attached via sp or sp^2 hybridised carbons; result of cross-coupling reactions). These nucleotides were used for the preparation of DNA modified by hydrophobic groups (Scheme 19b).[202] Reactions of vinyl modified nucleosides with thiol reagents have been described also by the group of Sasaki, where they used this reaction quite extensively during their studies of DNA interstrand cross-linking.[203–205] Interestingly, thiol-ene reaction also proceeded with 2-vinyladenosine nucleoside derivative, which was ultimately transformed into a corresponding phosphoramidite and incorporated into DNA by chemical synthesis.[206] On the other hand, DNA itself has not been modified via a thiol-ene radical reaction to the best of our knowledge. Thiol reactivity itself has been utilised for post-synthetic modification of DNA, namely through Michael addition of cysteine and cysteine-containing peptide and protein to a vinylsulfoneamide moiety.[33,207] Another example is the use of thiol group for nucleophilic displacement of chlorine atom at the chloroacetamide moiety of the modified DNA (Scheme 19c).[36] Also DNA modified by cysteine reacted with maleimide reagents affording products of cross-linking of DNA and peptides and proteins (Scheme 19d).[144] Since reactions of

thiols have been used for post-synthetic DNA modification, we chose thiol-ene reaction as a suitable candidate to test for post-synthetic modification of vinyl-linked DNA.



Scheme 19. Utilisation of thiols for DNA modification. a) Synthesis of nucleobase-containing polymer. b) Thiol-ene reaction on nucleotide used for enzymatic synthesis of DNA. c) Nucleophilic substitution of chlorine atom by thiol reagents on DNA. d) Reaction of thiol-modified DNA with maleimide reagent.

In order to be able to easily assess the progress of the reactions, we chose to perform the reactions with fluorescent labels. In this way the resulting products of click reaction will be fluorescent and therefore easily detectable by the newly acquired fluorescence properties. Commercially available cyanine 3 azide (**Cy3-N$_3$**, Figure 17a) was chosen as a reagent for CuAAC reaction with ethynyl-modified DNA (DNA1^{E}A or DNA4^{E}A). The reaction in the presence of CuBr, sodium ascorbate and Cu^{I} binding ligand TBTA [tris(benzyltriazolylmethyl)amine] in DMSO/H_2O/*t*-BuOH mixture proceeded smoothly to provide the fluorescent conjugates DNA1^{Cy3}A and DNA4^{Cy3}A (Figure 17b). The DNA products were isolated from the reaction mixture using silicon-base size-exclusion columns and the emission spectra of the acquired DNAs were measured. A signal of fluorescence corresponding to the newly attached cyanine fluorophore confirmed that the reaction took place. Natural DNA (DNA1A and DNA4A) was subjected to the same reaction conditions and purification in order to exclude the possibility that some of the unreacted azide is responsible for the fluorescence. Fluorescence intensity of this sample was comparable to that of the

starting ethynyl-modified DNA (Figure 17c). Moreover, the visualisation of the fluorescent products was also possible by direct irradiation of the DNA samples by UV (325 nm) (Figure 17d).

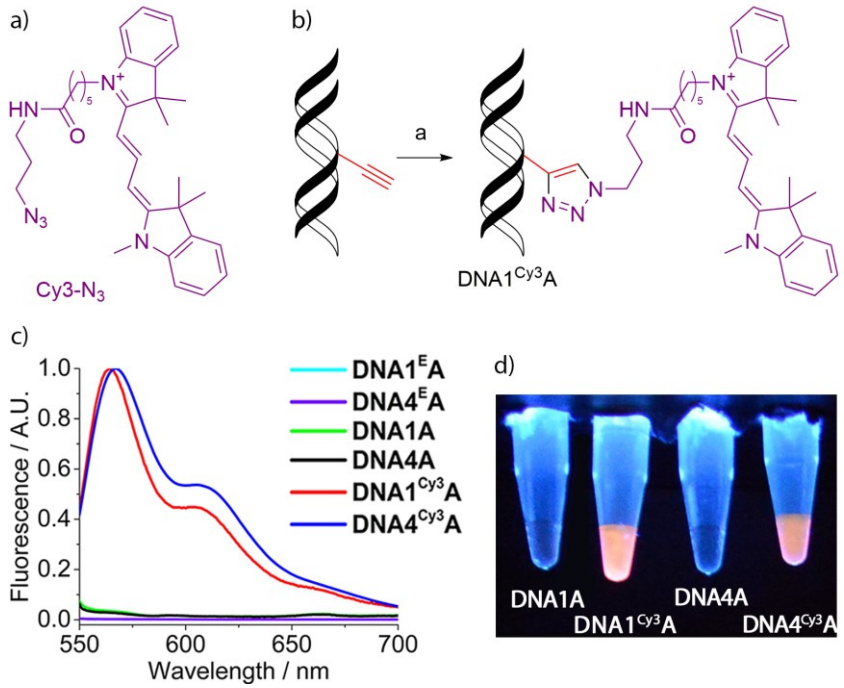


Figure 17. a) Structure of **Cy3-N_3**. b) Depiction of CuAAC between DNA bearing one ethynyl group and cyanine-linked azide providing the fluorescently labelled $DNA1^{Cy3}A$. c) Normalised fluorescence spectra of $DNA1^{E}A$ and $DNA4^{E}A$ before and after CuAAC reaction with **Cy3-N_3** compared to natural DNA treated with the same reagent. d) Photograph of the vials containing the fluorescent DNA ($DNA1^{Cy3}A$ and $DNA4^{Cy3}A$ in H_2O/glycerol mixture) compared to natural non-modified DNA treated with the same reagent upon UV irradiation (365 nm). Reaction conditions: a) **Cy3-N_3**, CuBr, sodium ascorbate, TBTA, DMSO/H_2O/*t*-BuOH, rt, 5 h.

Preparation of fluorescent DNA with utilisation of the vinyl group through thiol-ene reaction was tested with the use of known coumarinemethylthiol (**CM-SH**, Figure 18a).[208] From our experience, reactions between thiols and DNA proceed only when a large excess of the thiol reagent is used in the reaction (unless proximity effect accelerates the reaction).[33,36] Thiol-ene reaction is also described as highly efficient, when photoinitiation is used to promote the reaction. In our case however, direct irradiation of the reaction mixtures led to decomposition of the fluorophore (bleaching). Therefore the reaction was conducted with a large excess of **CM-SH** (12500 eq.) in TEAA buffer for three days where the vinyl-modified DNA ($DNA1^{V}A$ and $DNA4^{V}A$) participated in thiol-ene reaction resulting in coumarine-linked products ($DNA1^{CM}A$ and $DNA4^{CM}A$) (Figure 18b). Products of the reaction were again purified by size-exclusion columns and fluorescence spectra of the samples were measured. Once again

we observed a signal of the corresponding coumarine fluorophore, confirming that the reaction was successful. Only background fluorescence was measured in the case natural DNA was used for the same reaction (Figure 18c). Photograph of the vials containing the fluorescently labelled DNA showed clearly the formation of the desired products (Figure 18d). Formation of all products of click reactions was confirmed by MALDI-TOF analysis (Table 4). In the case of thiol-ene reaction of DNA4^{V}A, we observed the formation of the product of four reactions; however the most abundant product seemed to be the product of one or two vinyl group transformation (for copies of MALDI spectra, see Appendix 1).

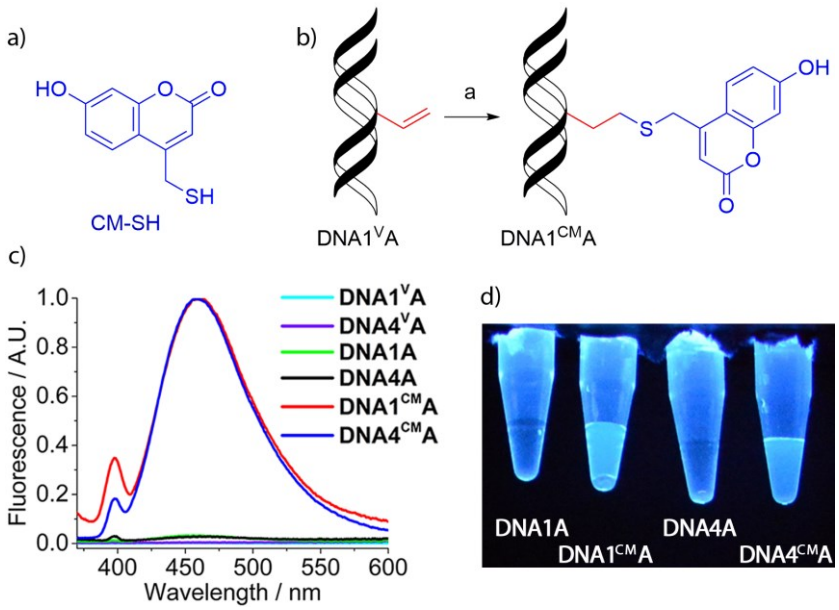


Figure 18. a) Structure of **CM-SH**. b) Depiction of thiol-ene reaction between DNA bearing one vinyl group and coumarinemethylthiol (**CM-SH**) providing the fluorescently labelled DNA1^{CM}A. c) Normalised fluorescence spectra of DNA1^{V}A and DNA4^{V}A before and after thiol-ene reaction with **CM-SH** compared to natural DNA treated with the same reagent. d) Photograph of the vials containing the fluorescent DNA (DNA1^{CM}A and DNA4^{CM}A) compared to natural non-modified DNA treated with the same reagent upon UV irradiation (365 nm). Reaction conditions: a) **CM-SH**, TEAA buffer, 37 °C, 3 days.

Although fluorescence confirmed that all reactions proceeded, this method only allows for a qualitative assessment of the reactions. In order to have an idea about the efficiency of the reactions, we needed a way to determine the conversions of the reactions. One possibility is to perform a PAGE analysis of the formed products. When a FAM labelled DNA is used, the direct comparison of the intensities of the spots on the gel will provide information about the conversion of the reactions. This is possible thanks to different electrophoretic mobility of the starting DNAs and the click reaction

products. Thus the click reactions were performed again, this time on a FAM-labelled minor-groove modified DNAs (this analysis was performed only for DNA1^{V}A and DNA1^{E}A). Purification followed by PAGE analysis shows that thiol-ene reaction gives the corresponding product in ca. 60% conversion. On the other hand, CuAAC proceeds smoothly and the desired product in formed with >90% conversion (Figure 19). Notably, the CuAAC product DNA1^{Cy3}A represents a more stable DNA duplex than that of the starting DNA1^{E}A (Table 5).

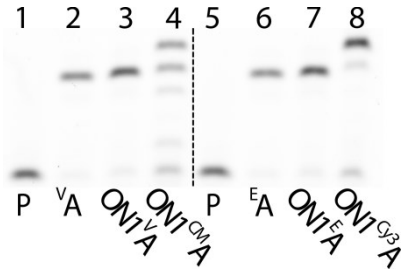
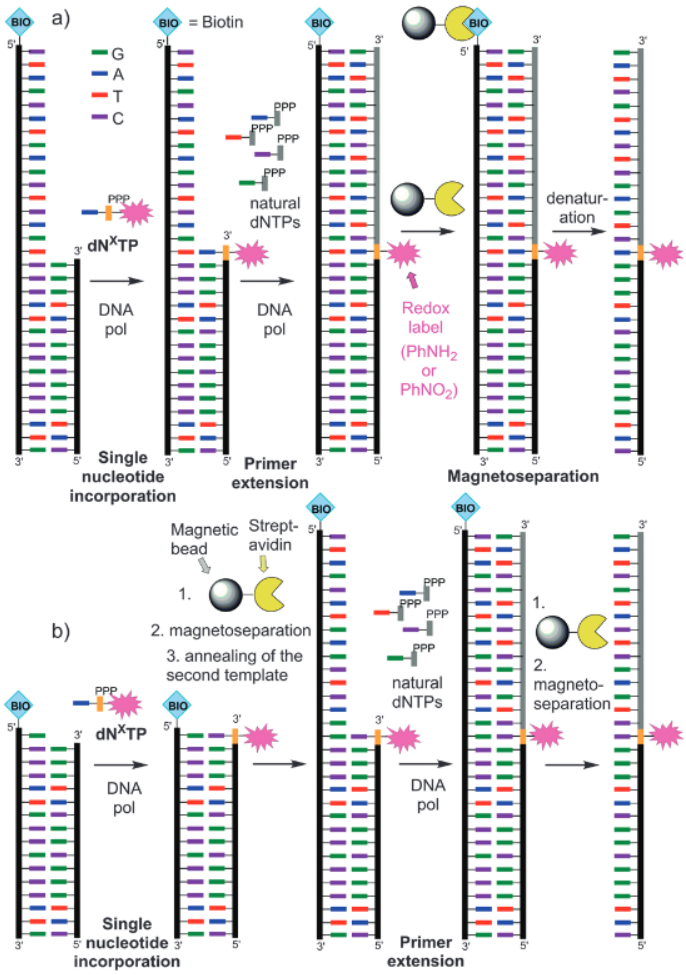


Figure 19. Denaturing PAGE showing results of a thiol-ene or CuAAC reaction of starting vinyl- or ethynyl-modified dsDNA (DNA1^{V}A or DNA1^{E}A) containing one modification in the sequence. Lanes 1 and 5, P: primer; lanes 2 and 6, ^{R}A: products of analytical PEX with dGTP and functionalized **d^{R}ATP**; lanes 3 and 7 **ON1^{R}A**: products of semi-preparative PEX with dGTP and functionalized **d^{R}ATP**, Prim15-FAM and Temp19A; lanes 4 and 8, **ON1^{X}A**: products of thiol-ene or CuAAC reaction respectively.

In conclusion we have shown that not only **d^{Cl}ATP** and **d^{NH_2}ATP**, but also other 2-substituted dATP derivatives bearing small C-substituents (methyl, vinyl ethynyl) are good substrates for various DNA polymerases. Phenyl group on the other hand, is already too big for minor-groove modification. DNA minor groove was then further modified by click reactions with fluorescent labels, achieving modification of the minor groove with bigger functional groups than would be possible to install using enzymatic synthesis.

3.2 2-Substituted dATP derivatives modified by allyl- and propargylamino groups as substrates for site-specific introduction of minor-groove modification into DNA

The enzymatic methods of synthesis of modified DNA based on polymerase extension of primer are typically not suitable for site-specific single modification, because the enzyme incorporates the modified nucleotide against the complementary base at every position. However, the site-specific modification is highly desirable (e.g. studies of DNA-protein cross-linking taking place at the recognition site vs. outside of this site, etc.).[33,34,36,207] Therefore, the Hocek group has previously developed a method based on single-nucleotide extension followed by PEX. The primer is first extended by incorporation of one (modified) nucleotide in single-nucleotide incorporation which is then followed by PEX with an excess of natural dNTPs. This is possible when the modified nucleobase is followed by a different nucleobase (Scheme 20a). In case the primer is supposed to be extended by two consecutive incorporation of the same nucleoside, firstly PEX with a biotinylated template one-nucleotide longer than the primer is conducted followed by magnetoseparation resulting in ssDNA with the incorporated modified nucleoside at the 3'-end. This is then reannealed with another longer template and PEX is performed again, this time with natural dNTPs (and the resulting DNA has two of the same nucleobase following each other with one of them being modified, Scheme 20b). This approach has been used for modification of major groove of DNA with redox labels for electrochemical detection of nucleic acids.[209] However, so far it was impossible to site-specifically introduce a single modification into the DNA minor groove.



Scheme 20. Synthesis of ssDNA bearing a single modification at a specific position utilising single nucleotide incorporation. a) Modified nucleobase is followed by different nucleobase. b) Modified nucleobase is followed by the same non-modified nucleobase. (Scheme taken from Ménová, P. et al., Chem. Commun. 2013, 49, 4652-4654.)

Previously we have shown that it is possible to prepare base-modified DNA functionalised in the minor groove enzymatically with the use of 2-substituted dATP derivatives (Chapter 3.1). Shortly thereafter, Gowda et al. published a study showing that N^2-substituted dGTPs are generally not good substrates for DNA polymerases, however human DNA polymerase κ was able to incorporate these triphosphates into DNA.[210] Both of these studies show the worth of further exploration of the possibilities of minor-groove modification.

We set out to prepare a series of 2-alkylamino-2'-deoxyadenosine triphosphates and test them as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA. Again we chose substituents of increasing size (methyl-, ethyl-, allyl- and propargylamino modifications) in order to determine the steric restrictions of the enzymatic synthesis. We realised that some of these groups are larger than the vinyl

and ethynyl groups that were used to modify the minor groove previously. On the other hand, alkyl chains are more flexible and therefore might not completely inhibit the DNA polymerase activity in this regards (compared to phenyl group used in the previous study). Moreover, since the presence of 2,6-diamonopurine nucleobase in the DNA stabilised the DNA duplex, we envisaged that the additional hydrogen bond with thymine might compensate for the (presumably destabilising) steric effect of these alkyl groups. In the end, the DNA could be again post-synthetically modified utilising click reactions. Minor-groove site of the nucleobases is necessary for the crucial Watson-Crick base-pairing, but it is also an important place of interactions with DNA polymerases during chain extension,[183] so this might be another problem that might arise from the use of these compounds (Figure 20). Nevertheless, we decided to prepare this series of **d^{R}ATP**s and test them as substrates for DNA polymerases in the synthesis of minor-groove modified DNA.

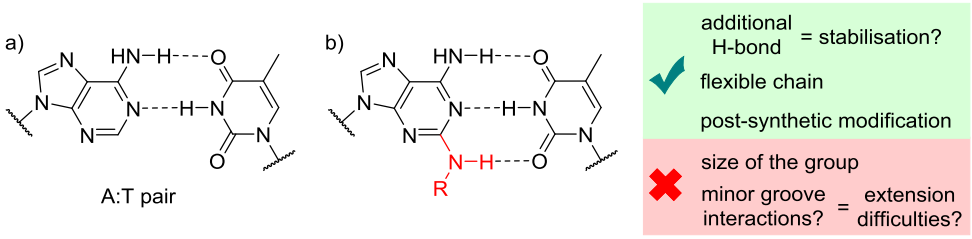
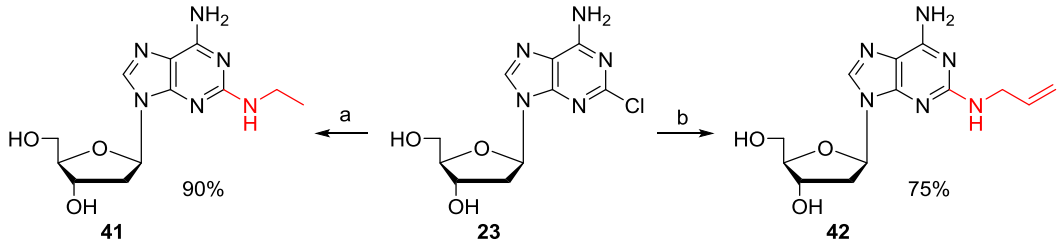


Figure 20. a) Typical Watson-Crick base-pairing of A:T pair. b) Base-pairing of 2-alkylamino modified adenine with thymine, its advantages and disadvantages.

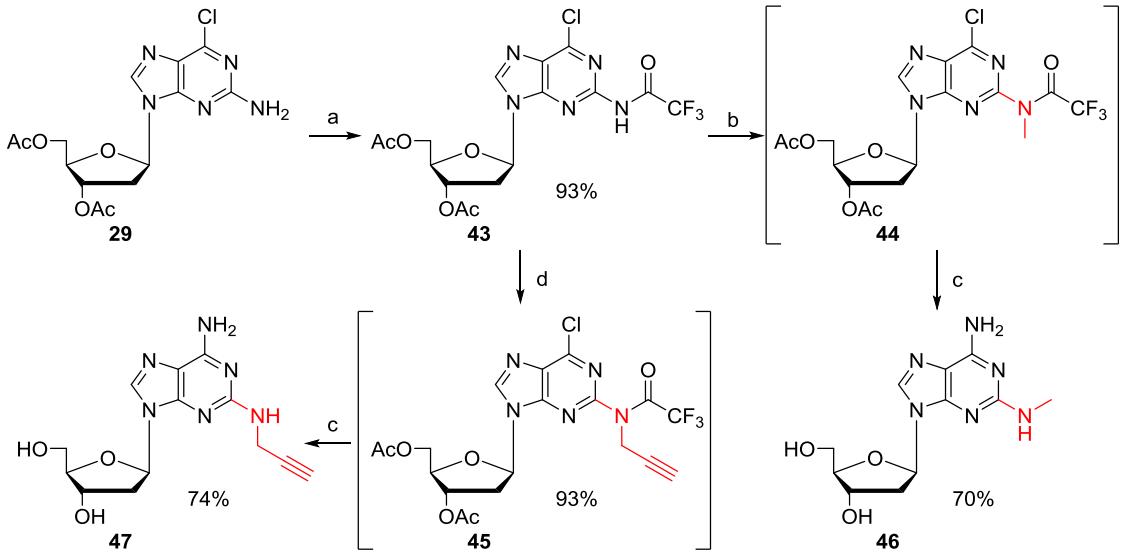
3.2.1 Synthesis of 2-alkylamino-2'-deoxyadenosine triphosphates d^{R}APTs

The synthesis began similarly to the previous study, by preparation of all modified nucleosides, which was followed by their triphosphorylation. Since we are dealing with 2-modified adenosine derivatives, some of the already prepared reaction intermediates can be utilised. 2-Chloro modified adenosine **23** was used as a starting compound for the synthesis of two required nucleosides. It reacted in nucleophilic aromatic substitution of the chlorine atom with appropriate primary amine. Thus, nucleoside **23** reacted with ethylamine in MeOH at elevated temperature for 24 hours, yielding the desired ethylamino modified nucleoside **41** in 90%. Similarly, the allylamino modified nucleoside **42** was prepared by the reaction of **23** with allylamine in MeOH in 75% yield (Scheme 21).



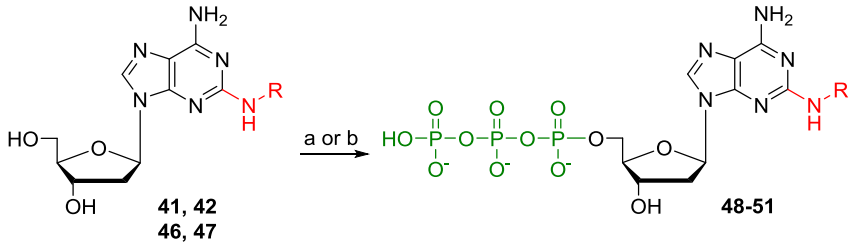
Scheme 21. Nucleophilic aromatic substitution reactions using primary amines. Reaction conditions: a) ethylamine, MeOH, 130 °C, 24 h; b) allylamine, MeOH, 130 °C, 24 h.

A different strategy had to be applied for the synthesis of the remaining two nucleosides (due to rapid decomposition of propargylamine at elevated temperature). Protected nucleoside **29** was first treated with trifluoroacetic anhydride and diisopropyl(ethyl)amine in CH_2Cl_2 to provide the **43** in 93% yield. This increases the acidity of the amide hydrogen, which makes it available for easy deprotonation and subsequent nucleophilic substitution can take place. Thus, **43** reacted with either methyl iodide or propargyl bromide upon treatment with K_2CO_3 in DMF. Since the trifluoroacetyl group was very easily cleavable on silica column, the protected methyl (**44**) and propargyl (**45**) intermediates were not isolated. Instead, the products of successful alkylation were subjected to global deprotection and amination reaction with methanolic ammonia at elevated temperature, yielding the 2-methylamino (**46**) and 2-propargylamino (**47**) modified nucleosides (70% and 74% respectively) (Scheme 22).



Scheme 22. Preparation of remaining alkylamino modified nucleosides. Reaction conditions: a) TFA_2O, DIPEA, DCM, rt, 1 h; b) methyl iodide, K_2CO_3, DMF, rt, 24 h; c) methanolic ammonia, 100 °C, overnight; d) propargyl bromide, K_2CO_3, DMF, rt, 24 h.

With all of the required modified nucleosides at hand, we moved on to preparation of corresponding nucleoside triphosphates. Yoshikawa's phosphorylation method was used again for the preparation of 2-methyl- (**48**, **d^{MA}ATP**) and 2-ethylamino- (**49**, **d^{EA}ATP**) derivatives in the yields of 20% for both of these reactions. The reaction conditions were again modified for the preparation of nucleotides bearing an unsaturated chains and proton sponge was added to the first step of the reaction. The desired nucleoside triphosphates bearing an allyl (**50**, **d^{AA}ATP**) or a propargyl group (**51**, **d^{PA}ATP**) were isolated in 34% and 24% yield respectively (Scheme 23). The reaction conditions and yields are summarised in Table 6.



Scheme 23. Triphosphorylation of 2-alkylamino modified nucleosides. a) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min; b) 1. proton sponge, $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min.

Table 6. Summarised results of the synthesis **d^{R}ATP**s.

Entry	Starting nucleoside → product	R substituent	Conditions	Yield [%]
1	**46 → 48**	methyl	a	20
2	**41 → 49**	ethyl	a	20
3	**42 → 50**	allyl	b	34
4	**47 → 51**	propargyl	b	24

3.2.2 2-Alkylamino-2'-deoxyadenosine triphosphates (d^RATPs) in enzymatic synthesis of minor-groove modified DNA; synthesis of site-specifically minor-groove modified DNA

Prepared **d^RATP**s were then tested as substrates for DNA polymerases in enzymatic synthesis of DNA. First we performed PEX with various DNA polymerases, Prim15-FAM and Temp19A (for sequences of ONs used in this study, see Table 7). Unfortunately, full-length products for all tested nucleotides were not reached with any of the tested DNA polymerases as shown by PAGE analysis (Figure 21). Out of the tested enzymes, KOD XL [and partially Vent(exo-)] DNA polymerase was the only one that was able to produce a full-length product in case **$d^{MA}ATP$** was used instead of natural dATP (Figure 21a). This is an expected result, since the size of methylamino group is similar to that of vinyl (2-vinyl modified dATP was a substrate for this enzyme in the previous study). Out of all the DNA polymerases, Therminator DNA polymerase was the one that showed the formation of longer products, however even in this case full-length products were not achieved (Figure 21b).

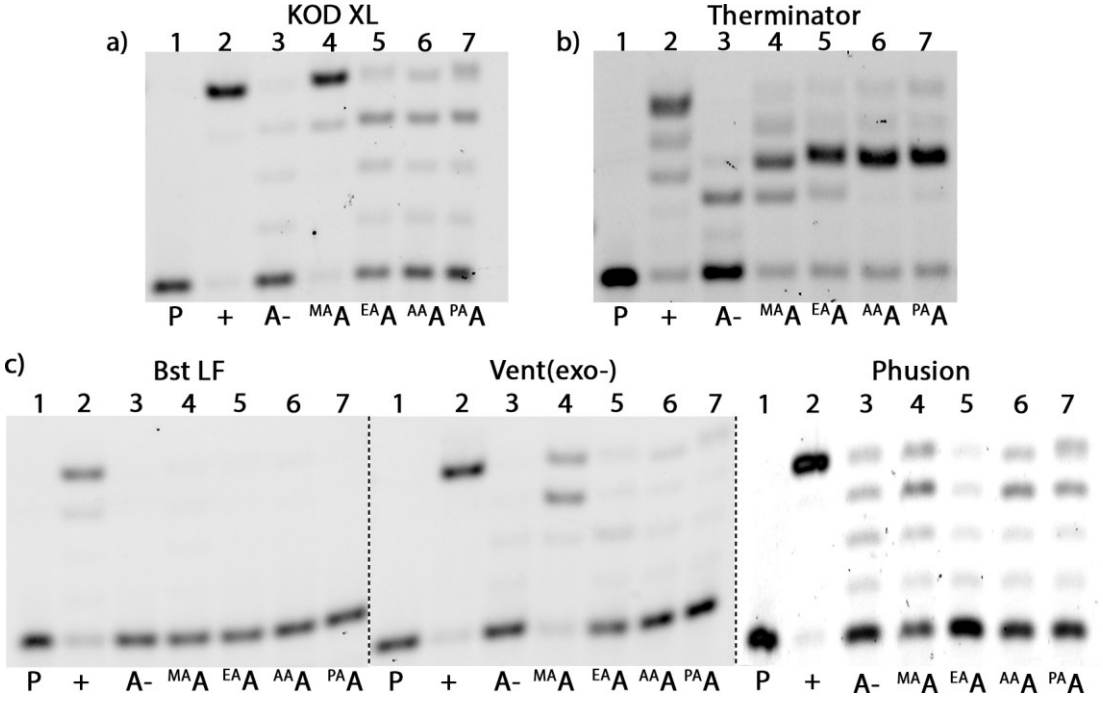


Figure 21. Denaturing PAGE of PEX experiment in presence of Prim15-FAM and Temp19A. and a) KOD XL, b) Therminator and c) Bst Large Fragment, Vent(exo-) and Phusion DNA polymerases. Lanes: 1, P: primer; 2, +: products of PEX with natural dNTPs; lane 3, A-: products of PEX with dTTP, dCTP, dGTP; lanes 4-7, ^RA: products of PEX with dTTP, dCTP, dGTP and functionalized **d^RATP** used instead of natural dATP.

Table 7. List of all oligonucleotides used in this study. [a] 6-Carboxyfluorescein (6-FAM) used for oligonucleotide labeling at 5'-end; [b] Primer sequences in template are underlined; [c] Template biotinylated at 5'-end; [d] Template labeled by *ortho*-TINA at 5'-end; [e] Template biotinylated at 3'-end and labeled by *ortho*-TINA at 5'-end.

Oligonucleotide	Sequence 5'→ 3'	Length
Prim15	CATGGGCGGCATGGG	15-mer
Prim15-FAM [(a)]	CATGGGCGGCATGGG	15-mer
Temp19A [(b)]	CCCTCCCATGCCGCCCATG	19-mer
Temp16A	TCCCATGCCGCCCATG	16-mer
Temp31_AC	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
= ON31_target		
Temp31_AC-bio [(c)]	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp31_AC-TINA [(d)]	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp31_AC-(bio)TINA [(e)]	CTAGCATGAGCTCAGTCCCATGCCGCCCATG	31-mer
Temp31_AT	CTAGCATGAGCTCAATCCCATGCCGCCCATG	31-mer
Temp31_AT-bio [(c)]	CTAGCATGAGCTCAATCCCATGCCGCCCATG	31-mer
Temp31_AG	CTAGCATGAGCTCACTCCCATGCCGCCCATG	31-mer
Temp31_AG-bio [(c)]	CTAGCATGAGCTCACTCCCATGCCGCCCATG	31-mer
Temp31_AA	CTAGCATGAGCTCATTCCCATGCCGCCCATG	31-mer
Temp31_AA-bio [(c)]	CTAGCATGAGCTCATTCCCATGCCGCCCATG	31-mer
revPrim19	CTAGCATGAGCTCAGTCCC	19-mer
revPrim19-FAM	CTAGCATGAGCTCAGTCCC	19-mer
revTemp31_AT	CATGGGCGGCATGGGACTGAGCTCATGCTAG	31-mer
revTemp31_AT-bio [(c)]	CATGGGCGGCATGGGACTGAGCTCATGCTAG	31-mer
ON1^{MA}A	CATGGGCGGCATGGG^{MA}**A**GGG	19-mer
ON31_1^{R}AC	CATGGGCGGCATGGG^{R}**A**CTGAGCTCATGCTAG	31-mer
ON31_1^{R}AG	CATGGGCGGCATGGG^{R}**A**GTGAGCTCATGCTAG	31-mer
ON31_1^{R}AT	CATGGGCGGCATGGG^{R}**A**TTGAGCTCATGCTAG	31-mer
ON31_1^{R}AA	CATGGGCGGCATGGG^{R}**A**ATGAGCTCATGCTAG	31-mer
ON31_2^{R}A	CATGGGCGGCATGGG^{R}**A**^{R}**A**TGAGCTCATGCTAG	31-mer
revON31_1^{R}AT	CTAGCATGAGCTCAGTCCC^{R}**A**TGCCGCCCATG	31-mer
ON16_1^{R}A	CATGGGCGGCATGGG^{R}**A**	16-mer

Since Therminator DNA polymerase was the enzyme that gave the most promising results, we decided to screen the conditions of PEX in the presence of Temp16A and Prim15-FAM with each of the modified **d^{R}ATP**s. In this experiment the modified adenosine triphosphate is the only one being incorporated into the extending

primer (single nucleotide extension, SNE). Surprisingly, PAGE analysis of the products showed formation of full-length products for all tested triphosphates (Figure 22).

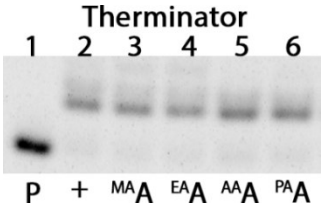


Figure 22. Denaturing PAGE of PEX experiment in presence of Prim15-FAM and Temp16A. and Therminator DNA polymerase. Lane: 1, P: primer; 2, +: product of PEX with natural dATP; lanes 3-6, ^{R}A: products of PEX with functionalized **d^{R}ATP** used instead of natural dATP.

Other DNA polymerases deemed most likely to achieve the same result in SNE were tested as well, however the results show that only Therminator DNA polymerase is able to incorporate these **d^{R}ATP**s, whereas KOD XL and Vent(exo-) DNA polymerases gave no products of extension as witness after PAGE analysis (Figure 23). (These experiments were conducted only with the allyl- and propargylamino modified nucleotides, since they are the most useful for further applications.)

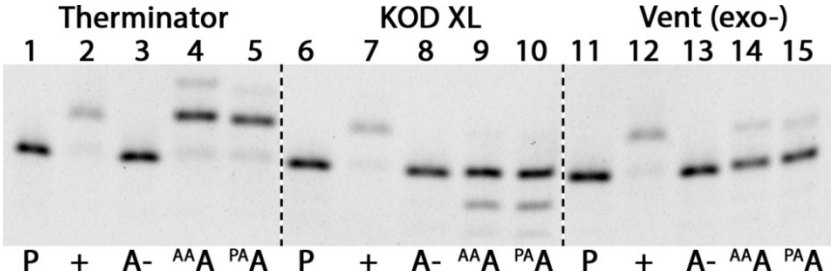


Figure 23. Denaturing PAGE of PEX experiment in presence of Prim15-FAM and Temp16A. and Therminator, KOD XL and Vent(exo-) DNA polymerases showing SNE only in the case of Therminator DNA polymerase. Lanes 1, 6, 11, P: primer; lanes 2, 7, 12, +: products of PEX with natural dATP; lanes 3, 8, 13, A-: products of PEX without the addition of any triphosphate (only starting material present); lanes 4-5, 9-10, 14-15, ^{R}A: products of PEX with functionalized **d^{R}ATP**.

Next, we wanted to test, whether it would be possible to repeat the same experiment using a longer template. The experiment could in principle provide a DNA modified in the minor groove at a specific location based on the sequences of primer and template (from here onward, the experiments were conducted using only **d^{AA}ATP** and **d^{PA}ATP**, unless stated otherwise). The first step was to optimise the conditions of the SNE using Therminator DNA polymerase, Prim15-FAM and Temp31_AC-TINA (template modified by TINA is used to ultimately minimise the non-templated extension of the primer;[191] for structure, see Figure 12a). Since site-specific modification of DNA

using enzymatic synthesis has been studied previously in Hocek group, we started with the same reaction conditions as were used before (1.3 equiv. of **d^{R}ATP** and low loading of DNA polymerase).[209] However, we soon realised that in the case of 2-alkylamino modified nucleotides, the conditions have to be changed, since these compounds are not good substrates for DNA polymerases (no extension was observed, Figure 24a). Thus the reaction conditions were optimised and full conversion of the primer to the product extended by incorporation of one **d^{R}ATP** (without significant formation of longer ONs) was achieved while using 50 equiv. of nucleotides (calculated for the amount of primer used) in 30 minutes as observed by PAGE analysis (Figure 24b).

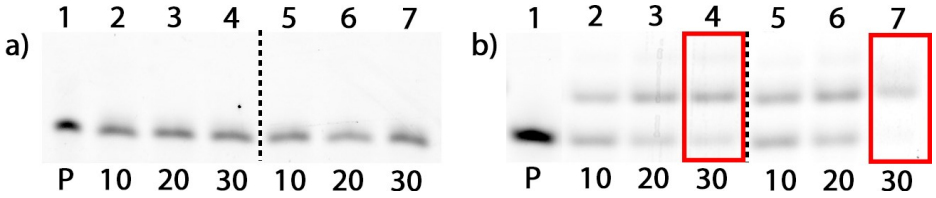


Figure 24. PAGE analysis of SNE with designated reaction time and a) 1.5 and b) 50 equiv. of **d^{R}ATP**s. Time intervals are given in minutes. Red rectangles denote the successful SNE. Lanes 1, P: primer; lanes 2-4: SNE reaction with **d^{AA}ATP** in 10, 20 or 30 minutes; lanes 5-7: SNE reaction with **d^{PA}ATP** in 10, 20 or 30 minutes.

The next step was to try to finish the synthesis using natural dNTPs. We repeated the SNE with the optimised conditions and then an excess of natural dNTPs (50 equiv.) was added to the same sample without any further purification. PAGE analysis of the reaction products showed formation of clean full-length products in both cases (Figure 25; lanes 9,10).

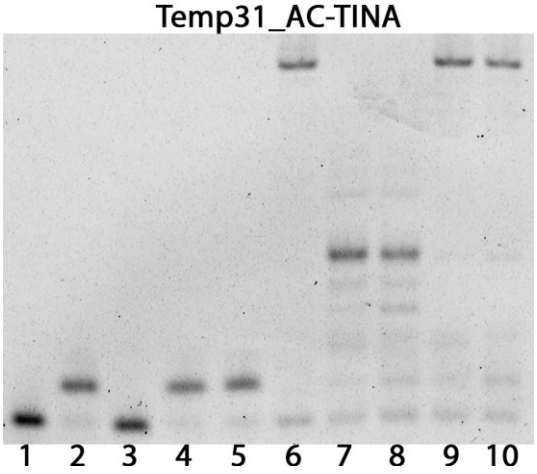


Figure 25. PAGE analysis of enzymatic synthesis of DNA site-specifically labelled in the minor groove using Temp31_AC-TINA template. Lane 1: primer; lane 2: 16-mer ON standard; lane 3: negative control for SNE (no dNTPs present); lanes 4, 5: SNE of **d^{R}ATP** (only **d^{AA}ATP** or **d^{PA}ATP** present in the reaction mixture); lane 6: positive control experiment – product after PEX with natural dNTPs; lanes 7, 8: negative control experiment for PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (extension of the SNE products in the presence of dGTP, dCTP and dTTP; absence of dATP); lanes 9, 10: product of PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (all natural dNTPs present for final extension).

In this particular case, the modified adenine (^{R}A) base is followed by a cytosine (C) in the newly synthesised DNA strand (Table 7; ON31_1^{R}AC). We tested the same reaction conditions for other sequences, where ^{R}A would be followed by a different nucleobase [thymine (T) and guanine (G)]. The reaction conditions were suitable for Temp31_AG (^{R}A followed by G), whereas the SNE was not finished using these conditions for Temp31_AT (^{R}A followed by T) (not shown). Therefore, the conditions had to be modified again and successful SNE was observed upon using 100 equiv. of **d^{R}ATP**s for the reaction with Temp31_AT. SNE was repeated with the optimised conditions for both templates and similarly to the previous example, an excess on natural dNTPs was used afterwards to reach the full-length products (Figure 26a,b). (The amount of dNTPs differed again based on the template used; 50 equiv. of dNTPs were enough for the experiment with Temp31_AG, while 100 equiv. had to be used for the reaction with Temp31_AT.)

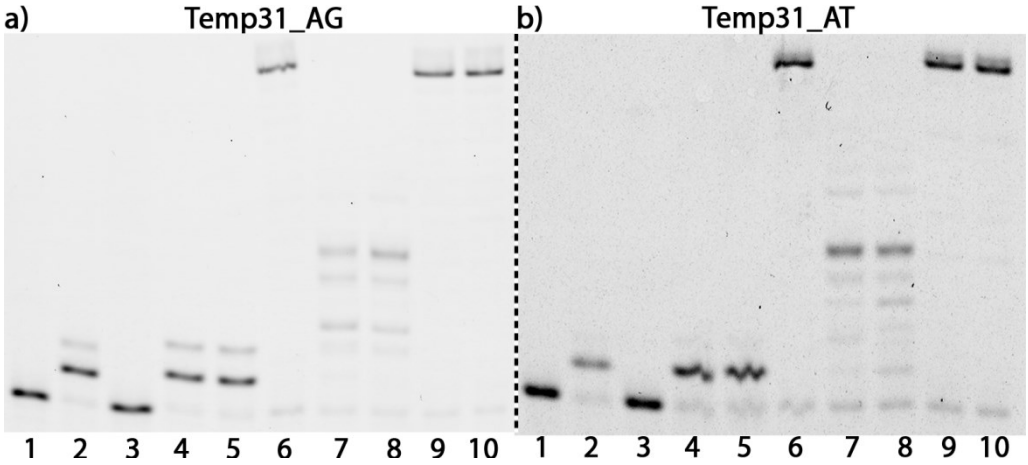


Figure 26. PAGE analyses of enzymatic synthesis of DNA site-specifically labelled in the minor groove using a) Temp31_AG and b) Temp31_AT template. Lanes 1: primer; lanes 2: 16-mer ON standard; lanes 3: negative control for SNE (no dNTPs present); lanes 4, 5: SNE of **d^{R}ATP** (only **d^{AA}ATP** or **d^{PA}ATP** present in the reaction mixture); lanes 6: positive control experiment – product after PEX with natural dNTPs; lanes 7, 8: negative control experiment for PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (extension of the SNE products in the presence of dGTP, dCTP and dTTP; absence of dATP); lanes 9, 10: product of PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (all natural dNTPs present for final extension).

Lastly, we wanted to perform the reaction with Temp31_AA, template which encodes for two adenines in a row in the synthesised DNA strand. Previously in the case of site-specific major-groove modification, this was achieved following a laborious procedure consisting of PEX with a biotinylated one-nucleotide longer template, ssDNA generation via magnetoseparation, reannealing with a longer template and another PEX (Scheme 20b). We tested the SNE of **d^{R}ATP**s using Temp31_AA and, surprisingly, we found that based on the amount of **d^{R}ATP**s used, it was possible to prepare the product containing one ^{R}A, but also the product of two consecutive incorporations of **d^{R}ATP**s. The first scenario was achievable using only 10 equiv. of **d^{R}ATP**s, while for the second one 75 equiv. of **d^{R}ATP**s were necessary. Following, the SNE was repeated with the optimised conditions and the synthesis was finished after the addition of natural dNTPs (50 equiv.) (Figure 27a,b).

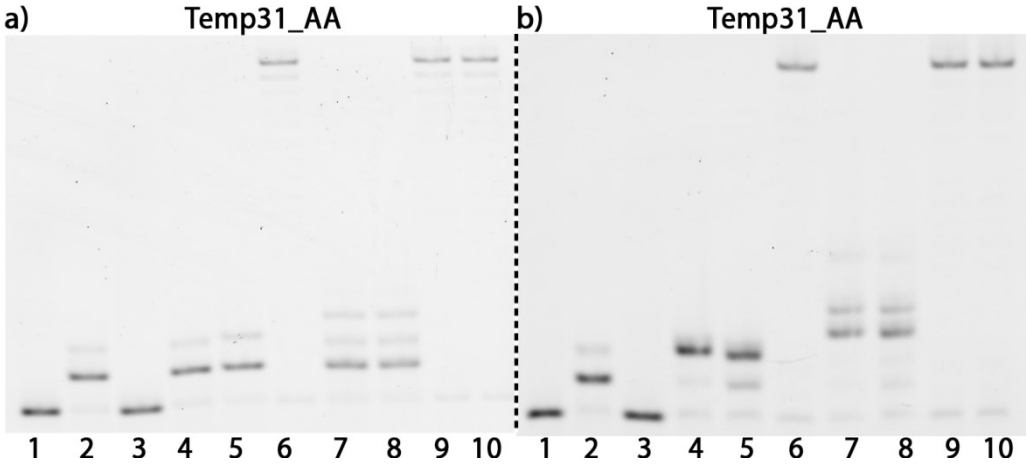
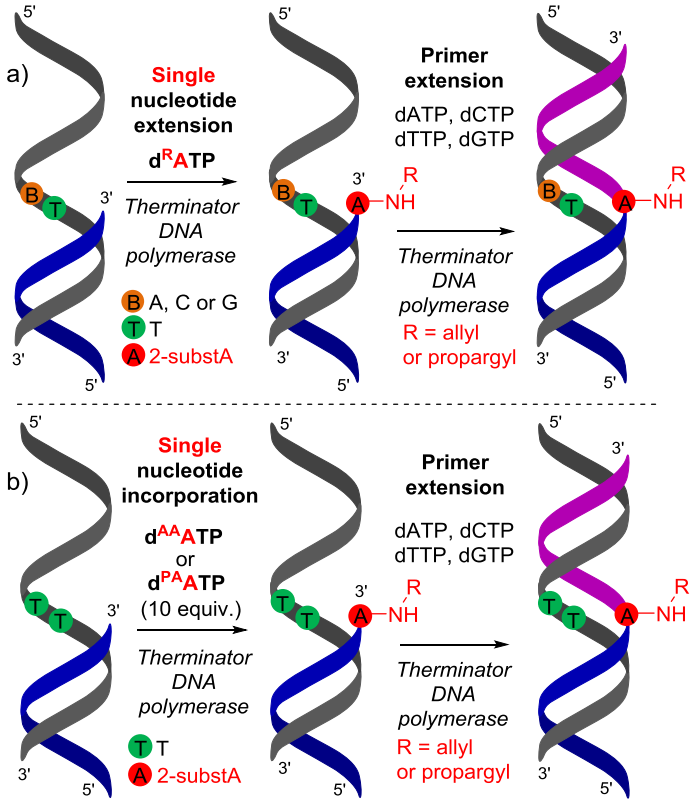


Figure 27. PAGE analyses of enzymatic synthesis of DNA site-specifically labelled in the minor groove using Temp31_AA, where either a) SNE or b) two consecutive **d^{R}ATP** incorporations were taking place. Lanes 1: primer; lanes 2: 16-mer ON standard; lanes 3: negative control for SNE (no dNTPs present); lanes 4, 5: a) SNE of **d^{R}ATP** (only **d^{AA}ATP** or **d^{PA}ATP** present in the reaction mixture) b) two consecutive **d^{R}ATP** incorporation; lanes 6: positive control experiment – product after PEX with natural dNTPs; lanes 7, 8: negative control experiment for PEX after a) SNE or b) two consecutive incorporation of either **d^{AA}ATP** or **d^{PA}ATP** (extension in the presence of dGTP, dCTP and dTTP; absence of dATP); lanes 9, 10: product of PEX after a) SNE or b) two consecutive incorporations of either **d^{AA}ATP** or **d^{PA}ATP** (all natural dNTPs present for final extension).

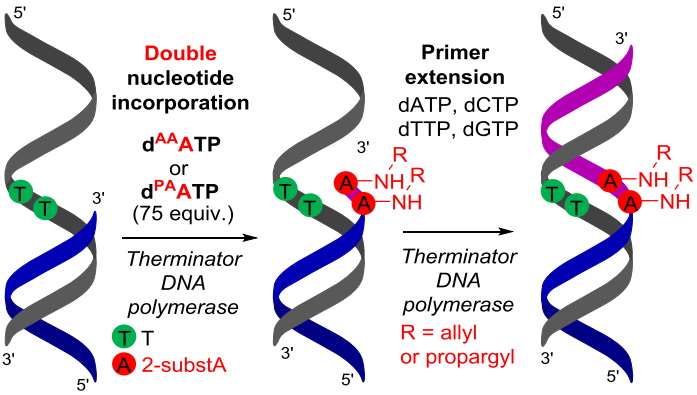
The optimised conditions are summarised in Table 8. Generally the conditions for these reactions were similar for different sequences, where quite a large excess of **d^{R}ATP**s was required for successful SNE to take place. The only difference was the SNE in the case of Temp31_AA, where just 10 equiv. of **d^{R}ATP**s was enough. The reaction time of the final extension step was notably short in some cases, as it was carefully optimised to minimise the non-templated extension (Temp31_AC-TINA was the only TINA labelled template used in the study). Realisation of these experiments is depicted in Schemes 24 and 25.

Table 8. Reaction conditions for SNE and following PEX on analytical scale. The amount of Therminator DNA polymerase was 0.1 U unless stated otherwise. [a] The amounts of dNTPs (**d^{R}ATP** or natural dNTPs) is calculated based on the amount of primer used in the reaction mixture. [b] The amount of Therminator DNA polymerase was 0.2 U.

Product	**d^{R}ATP** for SNE / equiv.[(a)]	SNE conditions	Final extension conditions (dNTPs equiv./temperature/time)
ON31_1^{AA}AC	50	60 °C / 30 min	50 / 60 °C / 15 min
ON31_1^{PA}AC	50	60 °C / 30 min	50 / 60 °C / 15 min
ON31_1^{AA}AT	100	60 °C / 60 min	100 / 60 °C / 5 min
ON31_1^{PA}AT	100	60 °C / 60 min	100 / 60 °C / 5 min
ON31_1^{AA}AG	50	60 °C / 30 min	50 / 60 °C / 5 min
ON31_1^{PA}AG	50	60 °C / 30 min	50 / 60 °C / 5 min
ON31_1^{AA}AA	10	60 °C / 30 min	50 / 60 °C / 5 min
ON31_1^{PA}AA	10	60 °C / 30 min	50 / 60 °C / 5 min
ON31_2^{AA}A	75	60 °C / 60 min	50 / 60 °C / 10 min
ON31_2^{PA}A	75	60 °C / 60 min	50 / 60 °C / 10 min
revON31_1^{AA}AT [(b)]	100	60 °C / 90 min	50 / 60 °C / 5 min
revON31_1^{PA}AT [(b)]	100	60 °C / 90 min	50 / 60 °C / 5 min



Scheme 24. Single nucleotide extension using modified **d^{R}ATP**s followed by PEX with natural dNTPs as performed for templates where a) a different nucleobase and b) another adenine is following the modified ^{R}A in the synthesised DNA strand.



Scheme 25. Two consecutive **d^{R}ATP** incorporations followed by PEX with natural dNTPs.

These PEX experiments were conducted on an analytical scale, meaning that the amount of DNA synthesised is enough for PAGE analysis. However, for any further application or analyses, it is necessary to prepare the DNA on a larger (semi-preparative) scale. Unfortunately, in this case it is not possible to simply multiply the amounts of reagents from the optimised reaction conditions, since the resulting reactions would have to be done in millilitres of water and an enormous amount of DNA polymerase would be required. The reactions are therefore usually conducted utilising a new set of conditions using more concentrated reaction mixtures. The semi-preparative scale reactions were screened for their optimal conditions in the same way as described above, first looking for conditions allowing for a successful SNE and afterwards conditions for PEX with natural dNTPs. These conditions are summarised in Table 9. Interestingly, more concentrated solutions allowed the SNE to proceed with significantly smaller amounts of **d^{R}ATP**s (generally 3-10 equiv.), thanks to the more concentrated reaction mixtures. On the other hand, the reaction time for the final extension using natural dNTPs needed to be significantly prolonged in order to achieve full-length products. The optimised conditions are summarised in Table 9.

Table 9. Reaction conditions for SNE and following PEX on semi-preparative scale. The amount of Therminator DNA polymerase was 1.07 U unless stated otherwise. [a] The amounts of dNTPs (**d^{R}ATP** or natural dNTPs) is calculated based on the amount of primer used in the reaction mixture. [b] Additional 0.2 U of Therminator DNA polymerase was added for the final extension step. [c] The amount of Therminator DNA polymerase was 2 U.

Product	**d^{R}ATP** for SNE / eq. (a)	SNE conditions	Final extension conditions (dNTPs equiv./temperature/time)
ON31_1^{AA}AC	3	60 °C / 60 min	30 / 60 °C / 30 min
ON31_1^{PA}AC	5	60 °C / 60 min	30 / 60 °C / 30 min
ON31_1^{AA}AT	10	60 °C / 60 min	75 / 60 °C / 60 min
ON31_1^{PA}AT	10	60 °C / 60 min	75 / 60 °C / 60 min
ON31_1^{AA}AG	3	60 °C / 60 min	75 / 60 °C / 60 min
ON31_1^{PA}AG	5	60 °C / 60 min	75 / 60 °C / 60 min
ON31_1^{AA}AA	3	60 °C / 60 min	50 / 60 °C / 60 min
ON31_1^{PA}AA	5	60 °C / 60 min	50 / 60 °C / 60 min
ON31_2^{AA}A	10	60 °C / 60 min	100 / 60 °C / 60 min
ON31_2^{PA}A	20	60 °C / 60 min	100 / 60 °C / 60 min (b)
revON31_1^{PA}AT (c)	25	60 °C / 60 min	30 / 60 °C / 30 min

The reactions were repeated using templates biotinylated at the 5'-end and ssDNA was obtained after magnetoseparation. Formation of all desired products (for sequences see Table 7) was confirmed by MALDI-TOF analysis (Table 10).

Having the methodology for the preparation of site-specifically minor-groove modified DNA in hand, we decided to study the effect of these alkylamino modifications on the stability of the DNA duplexes. Denaturing temperatures of the duplexes were measured and are summarised in Table 11. Even though **d^{AA}ATP** and **d^{PA}ATP** are worse substrates than previously studied dATPs bearing small substituents at position 2, the overall stability of the DNA duplexes was higher than previously (Table 5). This suggests that the steric effect of the alkylamino modification is mostly compensated for by the additional hydrogen bond with thymine nucleobase.

Table 10. MALDI data of prepared modified oligonucleotides. [a] Calculated for the desired product extended by one additional A at the 3'-end.

Product	Template used	M (calculated) / Da	M (found) [M+H] / Da
ON31_1^{AA}AC	Temp31_AC-bio	9672.3	9671.5
ON31_1^{PA}AC	Temp31_AC-bio	9670.3	9671.0
ON31_1^{CM}AC	Temp31_AC-bio	9881.5	9885.6
ON31_1^{Cy5}AC	Temp31_AC-bio	10235.7	10236.6
ON31_1^{AA}AT	Temp31_AT-bio	9687.3	9688.1
ON31_1^{PA}AT	Temp31_AT-bio	9685.3	9686.4
ON31_1^{AA}AG	Temp31_AG-bio	9712.3	9713.0
ON31_1^{PA}AG	Temp31_AG-bio	9710.3	9711.3
ON31_1^{AA}AA	Temp31_AA-bio	9696.3	9696.2
ON31_1^{PA}AA	Temp31_AA-bio	9694.3	9695.0
ON31_2^{AA}A	Temp31_AA-bio	9751.3	9752.3
ON31_2^{PA}A	Temp31_AA-bio	9747.3	9748.1
revON31_1^{PA}AT	revTemp31_AT-bio	9783.3 [(a)]	9784.2
revON31_1^{Cy3}AT	revTemp31_AT-bio	10323.3 [(a)]	10323.2
ON16_1^{Cy5}A	Temp31_AC-bio	5605.7	5606.6

Table 11. Denaturing temperatures of DNA duplexes. [(a)] $\Delta T_m = (T_{m\,mod} - T_{m+})/n_{mod}$

DNA	T_m / °C	ΔT_m / °C [(a)]	DNA	T_m / °C	ΔT_m / °C
DNA31_AC	82.2 ± 0.1	–	DNA31_AG	80.5 ± 0.1	–
DNA31_1^{AA}AC	82.8 ± 0.1	+ 0.6	DNA31_1^{AA}AG	81.0 ± 0.2	+ 0.5
DNA31_1^{PA}AC	82.7 ± 0.2	+ 0.5	DNA31_1^{PA}AG	80.4 ± 0.1	– 0.1
DNA31_1^{CM}AC	81.9 ± 0.2	– 0.3	DNA31_AA	79.9 ± 0.2	–
DNA31_1^{Cy5}AC	81.0 ± 0.2	– 1.2	DNA31_1^{AA}AA	79.9 ± 0.2	0
DNA31_1^{Cy3}A_1^{Cy5}A	80.2 ± 0.2	– 2.0	DNA31_1^{PA}AA	79.7 ± 0.2	– 0.2
DNA31_AT	79.4 ± 0.2	–	DNA31_2^{AA}A	79.8 ± 0.2	– 0.05
DNA31_1^{AA}AT	79.9 ± 0.3	+ 0.5	DNA31_2^{PA}A	79.1 ± 0.1	– 0.4
DNA31_1^{PA}AT	79.3 ± 0.3	– 0.1			

3.2.3 Post-synthetic modification of site-specifically minor-groove modified DNA and its application

Similarly to our previous study, we wanted to know, whether it would be possible to perform click reaction on the site-specifically minor-groove modified DNA. Based on our previous experience, we envisaged the allyl group for the thiol-ene reaction, whereas the propargyl group was planned to react in CuAAC. Reaction of modified DNA with fluorescent labels once again allowed for easy monitoring of the progress of the reaction. We chose the same fluorescent thiol as previously, the coumarinemethylthiol (**CM-SH**, Figure 28a) and commercially available cyanine 5 azide (**Cy5-N_3**). Thus, the allylamino modified DNA (DNA31_1^{AA}AC) reacted in thiol-ene reaction with an excess of **CM-SH** in TEAA buffer for 3 days resulting in blue-fluorescent conjugate (DNA31_1^{CM}AC, Figure 28a). The measurement of the emission spectra of the product, after purification by size-exclusion columns, showed a signal characteristic for the coumarine label, while the natural DNA (DNA31_AC) treated with the same reagent showed (after purification) only background level of fluorescence (Figure 28b). The fluorescently labelled DNA31_1^{CM}AC was visualised by UV irradiation (365 nm) (Figure 28c).

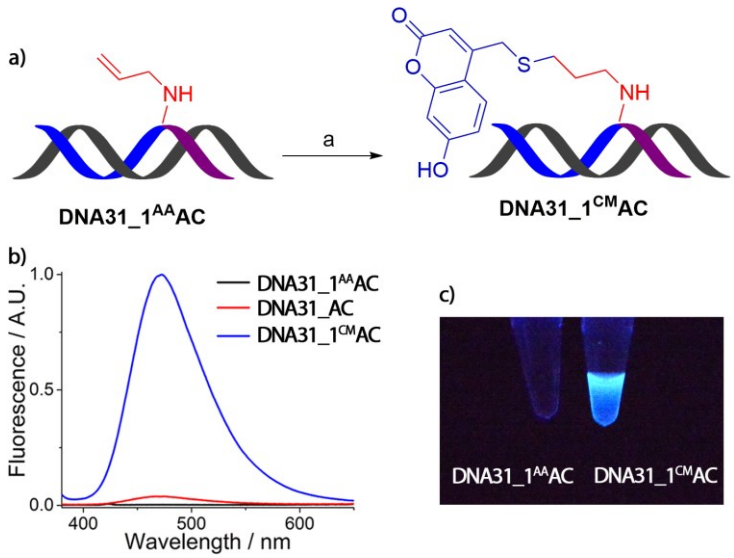


Figure 28. a) Depiction of thiol-ene reaction between DNA bearing an allylamino modification and coumarinemethylthiol (**CM-SH**) providing the fluorescently labelled DNA31_1^{CM}AC. b) Normalised fluorescence spectra of DNA31_^{AA}AC before and after thiol-ene reaction with **CM-SH** compared to natural DNA31_AC treated with the same reagent. c) Photograph of the vials containing the fluorescent DNA31_1^{CM}AC compared to non-fluorescent starting DNA31_1^{AA}AC upon UV irradiation (365 nm). Reaction conditions: a) **CM-SH**, TEAA buffer, 37 °C, 3 days.

The CuAAC reaction with propargylamino modified DNA (DNA31_1^{PA}AC) proceeded smoothly with **Cy5-N$_3$** (Figure 29a) in the presence of CuBr and Cu^{I} binding ligand TBTA in DMSO/H_2O/*t*-BuOH mixture in 24 hours (Figure 29b). We realised that in this case sodium ascorbate in not necessary for the reaction to take place (it is used to when reaction are conducted with Cu^{II} salts, such as $CuSO_4$). The reaction provided the fluorescently labelled DNA31_1^{Cy5}AC after purification. Once again, the fluorescence spectra of the prepared product were measured, confirming the presence of the cyanine label, while natural DNA (DNA31_AC), which was subjected to the same reaction conditions, did not exhibit any fluorescence (Figure 29c). Upon irradiation of the sample of DNA31_1Cy5AC by 590 nm light (based on the absorption spectra of the label) the red fluorescence of the product was visible (Figure 29d). Formation of both products of click reactions was also confirmed by MALDI-TOF analysis (Table 10).

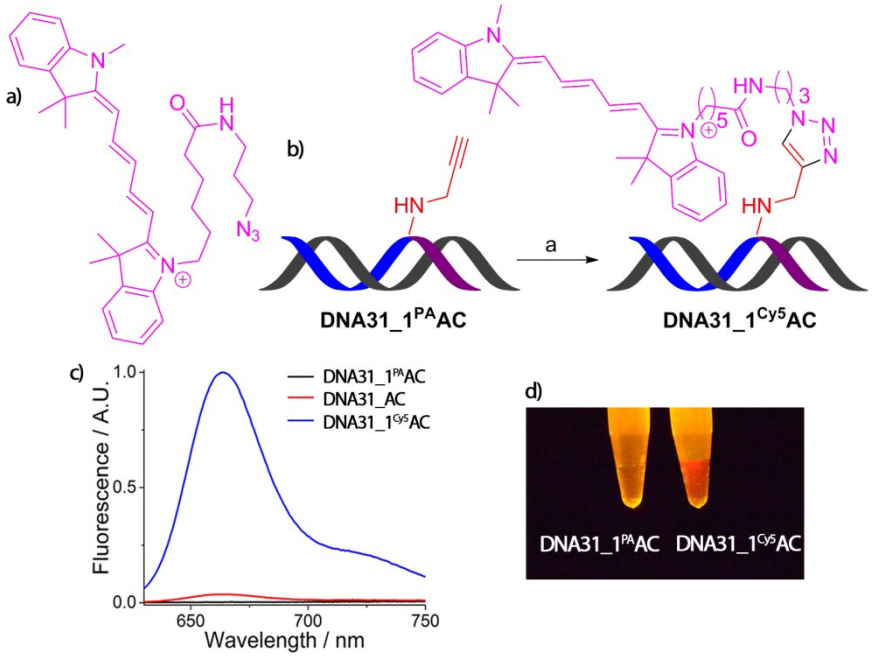


Figure 29. a) Structure of **Cy5-N$_3$**. b) Depiction of CuAAC reaction between DNA bearing an propargylamino modification and cyanine 5 azide (**Cy5-N$_3$**) providing the fluorescently labelled DNA31_1^{Cy5}AC. b) Normalised fluorescence spectra of DNA31_^{PA}AC before and after CuAAC reaction with **Cy5-N$_3$** compared to natural DNA31_AC treated with the same reagent. c) Photograph of the vials containing the fluorescent DNA31_1^{Cy5}AC compared to non-fluorescent starting DNA31_1^{PA}AC upon irradiation (590 nm). Reaction conditions: a) **Cy5-N$_3$**, CuBr, TBTA, DMSO/H_2O/t-BuOH, rt, 24 h.

We also performed PAGE analysis of the products of the click reactions in order to determine the conversion of the reaction (Figure 30). Both click reactions were repeated with FAM-labelled primer (Prim15-FAM) and the products were purified and

analysed by PAGE. The conversion of the CuAAC reaction reached completion (ON31_1^{Cy5}AC) similarly to the previous case. Surprisingly, also the thiol-ene reaction showed a complete conversion of the starting DNA to a product of lower electrophoretic mobility (ON31_1^{CM}AC). This is most probably the result of better access of the allyl group (compared to the vinyl group, Figure 19).

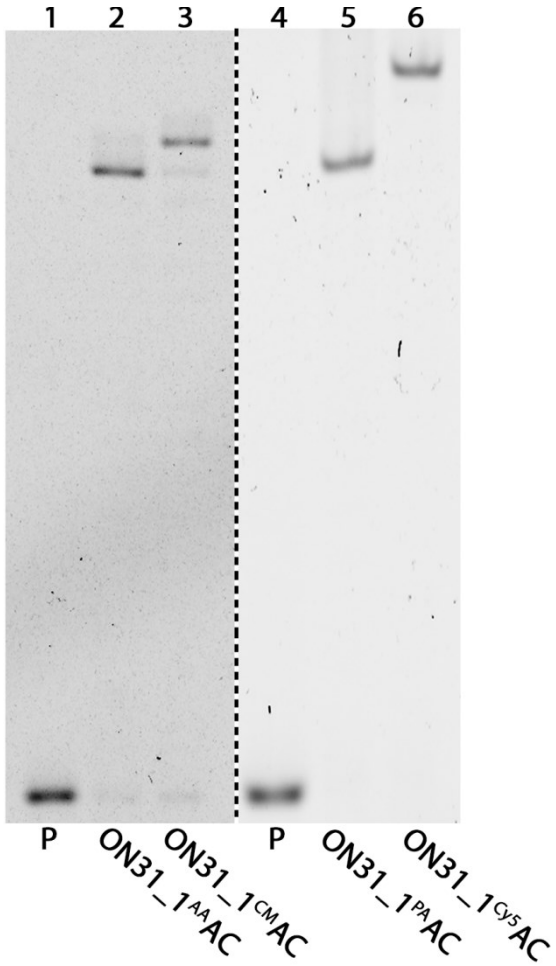


Figure 30. Denaturing PAGE showing results of a thiol-ene or CuAAC reaction of starting allyl- of propargylamino- modified dsDNA (DNA31_1^{AA}AC or DNA31_1^{PA}AC). Lanes 1 and 4 P: primer; lanes 2 and 5: products of SNE of either **d^{AA}ATP** or **d^{PA}ATP** followed by extension with natural dNTPs; lanes 3 and 6: products of thiol-ene (ON31_1^{CM}AC) or CuAAC (ON31_1^{Cy5}AC) reaction respectively.

We have shown that **d^{R}ATP**s from this study are bad substrates for DNA polymerases. However, during the SNE an excess of **d^{R}ATP**s is used (generally 3-10 equiv. in semi-preparative scale). Since no additional purification step is taking place after the SNE is finished and before natural dNTPs are added, the unreacted **d^{R}ATP**s are still present in the reaction mixture. One can therefore dispute that even though these compounds are bad substrates, there is still a possibility of incorporation of the

modified nucleotides somewhere along the synthesised DNA strand instead of natural dATP. Although unlikely, we still needed to design an experiment that would prove it. Since we observed full conversion of the click reactions, we can expect that also any allyl or propargyl group incorporated outside of the expected site would react in these reactions. We therefore decided to use click reactions for this experiment. Thus, the semi-preparative SNE with optimised conditions was repeated with Temp31_AC and Prim15 and either 3 equiv of **d^{AA}ATP** (Figure 31a) or 5 equiv. of **d^{PA}ATP** (Figure 31b). Additionally, the optimised amount of dNTPs, normally used for the second step of the reaction, was added immediately. The reactions were heated at 60 °C for 30 minutes (time of the final extension step, i.e. the time the unreacted **d^{R}ATP**s are normally still present in the reaction mixture with natural dNTPs). The products of these reactions (DNA31_X^{AA}AC; Figure 31a and DNA31_X^{PA}AC; Figure 31b), with potentially some allyl- or propargylamino groups, were then subjected to click reactions based on optimised conditions. Fluorescence of these potential click reaction products (DNA31_X^{CM}AC and DNA31_X^{Cy5}AC) was recorded and compared to the spectra of the natural DNA (DNA31_AC) treated with the same reagents and also with the products of successful click reactions (DNA31_1^{CM}AC and DNA31_1^{Cy5}AC). The results showed that the fluorescence of DNA31_X^{AA}AC and DNA31_X^{PA}AC was on the same level as that of natural DNA31_AC, meaning that only the residual fluorescent labels still remaining in the sample after purification were responsible for the fluorescence (the fluorescence of the click products was at least 20x higher) and only natural DNA was present in the samples containing DNA31_X^{AA}AC and DNA31_X^{PA}AC (Figure 32). It was concluded that no (significant) incorporation of **d^{R}ATP**s is taking place in the presence of natural dATP. (The same result was observed when the enzymatic reaction described above was conducted with biotinylated template and the finished PEX product was submitted to MALDI-TOF analysis; no allyl or propargylamino modified DNA signal was found; for copies of MALDI spectra, see Appendix 2.)

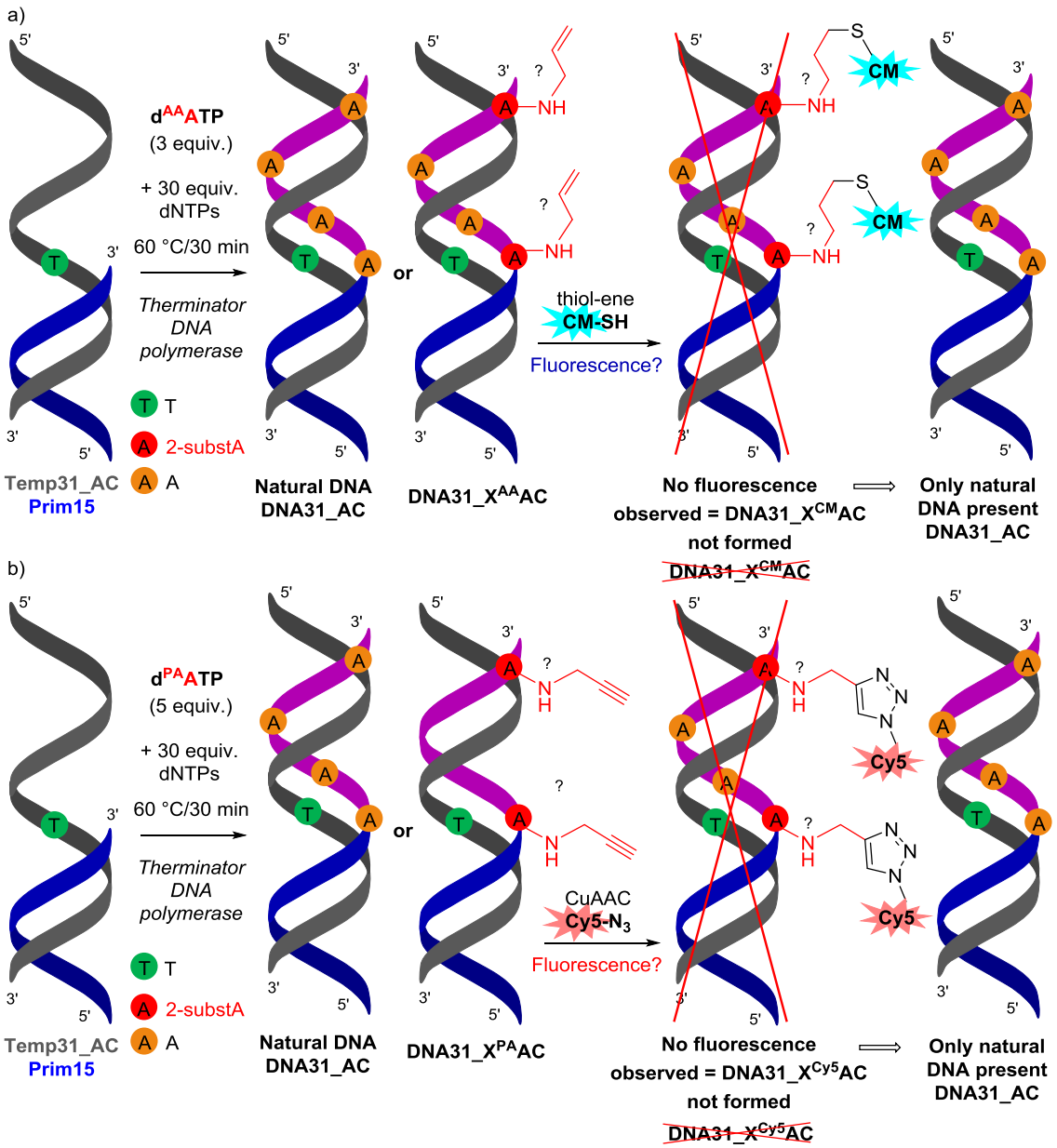


Figure 31. PEX in the presence of *Therminator* DNA polymerase, Temp31_AC, Prim15, excess of natural dNTPs and a) 3 equiv. of **d^{AA}ATP** or b) 5 equiv. of **d^{PA}ATP**, followed by a) thiol-ene reaction with **CM-SH** or b) CuAAC reaction with **Cy5-N$_3$** to test whether any partial incorporation of **d^{R}ATP**s takes place in the presence of natural dATP.

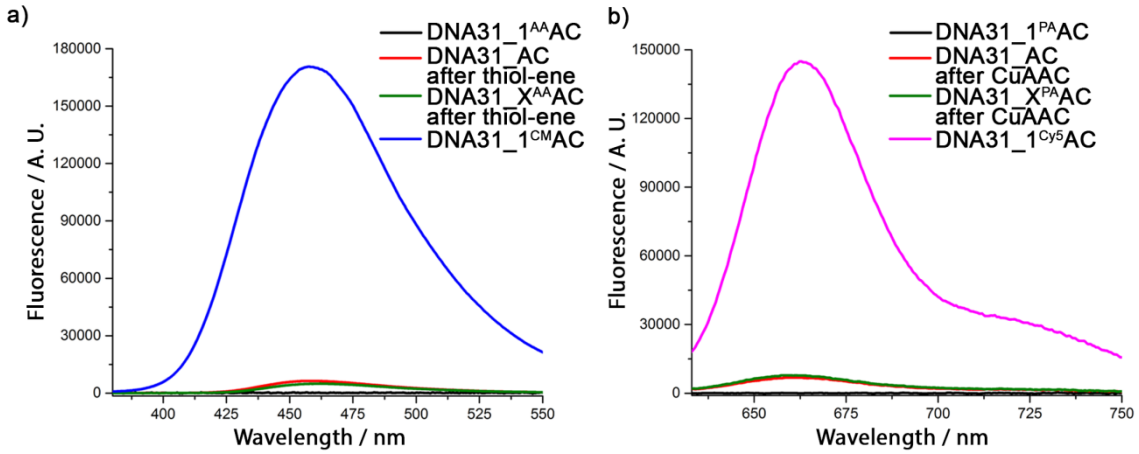


Figure 32. Corresponding fluorescence spectra to the experiments described in Figure 31. a) Fluorescence spectra of the products of PEX with natural dNTPs in presence of **d^{AA}ATP** followed by thiol-ene reaction (green curve) in comparison with natural DNA (red curve) and a 31-mer ON containing one CM-modified adenine **DNA31_1^{CM}AC** (blue curve). b) Fluorescence spectra of the products of PEX with natural dNTPs in presence of **d^{PA}ATP** followed by CuAAC reaction with **Cy5-N_3** (green curve) in comparison with natural DNA (red curve) and a 31-mer ON containing one Cy5-modified adenine **DNA31_1^{Cy5}AC** (magenta curve).

Finally, we decided to present a possible application of this newly developed method for DNA modification. Having already prepared fluorescently labelled DNA, we decided to prepare a double stranded DNA (dsDNA) bearing one fluorophore in each strand. This setup can in principle provide an interaction between the two fluorophores, pending their relative proximity. Förster resonance energy transfer (FRET) is a mechanism of energy transfer between two light-sensitive molecules. One of the molecules represents an energy donor (D), while the other one accepts this energy (acceptor; A). In the case of fluorophores, upon excitation of the D molecule by the light of appropriate wavelength, the energy is exhibited (among others) by emission of light of higher wavelength. If this light is then able to excite the A molecule (emission spectra of D and absorption spectra of A have to overlap), an energy transfer between D and A is occurring. A then releases this energy again in the form of fluorescence. Therefore the result of the experiment is that by excitation of D signal of fluorescence of A should be recorded. FRET has been used quite extensively, both in the nucleic acid research[211] and also for intracellular applications.[212] Even minimal changes in the distances between D and A are easily detectable, since the transferred energy is dependent on the distance between the two fluorophores to the power of six (Figure 33).

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

Figure 33. Formula for the calculation of FRET efficiency (E). r is the distance between D and A and R_0 is the Förster distance of the two fluorophores (distance at which the efficiency of the energy transfer is 50%; intrinsic quality of the two fluorophores used, i.e. a constant).

This phenomena has been used for the construction of DNA probes able to monitor the binding of small molecules to DNA,[213] to monitor the DNA origami assembly[214] or for DNA probes with the potential use in sensing and diagnostics.[215,216] It is apparent that the distance between D and A Is crucial for a successful energy transfer to take place. Out methodology of site-specific DNA modification is therefore useful for the construction of DNA FRET probes. We chose to prepare two complementary ONs, one bearing cyanine 3 and one bearing cyanine 5. These fluorophores were chosen based on their previously described use in FRET experiments[217,218] and also because the CuAAC with the corresponding azides (**Cy3-N_3** and **Cy5-N_3**) is more efficient (and faster) than the thiol-ene reaction. Thus we prepared two DNAs modified by propargylamino groups (for sequences see Table 7), where the first one was synthesised following the already optimised conditions of SNE with Temp31_AC-bio followed by magnetoseparation (DNA31_1^{PA}AC). The other ON needed to be complementary, so a corresponding template was designed (revTemp31_AT) and the reaction conditions were optimised for a successful SNE and extension on an analytical scale (for completion, the reaction conditions were optimised for both **d^{AA}ATP** and **d^{PA}ATP**; Figure 34), and also on semi-preparative scale, where the reaction was performed with a biotinylated template, preparing the required DNA (revDNA31_1^{PA}AT). (For conditions, see Tables 8,9.)

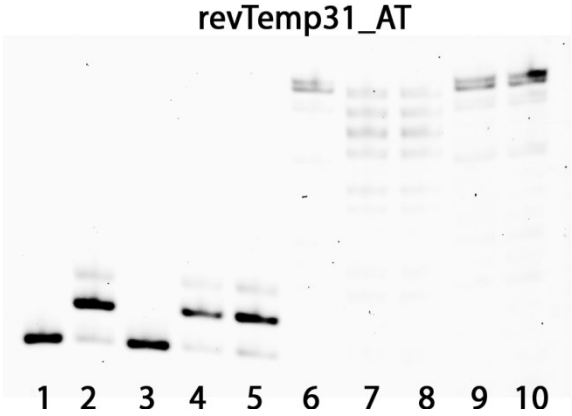


Figure 34. PAGE analysis of polymerase synthesis of DNA site-specifically labelled in the minor groove for revTemp31_AT template. Lane 1: primer; lane 2: 16-mer ON standard; lane 3: negative control for SNE (no dNTPs present); lanes 4, 5: SNE of **d^{R}ATP** (only **d^{AA}ATP** or **d^{PA}ATP** present in the reaction mixture); lane 6: positive control experiment – product after PEX with natural dNTPs; lanes 7, 8: negative control experiment for PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (PEX in the presence of dGTP, dCTP and dTTP; absence of dATP); lanes 9, 10: product of PEX after SNE of either **d^{AA}ATP** or **d^{PA}ATP** (all natural dNTPs present for final extension).

Next, we performed CuAAC reactions with propargylamino modified DNAs followed by magnetoseparation. As previously, DNA31_1^{PA}AC reacted with **Cy5-N$_3$** ultimately giving ON31_1^{Cy5}AC as a product, whereas revDNA31_^{PA}AT reacted with **Cy3-N$_3$** which provided revON31_1^{Cy3}AT after magnetoseparation (identity of all ONs used in these studies was confirmed by MALDI-TOF, Table 10). The principle of the performed experiment is outlined in Figure 35a. We recorded the fluorescence spectra of the cyanine 3 labelled ON caused by irradiation by 535 nm light (Figure 35b, black curve) and afterwards added the same amount of the cyanine 5 labelled ON. Heating and cooling of the sample (annealing) afforded the DNA31_1^{Cy3}A_1^{Cy5}A. The denaturing temperature of this DNA duplex was approximately 80 °C and it did not significantly differ from that of the corresponding natural DNA (Table 11). Finally, we measured the emission spectra of this duplex upon irradiation by 535 nm light and FRET was observed (emission at 665 nm, Figure 35b, green curve). We then proceeded to heat the sample to the measured denaturing temperature of the duplex, upon which we observed (partial) ceasing of the FRET (disappearance of the signal at 665 nm, rising of the signal at 565 nm, Figure 35b, blue curve). After cooling of the sample, the ONs reannealed and FRET was restored (Figure 35b, red curve).

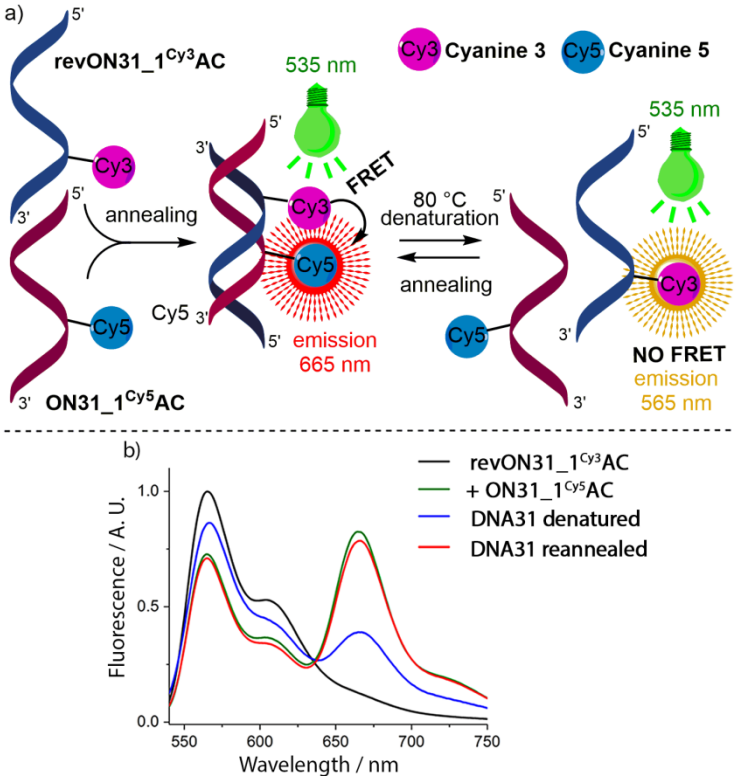


Figure 35. a) FRET during denaturation and reannealing of Cy3- and Cy5-linked complementary ONs. b) Corresponding emission spectra.

Since the first (proof-of-concept) experiment worked, we decided to test, whether it would be possible to use FRET for the detection of ONs. The idea of the experiment was that disappearance of FRET would be observed when a more stable DNA duplex is formed (Figure 36a). To this end, we repeated the synthesis of revON31_1^{Cy3}AT and prepared a new shorter complementary strand ON16_1^{Cy5}A. It was prepared by performing SNE with **d^{PA}ATP** and Temp31_AC-bio without subsequent extension by natural dNTPs, followed by CuAAC with **Cy5-N$_3$** and magnetoseparation. Prepared ONs were annealed together and the fluorescence spectra was recorded (Figure 36b, green curve) showing FRET and emission signal at 665 nm (Figure 36b, green curve). Upon titration of the sample by 0.5 equiv. of ON31_target (= Temp31_AC), heating and annealing, partial loss of the emission at 665 nm was observed (Figure 36b, blue curve). Addition of another 0.5 equiv. of the target ON resulted in complete disappearance of the 665 nm signal and ceasing of the FRET (Figure 36b, red curve), suggesting that the short ON16_1^{Cy5}A was fully replaced by the full-length complementary ON31_target. (Absorption spectra of the samples used for both experiments were measured in order to confirm the same concentration of DNA; see Appendix 3.)

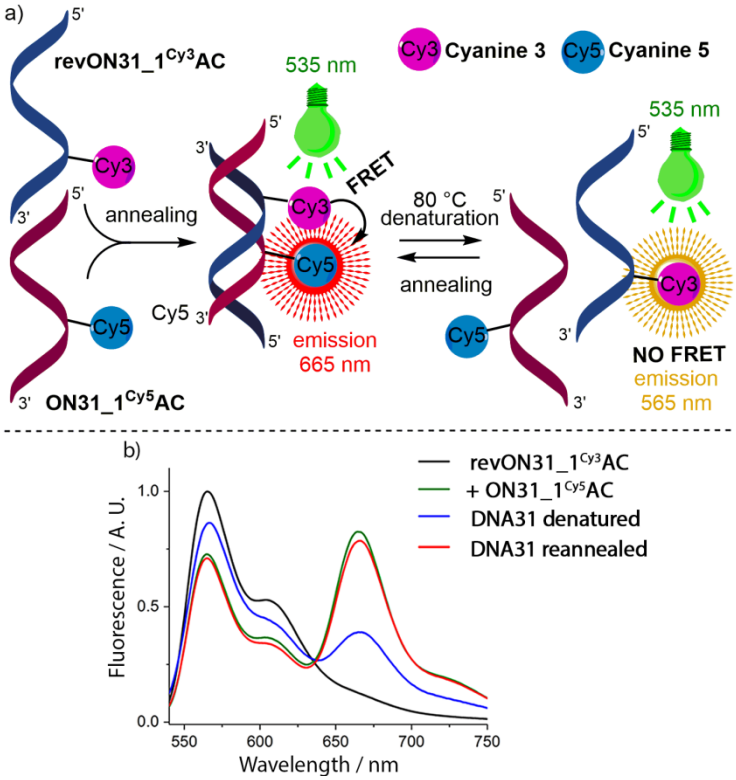


Figure 36. a) FRET during of Cy3- and Cy5-linked complementary ONs and its disappearance upon rehybridisation of the Cy3-modified ON with the full-length complementary ON. b) Corresponding emission spectra.

To conclude, we prepared a series of 2-alkylamino modified dATPs and tested them as substrates for DNA polymerases in enzymatic synthesis of DNA modified in the minor groove. Apart from **d^{MA}ATP**, none of the **d^{R}ATP**s were incorporated, however Therminator DNA polymerase was able to perform single nucleotide extensions using **d^{AA}ATP** and **d^{PA}ATP** and different templates. Addition of natural dNTPs afforded the full-length DNAs modified at a specific position (based on the primer and template used). The conditions had to be optimised for each sequence and also for both reactions on analytical scale and on semi-preparative scale. Allyl- and propargylamino-modified DNAs were used for post-synthetic modification of DNA minor groove using click reactions with fluorescent labels. This approach was then used for the construction of FRET probes for the detection of ONs.

3.3 2-Substituted 2'-deoxyinosine triphosphates as substrates for DNA polymerases in enzymatic synthesis of minor-groove modified DNA; effect of minor-groove modifications on cleavage of DNA by restriction endonucleases

Enzymatic synthesis of minor-groove modified DNA has become one of the ways of preparation of (base-)modified DNA. We have shown that 2-substituted 2'-deoxyadenosine triphosphates bearing small substituents (chloro, amino, methyl, vinyl and ethynyl) are good substrates for DNA polymerases. Later, Gowda et al. described that N^2-substituted 2'-deoxyguanosine nucleotides were substrates for human DNA polymerase κ.[210] We then developed a methodology for site-specific minor-groove modified DNA enzymatic synthesis utilising 2-alkylamino modified dATP derivatives. Since enzymatic synthesis of DNA using 2-substituted dATP derivatives was developed, we wanted to extend the pool of possible DNA polymerase substrates also to dGTP analogues. To this end, we designed a series of 2-substituted 2'-deoxyinosine triphosphates (**d^{R}ITP**s) to be tested for enzymatic synthesis of minor-groove modified DNA. The resulting modified hypoxanthines (^{R}H) should represent guanine base surrogates and base-pair with C in the synthesised DNA (Figure 37).

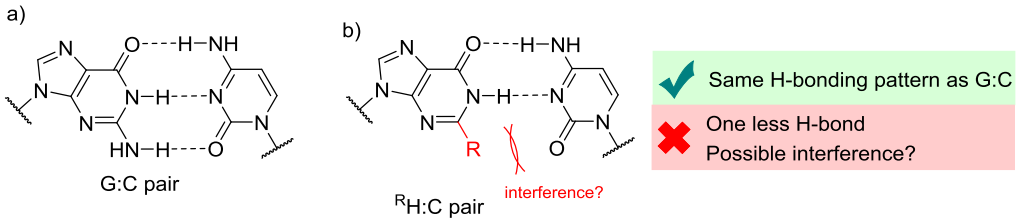


Figure 37. Watson-Crick hydrogen bonding between cytosine and a) guanine or b) modified hypoxanthine nucleobase.

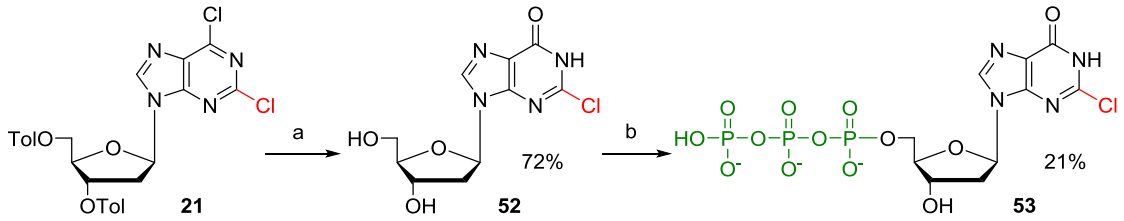
Type II restriction endonucleases (REs) are enzymes that recognise palindromic DNA sequences and cleave the DNA. They represent an important tool is gene analysis cloning.[161,219] They recognise the target site and bind to DNA mostly through interactions with the DNA major groove. The studies of the effect of modification on the RE cleavage were scarce,[162–165] until a comprehensive series of papers from the Hocek group described the effects of major-groove modifications on cleavage of DNA by REs.[166–169] The studies identified modifications which were tolerated by some REs and concluded that modifications at the C:G pair are not allowed, while it is possible to modify the A:T pair by small substituents. These finding were utilised in the

development of transient protection strategies against RE cleavage using silylation[170] or photocaging.[171] These studies were then used as a basis for the study of transcription; a more complex biological mechanism. Study of the effect of major-groove modification of DNA on bacterial transcription once again revealed that some modifications are tolerated during this process.[220] Later, transcription switches have been developed, allowing for switching of off the transcription using CuAAC click chemistry[221] or switching on of the transcription upon photodeprotection (the transcription was then switched off again by enzymatic phosphorylation).[174]

Studies of the effect of various DNA modifications on RE cleavage were useful as a basis for the study of a more complex biological process. Therefore we set out to explore the effects of minor-groove modifications on the cleavage of DNA by REs. Although REs bind to DNA mainly through interactions in the DNA major groove, a small number of individual studies showed that also some modifications at the minor-groove site of the nucleobases affected the RE cleavage (partial or complete inhibition of DNA cleavage).[175–182] We set out to prepare DNAs modified in the RE recognition sites by minor-groove modifications. To this end we wanted to utilise the already prepared **d^{R}ATP**s that were good substrates for DNA polymerases, but also the newly prepared **d^{R}ITP**s, representing analogues of dGTP enzymatic synthesis of DNA.

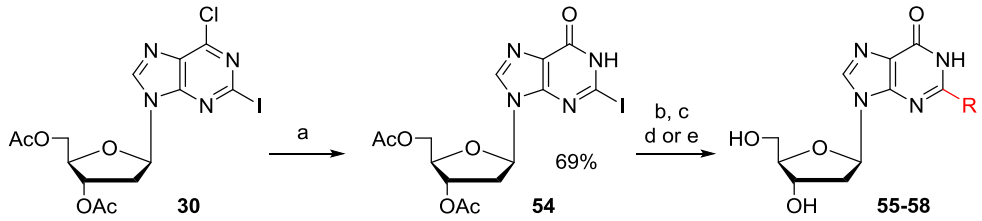
3.3.1 Synthesis of 2-substituted 2'-deoxyinosine triphosphates (d^{R}ITPs)

The synthesis started similarly to previous studies by the preparation of corresponding nucleosides. The chloro substituted derivative **52** was prepared from the already described per-toluoylated purine nucleoside **21**. The hydrolysis of the chlorine atom at position 6 was accomplished by treatment of **21** with DABCO, CsOAc and Et_3N in DMF, which was followed by deprotection of the ester protecting groups by K_2CO_3 in MeOH, providing **52** in 72% yield (it was mentioned in the literature, however, no synthetic or characterisation data is available).[68] The nucleoside was then phosphorylated using standard phosphorylation conditions and the 2-chloro modified nucleotide **53** (**d^{Cl}ITP**) was isolated in 21% yield (Scheme 26).



Scheme 26. Synthesis of 2-chloro-2'-deoxyinosine triphosphate. Reaction conditions: a) 1. DABCO, CsOAc, Et_3N, DMF, rt, overnight; 2. K_2CO_3, MeOH, rt, 1 h; b) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min.

2-Iodo modified purine nucleoside **54** was used as a common intermediate for the synthesis of remaining nucleosides. It was prepared from the already described nucleoside **30** following the DABCO-mediated hydrolysis described above in 69% yield. The desired nucleosides were then prepared from intermediate **54** in two steps; a Pd-catalysed cross-coupling reaction followed by deprotection. First, reaction of **54** with Me_3Al and $Pd(PPh_3)_4$ in THF provided followed by deprotection afforded the 2-methyl derivative **55** in 97% yield (nucleoside was claimed by a patent with no characterisation or synthetic data available).[222] Suzuki-Miyaura cross-coupling of **54** with an excess of either potassium vinyltrifluoroborate or phenylboronic acid in the presence of Cs_2CO_3, $Pd(OAc)_2$ and TPPTS in aqueous acetonitrile followed by deprotection provided the 2-vinyl (**56**) and (previously synthesised)[223] 2-phenyl (**57**) modified inosines in the yields of 74% and 92% respectively. Sodium methoxide in MeOH was used for the deprotection in all three cases. Finally, nucleoside **54** reacted in Sonogashira cross-coupling reaction with trimethylsilylacetylene in the presence of $PdCl_2(PPh_3)_2$, CuI and Et_3N in DMF, followed by treatment with methanolic ammonia to afford the 2-ethynyl derivative **58** in 73% yield (Scheme 27). The reaction conditions and yields of the reactions providing desired nucleosides are summarised in Table 12.

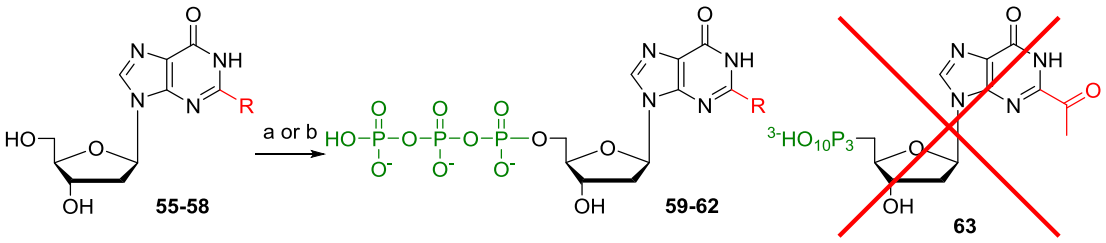


Scheme 27. Synthesis of 2-modified 2'-deoxyinosines using cross-coupling reactions. Reaction conditions: a) 1. DABCO, CsOAc, Et_3N, DMF, rt, overnight; b) 1. Me_3Al, $Pd(PPh_3)_4$, THF, 60 °C, 3.5 h; 2. MeONa, MeOH, rt, overnight; c) 1. potassium vinyltrifluoroborate, Cs_2CO_3, $Pd(OAc)_2$, TPPTS, CH_3CN/H_2O, 80 °C, 2.5 h; 2. MeONa, MeOH, rt, overnight; d) $PhB(OH)_2$, $Pd(OAc)_2$, Cs_2CO_3, TPPTS, CH_3CN/H_2O, 80 °C, 1.5 h; 2. MeONa, MeOH, rt, overnight; e) 1. trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, DMF, rt, 3 h; 2. methanolic ammonia, rt, overnight.

Table 12. Summarised results of preparation of 2-modified 2'-deoxyinosines.

Entry	Product	R substituent	Conditions	Yield [%]
1	**55**	methyl	b	97
2	**56**	vinyl	c	74
3	**57**	phenyl	d	92
4	**58**	ethynyl	e	73

Prepared nucleosides were then subjected to phosphorylation reaction affording the corresponding nucleoside triphosphates. The 2-methyl (**59**, **d^{Me}ITP**), 2-vinyl (**60**, **d^{V}ITP**) and 2-phenyl (**61**, **d^{Ph}ITP**) modified derivatives were prepared following the standard phosphorylation procedure in 56%, 31% and 39% yields respectively. The reaction conditions had to be modified for the preparation of 2-ethynyl modified nucleotide (**62**, **d^{E}ITP**) due to previously described[169] reactivity issues of the ethynyl group attached to the guanine base at position 7. Previously, transformation of the ethynyl group to acetyl has been observed, however in our case the acetyl derivative **63** was never isolated, only a complex mixture of products was obtained following the standard phosphorylation conditions. The reaction was therefore performed with the addition of proton sponge in the first step of the reaction and additionally 1 M NH_4HCO_3 was used to neutralise the reaction instead of 2 M TEAB. A solution of NH_4HCO_3 was also used during the HPLC purification of the final nucleotide **62**, after which it was isolated in 17% yield (Scheme 28). The phosphorylation conditions and yields are summarised in Table 13.



Scheme 28. Synthesis of **d^{R}ITP**s. Reaction conditions: a) 1. $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 2M TEAB, 0 °C – rt, 1 min; b) 1. proton sponge, $PO(OMe)_3$, $POCl_3$, 0 °C, 3 h; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0°C, 1 h; 3. 1M NH_4HCO_3, 0 °C – rt, 1 min.

Table 13. Summarised results of phosphorylation of 2-modified 2'-deoxyinosines.

Entry	Starting nucleoside → product	R substituent	Conditions	Yield [%]
1	**55 → 59**	methyl	a	56
2	**56 → 60**	vinyl	a	31
3	**57 → 61**	phenyl	a	39
4	**58 → 62**	ethynyl	b	17

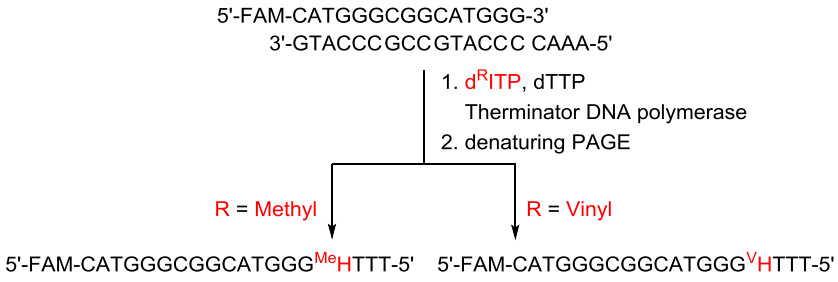
Notably, although the identity of the desired product was confirmed by NMR spectra and HRMS analysis, the ^{13}C NMR spectra of **62** did not show all of the signals of aromatic carbons (most probably due to tautomerism) even after 10 000 scans (see Appendix 4 for the copies of NMR and HRMS spectra of **62**).

3.3.2 d^{R}ITPs in enzymatic synthesis of minor-groove modified DNA

All of the synthesised **d^{R}ITP**s were then tested as substrates in enzymatic synthesis of minor-groove modified DNA. First, we performed PEX in the presence of different DNA polymerases, Prim15-FAM and Tem19G (for sequences of ONs used in this study, see Table 14). Therminator DNA polymerase was able to efficiently synthesise the full-length product bearing one modification in the newly synthesised DNA strand using **d^{Me}ITP** and **d^{V}ITP** (DNA19_1^{R}H, denotation ^{R}H is used, since modified hypoxanthine bases are being incorporated into DNA) (Scheme 29). Unfortunately, none of the other **d^{R}ITP**s were substrates for this DNA polymerase as shown by PAGE analysis (Figure 38). Other DNA polymerases were also tested, however these enzymes were not able to synthesise any full-length products using **d^{R}ITP**s (Figure 39).

Table 14. List of ONs used in this study. [a] 6-carboxyfluorescein (6-FAM) used for oligonucleotide labelling at 5'-end; [b] primer sequences in template are in italic; [c] template biotinylated at 5'-end; [d] template labeled by *ortho*-TINA at 5'-end; [e] template labeled by *ortho*-TINA at 5'-end and biotinylated at 3'-end; [f] target palindromic sequence for the restriction enzyme are in bold (position of the modification in the product is underlined)

Oligonucleotide	Sequence 5'→ 3'	Length
Prim15	CATGGGCGGCATGGG	15-mer
Prim15-FAM [(a)]	CATGGGCGGCATGGG	15-mer
Prim16-FAM	GGAGAAGTGAAAGTGG	16-mer
Temp19G [(b)]	AAAC *CCCATGCCGCCCATG*	19-mer
Temp19G-bio [(c)]	AAAC *CCCATGCCGCCCATG*	19-mer
Temp31-TINA [(d)]	CTAGCATGAGCTCAGT *CCCATGCCGCCCATG*	31-mer
Temp31-(bio)TINA [(e)]	CTAGCATGAGCTCAGT *CCCATGCCGCCCATG*	31-mer
Temp16G	C *CCCATGCCGCCCATG*	16-mer
Temp26-bio	GAGGAATTTC *CCACTTTCACTTCTCC*	16-mer
Prim_KpG-FAM	CATGGGCGGCATGGGG	16-mer
Temp_AfA [(f)]	AACGACGAC**AGCGC<u>T</u>** *CCCATGCCGCCCATG*	30-mer
Temp_AfG	AAGTAGTAG**AG<u>C</u>G<u>C</u>T** *CCCATGCCGCCCATG*	30-mer
Temp_EcA	AACGACGAC**GAA<u>TT</u>C** *CCCATGCCGCCCATG*	30-mer
Temp_EcG	AAGTAGTAG**GAATT<u>C</u>** *CCCATGCCGCCCATG*	30-mer
Temp_KpA	AACGACGAC**GG<u>T</u>ACC** *CCCATGCCGCCCATG*	30-mer
Temp_KpG	AAGTAGTAG**GGTA<u>C</u>** ***C****CCCATGCCGCCCATG*	30-mer
Temp_PsA	AACGACGAC**C<u>T</u>GCAG** *CCCATGCCGCCCATG*	30-mer
Temp_PsG	AAGTAGTAG**<u>C</u>TG<u>C</u>AG** *CCCATGCCGCCCATG*	30-mer
Temp_PvA	AACGACGAC**CAGC<u>T</u>G** *CCCATGCCGCCCATG*	30-mer
Temp_PvG	AAGTAGTAG**<u>C</u>AG<u>C</u>TG** *CCCATGCCGCCCATG*	30-mer
Temp_RsA	AACGACGACG**G<u>T</u>AC**G *CCCATGCCGCCCATG*	30-mer
Temp_RsG	AAGTAGTAGT**GTA<u>C</u>**A *CCCATGCCGCCCATG*	30-mer
Temp_SaA	AACGACGAC**GAGC<u>T</u>C** *CCCATGCCGCCCATG*	30-mer
Temp_SaG	AAGTAGTAG**GAG<u>C</u>T<u>C</u>** *CCCATGCCGCCCATG*	30-mer
Temp_ScA	AACGACGAC**AG<u>T</u>AC<u>T</u>** *CCCATGCCGCCCATG*	30-mer
Temp_ScG	AAGTAGTAG**AGTA<u>C</u>T** *CCCATGCCGCCCATG*	30-mer
ON19_1^{R}H	CATGGGCGGCATGGG$^{\mathbf{R}}$**H**TTT	19-mer
ON31_4^{R}H	CATGGGCGGCATGGGACT$^{\mathbf{R}}$**H**A$^{\mathbf{R}}$**H**CTCAT$^{\mathbf{R}}$**H**CTA$^{\mathbf{R}}$**H**	31-mer
ON26_1^{V}H	GGAGAAGTGAAAGTGG$^{\mathbf{V}}$**H**AAATTCCTC	26-mer



Scheme 29. Depiction of successful PEX in the presence of Therminator DNA polymerase, Prim15-FAM, Temp19G, dTTP and modified **d^{R}ITP**.

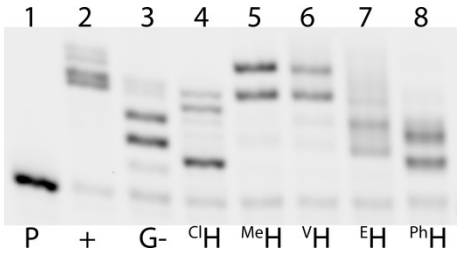


Figure 38. Denaturing PAGE of PEX experiment in presence of Therminator DNA polymerase, Prim15-FAM and Temp19G. Lane: 1, P: primer; lane 2, +: product of PEX with natural dNTPs; lane 3, G-: product of PEX with dTTP, dCTP, dATP; lanes 4-8, ^{R}H: products of PEX with dTTP, dCTP, dATP and functionalized **d^{R}ITP** used instead of natural dGTP.

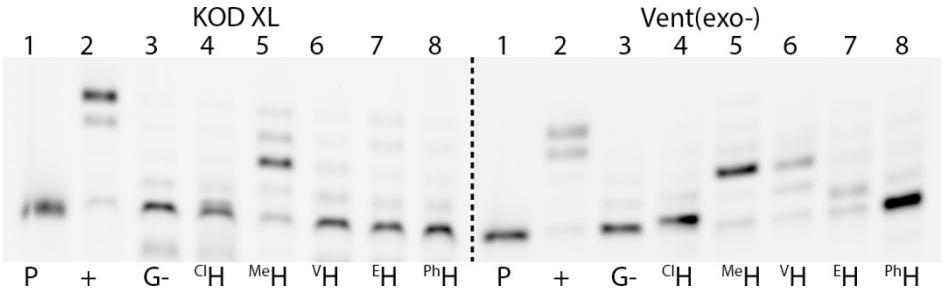


Figure 39. Denaturing PAGE of PEX experiment in presence of either KOD XL or Vent(exo-) DNA polymerases, Prim15-FAM and Temp19G. Lanes: 1, P: primer; lanes 2, +: products of PEX with natural dNTPs; lanes 3, G-: products of PEX with dTTP, dCTP, dATP; lanes 4-8, ^{R}H: products of PEX with dTTP, dCTP, dATP and functionalized **d^{R}ITP** used instead of natural dGTP.

Having established that **d^{R}ITP**s are worse substrates that corresponding **d^{R}ATP**s for DNA polymerases, we also performed PEX with Therminator DNA polymerase, Prim15-FAM and Temp31-TINA (for structure of TINA modification, see Figure 12a), which encodes for four modified hypoxanthine bases to be incorporated in the synhtesised DNA strand. PAGE analysis showed full-length products for the cases where **d^{Me}ITP** and **d^{V}ITP** were used instead of natural dGTP. Once again, none of the other experiments resulted in full-length products (Figure 40). Identity of all synthesised products was confirmed by repeating of the PEX with biotinylated templates,

magnetoseparation and MALDI-TOF analysis of the acquired ONs (ON19_1^{R}H and ON31_4^{R}H, Table 15).

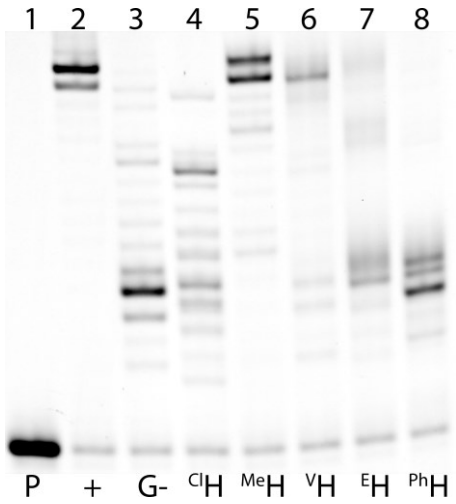


Figure 40. Denaturing PAGE of PEX experiment in presence of Therminator DNA polymerase, Prim15-FAM and Temp31-TINA. Lane: 1, P: primer; lane 2, +: product of PEX with natural dNTPs; lane 3, G-: product of PEX with dTTP, dCTP, dATP; lanes 4-8, ^{R}H: products of PEX with dTTP, dCTP, dATP and functionalized **d^{R}ITP** used instead of natural dGTP.

We also studied how efficiently are the **d^{Me}ITP** and **d^{V}ITP** being incorporated into DNA compared to natural dGTP. We conducted a simple kinetic study and compared the rates of the reactions for different nucleotides (Figure 41). PEX was performed using Therminator DNA polymerase, Prim15-FAM and Temp16G for the designated time interval followed by immediate denaturation by heating. We observed that the rate of incorporation of **d^{Me}ITP** is similar to that of natural dGTP (about 5 minutes to reach completion), while the DNA polymerase needed significantly more time to synthesise the DNA bearing the vinyl modified hypoxanthine ^{V}H (> 10 minutes).

Table 15. MALDI data of prepared modified oligonucleotides. [(a)] product of reaction of two vinyl groups; [(b)] product of reaction of one vinyl group, found as a $[M+K]^+$

Product	Template used	M (calculated) / Da	M (found) [M+H] / Da
ON19_1^{Me}H	Temp19G-bio	5914.9	5916.0
ON19_1^{V}H	Temp19G-bio	5926.9	5929.0
ON31_4^{Me}H	Temp31-bio	9613.3	9614.6
ON31_4^{V}H	Temp31-bio	9661.3	9662.8
ON19_1^{CM}H	Temp19G-bio	6134.9	6137.6
ON31_4^{CM}H [(a)]	Temp31-bio	11037.6	11038.3
ON19_1^{Cys}H	Temp19G-bio	6049.0	6050.5
ON31_4^{Cys}H [(b)]	Temp31-bio	10448.4	10448.7
ON26_1^{V}H	Temp26-bio	9652.8	9650.6
ON26_1^{pept_1}H	Temp26-bio	10060.5	10058.4

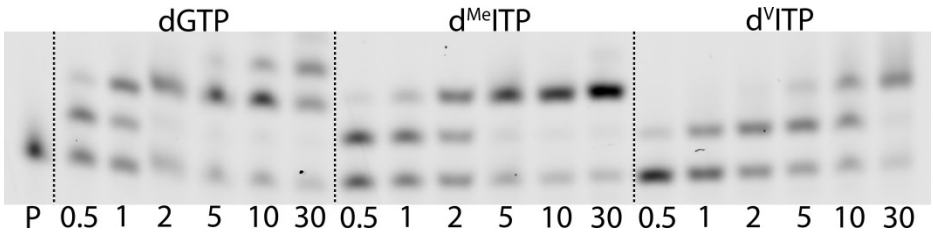


Figure 41. PAGE analyses of kinetic single nucleotide extension experiments with Temp16G using Therminator DNA polymerase and **d^{R}ITP**s in comparison with natural dGTP. Time intervals are given in minutes.

Having all of the modified ONs in hand, we also studied the denaturing temperatures of the corresponding DNA duplexes. Both methyl and vinyl modifications destabilised the DNA duplex equally, which is caused mainly by the missing hydrogen bond normally present in the G:C pair and possibly by some small local disturbances to the B-DNA duplex structure[71] (Table 16).

Table 16. Denaturing temperatures of synthesised DNA duplexes. [(a)] $\Delta T_m = (T_{m\,mod} - T_{m+})/n_{mod}$.

DNA	T_m / °C	ΔT_m / °C [(a)]
DNA19_1G	70.9 ± 0.2	–
DNA19_1^{Me}H	67.7 ± 0.1	– 3.2
DNA19_1^{V}H	67.6 ± 0.1	– 3.3
DNA31_4G	78.6 ± 0.1	–
DNA31_4^{Me}H	70.0 ± 0.2	– 2.2
DNA31_4^{V}H	69.9 ± 0.1	– 2.2

We also performed PCR in the presence of **d^{R}ITP**s with the expected result being DNA modified in both strands. Therminator DNA polymerase, Prim_FOR, Prim_REV and Temp98 (for sequences, see Table 2) reacted in PCR (35 cycles) with **d^{Me}ITP** and **d^{V}ITP**. Agarose gel analysis and GelRed fluorescent staining revealed that these nucleotides are not substrates for this DNA polymerase in PCR, since no product of equal mobility to that of the synthesised natural DNA was observed (Figure 42).

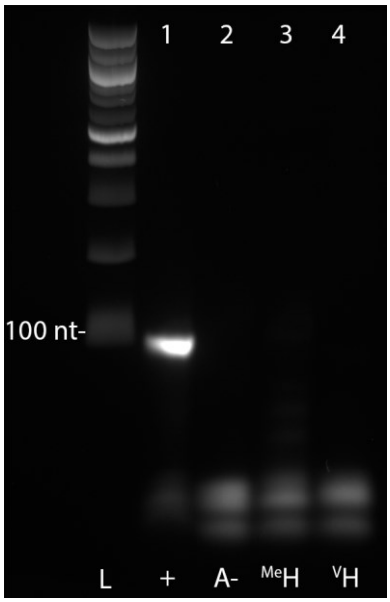


Figure 42. Agarose gel analysis of PCR products amplified by Therminator DNA polymerase in the presence of **d^{R}ITP**s. Lanes: L: 100-nt ladder; 1, +: PCR in the presence of natural dNTPs; 2, A-: PCR in the presence of dCTP, dTTP and dATP; 2,4, ^{R}H: PCR in the presence of corresponding **d^{R}ITP** instead of natural dGTP.

3.3.3 Post-synthetic DNA modification utilising thiol-ene reaction of vinyl-modified DNA

Having a secured synthesis method for the preparation of minor-groove modified DNA, we decided to test the viability of the vinyl group for post-synthetic modification of DNA via thiol-ene reaction with thiols. Similarly to our previous studies, we chose to perform the reaction with fluorescent thiol **CM-SH** in order to be able to easily monitor progress of the reaction (Figure 43a). Thus, the vinyl-modified DNAs (DNA19_1^{V}H and DNA31_4^{V}H) reacted in thiol-ene reaction with an excess of **CM-SH** in TEAA buffer for 3 days. Products of the reaction were purified using size-exclusion columns and fluorescence spectra of the purified products of thiol-ene reaction (DNA19_1^{CM}H and DNA31_4^{CM}H) were recorded. We observed a fluorescence signal characteristic for the attached coumarine label. When we recorded the fluorescence spectrum of natural DNA (DNA19_1G or DNA31_4G), that was subjected to the same reaction protocol and was then purified, we observed only background level of fluorescence, for which the residual coumarine reagent from the reaction was responsible (Figure 43b). Formation of fluorescent products was also possible to visualise by direct UV irradiation (365 nm) of the click reaction products (Figure 43c).

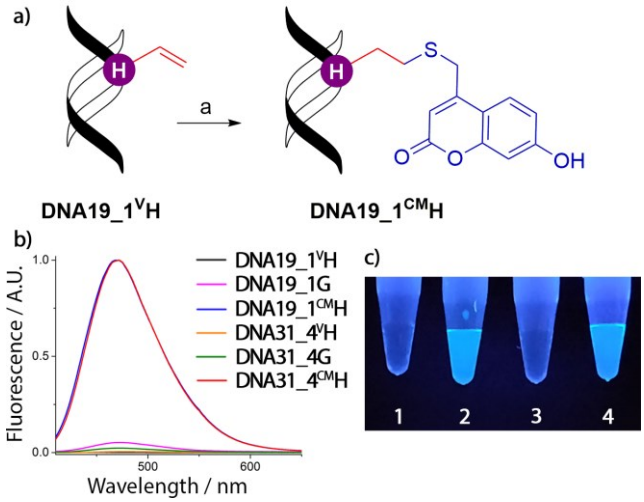


Figure 43. a) Depiction of thiol-ene reaction between DNA19_1^{V}H and coumarinemethylthiol (**CM-SH**) providing the fluorescently labelled DNA19_1^{CM}H. b) Normalised fluorescence spectra of DNA19_1^{V}H before and after thiol-ene reaction with **CM-SH** compared to natural DNA19_1H treated with the same reagent. c) Photograph of the vials containing the fluorescent DNA19_1^{CM}H and DNA31_4^{CM}H (vials 2 and 4 respectively) compared to non-fluorescent starting DNA31_1G and DNA31_4G (vials 1 and 3 respectively) upon UV irradiation (365 nm). Reaction conditions: a) **CM-SH**, TEAA buffer, 37 °C, 3 days.

Additionally, we also performed the thiol-ene reaction with L-cysteine as a model amino acid reagent containing a thiol group (Figure 44a). Similarly, the vinyl-linked DNA reacted with an excess of the amino acid in TEAA buffer for 3 days. The conversion of the reaction was analysed by PAGE analysis of the product (DNA19_1^{Cys}H) after purification together with product of the thiol-ene reaction with **CM-SH** (the DNA for these reactions and analyses was prepared using Prim15-FAM). The analysis showed that in both cases the conversion of the reaction was ca. 60% (Figure 44b), corresponding to the yield of the reaction when modified adenine reacted instead of hypoxanthine (see Figure 19). Reaction with L-cysteine was also performed with DNA31_4^{V}H yielding DNA labelled by the amino acid, as witnessed after MALDI-TOF analysis of the products. In fact, formation of all products of the thiol-ene reactions was confirmed by MALDI analysis (Table 15). Notably, the spectra did not show the formation of the product of the reaction of all four vinyl groups in the case of DNA31_4^{V}H (products of reaction of one or two vinyl groups were the major peaks), however, at least in the case of fluorescent labelling, even this conversion is satisfactory for visualisation of the products (for copies of MALDI spectra, see Appendix 5).

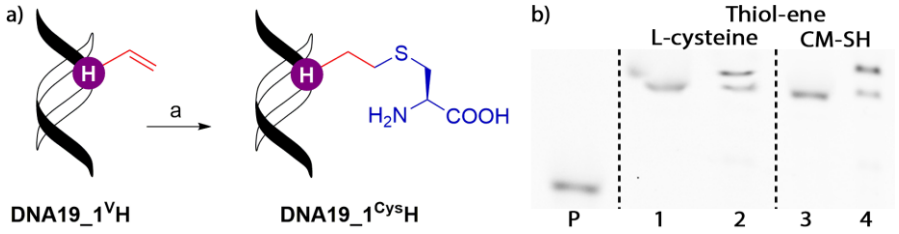


Figure 44. a) Depiction of thiol-ene reaction between DNA19_1^{V}H and L-cysteine providing DNA19_1^{Cys}H . b) PAGE analysis of products of thiol-ene reactions. Lanes: P: primer; lanes 1, 3: ON bearing one vinyl group (**ON19_1^{V}H**); lanes 2, 4: products of subsequent thiol-ene reaction with the corresponding reagent (**ON19_1^{Cys}H** and **ON19_1^{CM}H**). Reaction conditions: a) L-cysteine, TEAA buffer, 37 °C, 3 days.

Following these results, we were curious whether it would also be possible to perform a reaction of the vinyl-linked DNA with a minor-groove binding peptide. A dodecapeptide pept_1-SH (Figure 45) was chosen as a substrate, which contains a truncated sequence of HMG-I protein, known to bind to the minor groove of DNA.[224] The peptide contains multiple positively charged amino acids (arginines and lysines) essential for successful binding to the negatively charged DNA molecule. In our case, we attached cysteine to the C-terminus of the peptide to allow a possible reaction between pept_1-SH and vinyl-modified DNA to occur (Scheme 30a). We also designed

a second dodecapeptide, pept_2-SH, in which the lysines and arginines were replaced by negatively charged animo acids (glumatic and aspartic acid) in order to potentially cause an electrostatic repulsion between the two negatively charged molecules and in turn prohibit the reaction (Scheme 30b).

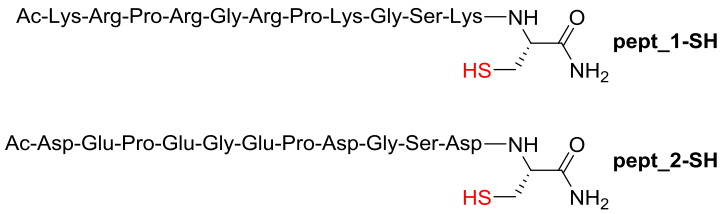
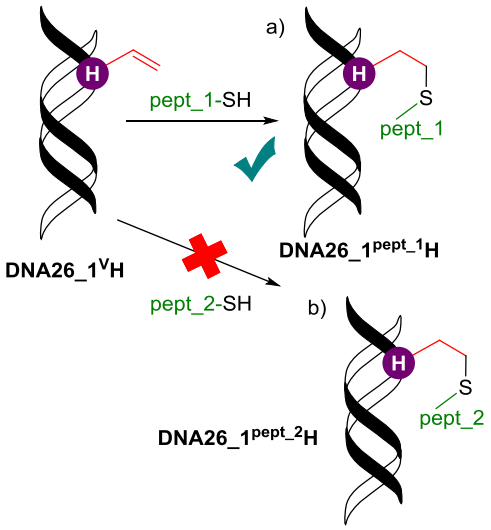


Figure 45. Structures of dodecapeptides used in the following experiment.



Scheme 30. Expected outcomes of the reactions between DNA26_1^{V}H and dodecapeptides. a) Successful and b) prohibited reaction.

We designed a DNA sequence described to allow the pept_1-SH binding and thus performed PEX with Therminator DNA polymerase, Prim16-FAM, Temp26 and **d^{V}ITP**. PAGE analysis of the products of PEX reaction showed formation of full-length products (DNA26_1^{V}H, Figure 46).

Figure 46. PAGE analysis of polymerase synthesis of DNA26_1^{V}H using Prim16-FAM and Temp26. Lane 1, P: primer; lane 2, +: PEX in the presence of natural dNTPs; lane 3, G-: negative control experiment, no dGTP used during PEX; lane 4, ^{V}H: PEX in the presence of dATP, dCTP, dTTP and **d^{V}ITP** used instead of dGTP.

The reactions were performed first with an increasing amount of peptides. Thus the vinyl-modified DNA reacted with the peptides at 37 °C for 24 hours. The products of the reaction were then analysed by PAGE (Figure 47). As is apparent from the PAGE gel, the reaction of DNA26_1^{V}H with pept_1-SH proceeded with as little as 2 equiv. of peptide providing a product of reduced electrophoretic mobility (DNA26_1^{pept_1}H). This can be most probably attributed to a proximity effect taking place upon binding of the dodecapeptide to the DNA molecule. Compared to the reaction conditions used previously for thiol-ene reactions with thiols (the use of 12 500 equiv. of thiol in the reaction) this is a significant improvement of the reaction rate. The best result was obtained when 10 equiv. of pept_1-SH were used, when the conversion of the reaction reached ca. 40%. Increasing amount of the peptide did not significantly improve the reaction yield and additionally, reaction of DNA26_1^{V}H and pept_2-SH started to take place (product DNA26_1^{pept_2}H), when more than 50 equiv. of peptide were used in the reaction (Figure 47). Formation of all products was also confirmed by MALDI-TOF analysis (Table 15).

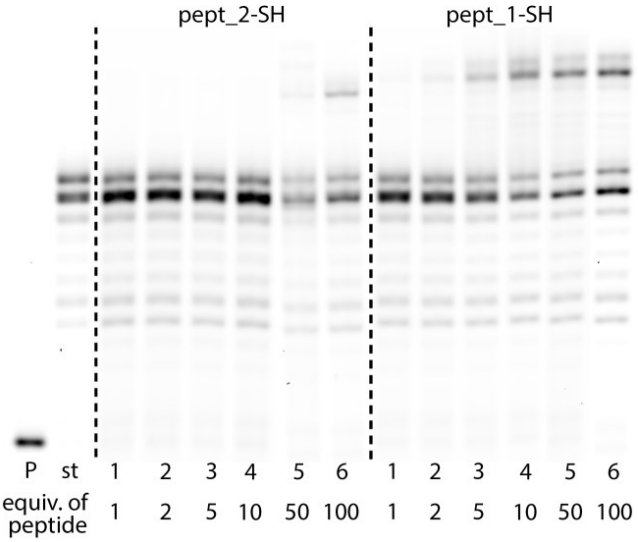


Figure 47. Denaturing PAGE showing results of optimization of the reaction between peptides containing cysteine and vinyl modified DNA (DNA26_1^{V}H). Lanes: P: primer; st: starting DNA26_1^{V}H; 1-6: products of reaction of vinyl modified DNA and corresponding peptide with increasing amount of the peptide.

We performed the reaction between DNA26_1^{V}H and pept_1-SH again using the condition where the highest conversion was observed (i.e. 10 equiv. of peptide) and tested whether the conversion of the reaction would approve with prolonged reaction time. PAGE analysis revealed that the conversion of the reaction after 3 day at 37 °C did not improve compared to the first reaction (Figure 48).

Figure 48. Denaturing PAGE comparing the results of the reactions between pept_1-SH and DNA26_1^{V}H with increasing reaction time. Lanes: P: primer; 1: starting DNA26_1^{V}H; 2: product of reaction between pept_1-SH and DNA26_1^{V}H after 24 hours; 3: product of reaction between pept_1-SH and DNA26_1^{V}H after 3 days.

3.3.4 Study of the influence of minor-groove modifications on cleavage of DNA by type II restriction endonucleases

Finally, we studied the effect of minor-groove modifications on the ability of restriction endonucleases to cleave the modified DNA. We decided to not only use the newly synthesised **d^{R}ITP**s, but also previously prepared **d^{R}ATP**s (Figure 49).

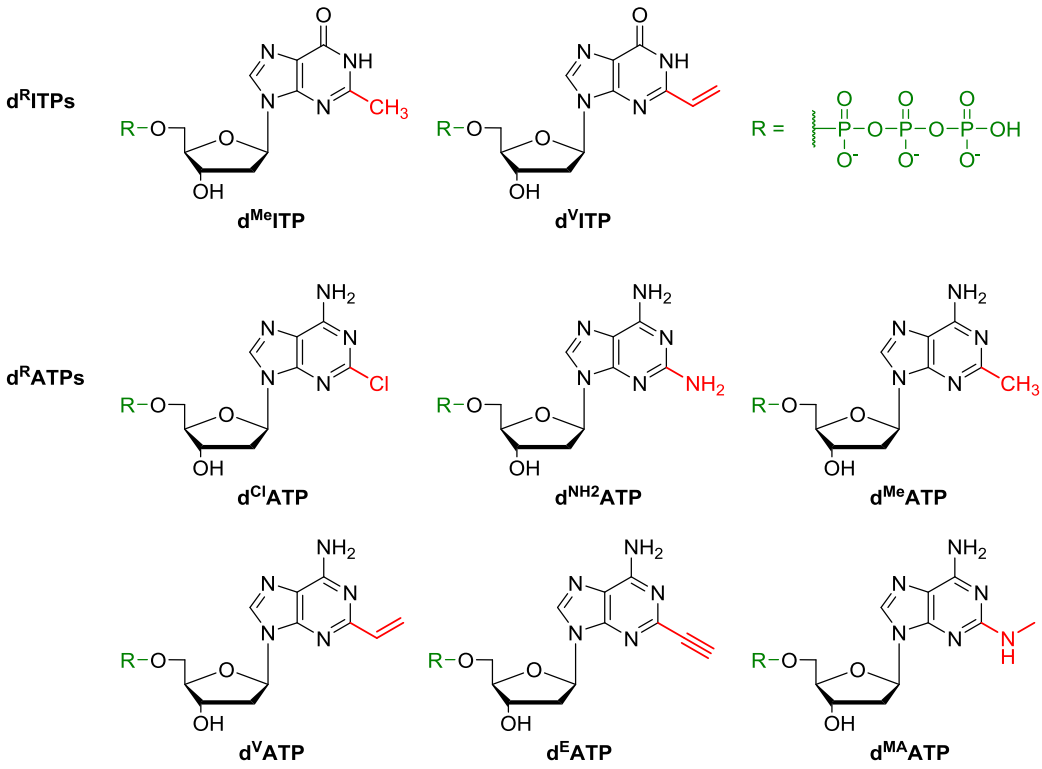
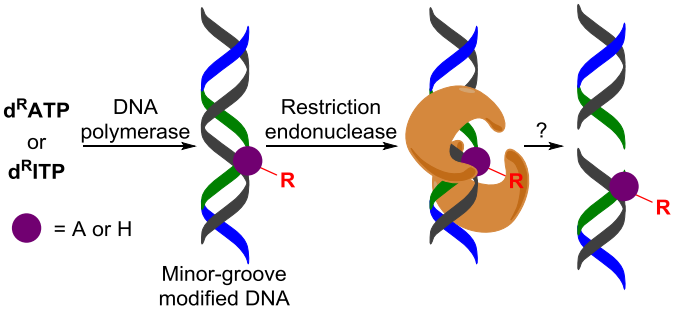


Figure 49. Structures of modified dNTPs used to study the influence of minor-groove modifications on the cleavage of DNA by type II restriction endonucleases.

First of all, we needed to prepare dsDNAs containing the modified ^{R}A or ^{R}H nucleobases inside the recognitions sites of different REs (for sequences, see Table 14). We conducted PEX with either KOD XL DNA polymerase or Therminator DNA polymerase and either **d^{R}ITP**s or **d^{R}ATP**s. PAGE analysis revealed formation of full-length products in all cases. We chose eight type II restriction endonucleases (AfeI, EcoRI, KpnI, PstI, PvuII, RsaI, SacI and ScaI) to be tested for their ability to cleave the prepared minor-groove modified DNAs (Scheme 31).



Scheme 31. Enzymatic synthesis of minor-groove modified DNA and its cleavage by restriction endonucleases.

The cleavage experiments were performed directly on the PEX products without further purification and thus the minor-groove modified DNA was incubated with the RE at 37 °C for 1 hour and the products of cleavage were analysed ba PAGE (Figures 50,51; lanes 4,6,8,10,12,14,16).

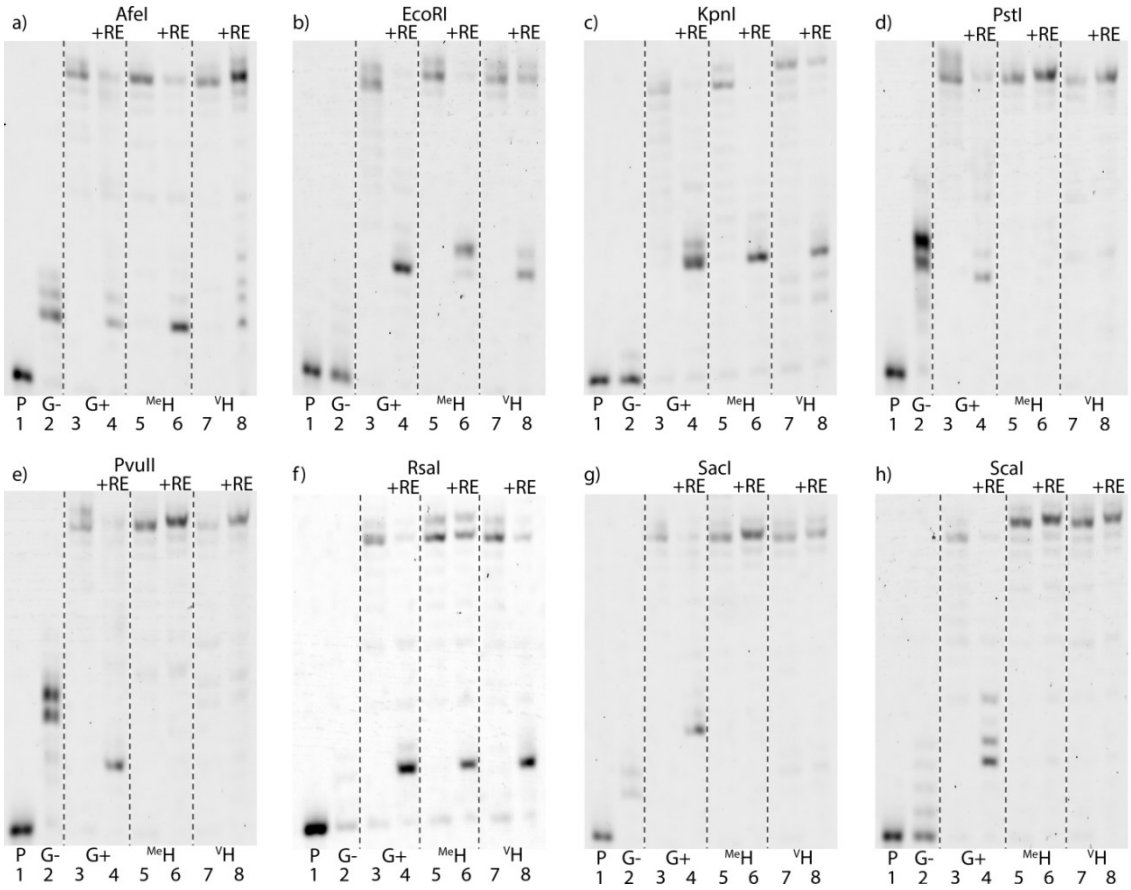
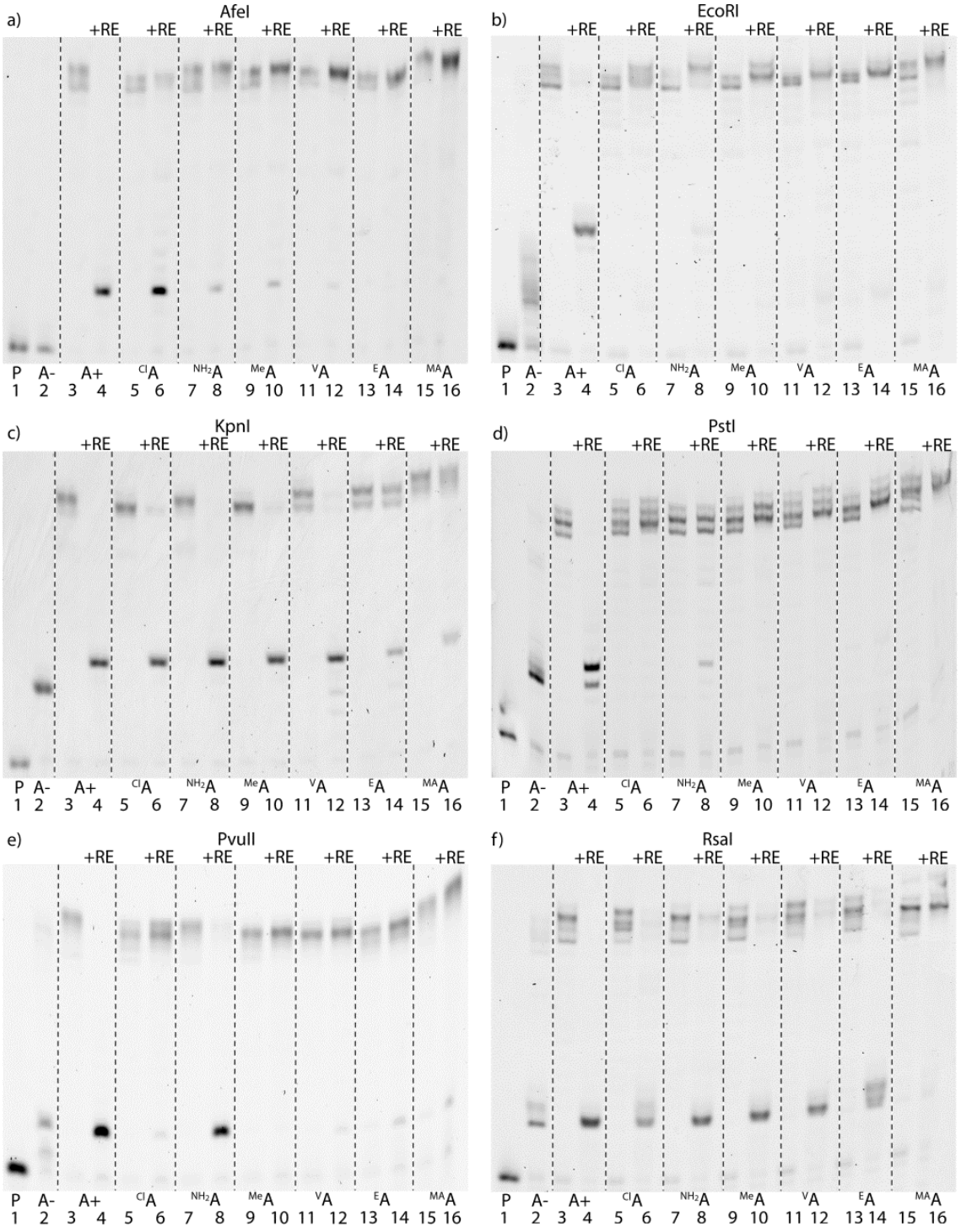


Figure 50. PAGE analysis of DNAs with "modified Gs" (**d^{R}ITPs** used in PEX) inside the recognition site of restriction enzymes. Lanes: 1, P: primer; 2, G-: PEX in the presence of dCTP, dATP and dTTP; 3, 5, 7, G+ or ^{R}H: PEX in the presence of dCTP, dATP, dTTP and either dGTP (G+) or corresponding modified **d^{R}ITP**; 4, 6, 8, +RE: cleavage of DNAs by corresponding restriction enzymes.


a)
AfeI
b)
EcoRI
c)
KpnI
d)
PstI
e)
PvuII
f)
RsaI
+RE
P A- A+ ClA NH2A MeA VA EA MAA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

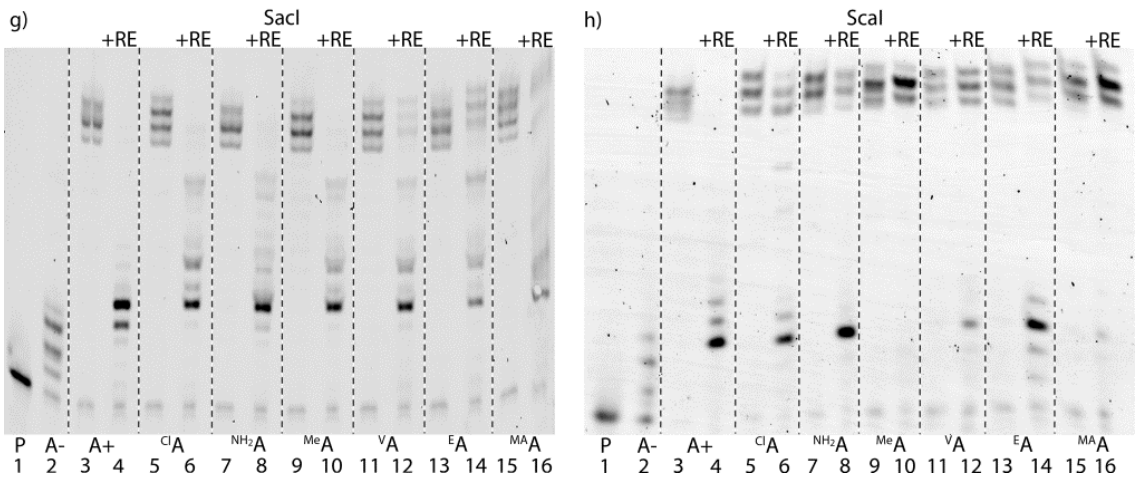


Figure 51. PAGE analysis of DNAs with modified As (**d^{R}ATPs** used in PEX) inside the recognition site of restriction enzymes. Lanes: 1, P: primer; 2, A-: PEX in the presence of dCTP, dGTP and dTTP; 3, 5, 7, 9, 11, 13, 15, A+ or ^{R}A: PEX in the presence of dCTP, dGTP, dTTP and either dATP (A+) or corresponding modified **d^{R}ATP**; 4, 6, 8, 10, 12, 14, 16, +RE: cleavage of DNAs by corresponding restriction enzymes.

The results of these studies show that, compared to the effect of major-groove modifications,[166–169] the effect of minor-groove modifications is not as general. We found enzymes that tolerated the minor-groove modifications, but also enzymes that were inhibited by them. PstI was the only RE that did not allow any of the tested modifications to be located in the recognition site. Modified adenine bases mostly inhibited the activity of AfeI, EcoRI and PvuII (PvuII was able to cleave the DNA containing ^{NH_2}A base), however AfeI and EcoRI were able to cleave the DNA containing modified hypoxanthine bases. Notably, ^{Me}H and ^{V}H were the only modified nucleobases that were allowed by EcoRI RE in this study and also considering previous studies. On the other hand, ScaI was able to cleave DNAs containing some of the modified adenines and SacI also cleaved the DNAs with modified adenines efficiently, but no modified hypoxanthines were allowed in both cases. RsaI allowed almost every modification and was only inhibited by the methylamino modified adenine base. KpnI was the only enzyme that cleaved all of the modified DNAs (some partially). Summarised results of the cleavage experiments are presented in Table 17.

Table 17. Summary of the results of RE cleavage of DNA modified in the minor groove. [a] Approximate yields of cleavage: – = 0-25%; + = 25-50%; ++ = 50-75%; +++ = 75-100%.

Enzyme	^{Cl}**A**	^{NH_2}**A**	^{Me}**A**	^{V}**A**	^{E}**A**	^{MA}**A**	^{Me}**H**	^{V}**H**
AfeI	++ [(a)]	–	–	–	–	–	+++	+
EcoRI	–	–	–	–	–	–	+++	++
KpnI	+++	+++	+++	+++	+	+	+++	+++
PstI	–	–	–	–	–	–	–	–
PvuII	–	+++	–	–	–	–	–	–
RsaI	+++	+++	+++	+++	+++	–	++	+++
SacI	+++	+++	+++	+++	++	++	–	–
ScaI	++	++	–	+	++	–	–	–

Even though restriction endonucleases recognise and bind to DNA through interactions in the major groove, minor-groove modifications also affect the ability of the enzymes to cleave the DNA. This is most probably caused by modulation of the hydrogen bonding of the minor groove or alternatively by conformational changes to the structure of B-DNA, which are suggested by the measured denaturing temperatures of the duplexes and also by related literature data.[71]

In conclusion, we synthesised a series of 2-substituted 2'-deoxyinosine triphosphates and tested them as substrates in enzymatic synthesis of minor-groove modified DNA. Therminator DNA polymerase was the only enzyme capable of synthesising full-length products while using the corresponding **d^{R}ITP**s. The vinyl modified DNA reacted in post-synthetic DNA modification via thiol-ene reaction with thiols. DNA labelled by fluorescent labels or by cysteine was obtained and furthermore, the vinyl DNA was shown to react with cysteine-containing minor-groove-binding dodecapeptide. The study of the effect of minor-groove modifications on the ability of various type II REs to cleave DNA was conducted and the results have shown that although REs recognise the DNA through interactions in the major groove, also minor-groove modifications can affect the recognition and cleavage.

4 Conclusions

In conclusion, the aim of the thesis was to develop enzymatic synthesis of minor-groove modified DNA. To this end, multiple 2-modified purine 2'-deoxynucleotides were designed and synthesised.

In the first part of the thesis, six 2-substituted dATPs (**d^{R}APT**s) bearing small modifications (chloro, amino, methyl, vinyl, ethynyl and phenyl) were prepared. Their synthesis involved triphosphorylation of the parent nucleosides. A systematic study of incorporation of **d^{R}APT**s into DNA depending on the size of the substituents was performed. Primer extension experiments showed that the 2-phenyl modified dATP was not a substrate for any of the tested DNA polymerases. The phenyl group is therefore already too big to be incorporated. All of the other modified nucleotides were good substrates KOD XL, Vent(exo-) and Bst LF DNA polymerases in PEX. It was also possible to incorporate **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** into DNA in PCR. Moreover the introduction of minor-groove modification did not interfere with the crucial Watson-Crick base-pairing with T, since only mild destabilisation of the modified DNA duplexes was observed.

In the second part, a series of dATPs with 2-alkylamino substituents was prepared. We found that these nucleotides are poor substrates for DNA polymerases in PEX. However, Therminator DNA polymerase was able to perform single nucleotide extension (SNE) using these triphosphates. When SNE was followed by PEX using natural dNTPs, the resulting synthesised DNA possessed one single minor-groove modification. Conditions of SNE and following PEX were optimised for different templates where adenine is followed either by a different nucleobase, but also by another adenine. Using this procedure it is therefore possible to prepare site-specifically minor-groove modified DNA. The alkylamino chain had a stabilising effect on the DNA duplex thanks to an additional hydrogen bond with thymine.

In the last part of the thesis we wanted to extend the scope of nucleotides used for enzymatic synthesis of minor-groove modified DNA also to analogues of dGTP. Thus a series of 2-substituted 2'-deoxyinosine triphosphates (**d^{R}ITP**) was synthesised possessing a modified hypoxanthine base as a surrogate of guanine. Only the 2-methyl and -vinyl derivatives were incorporated into DNA by Therminator DNA polymerase. All of the other tested 2-modified dITP (2-chloro, 2-ethynyl and 2-phenyl) were not substrates for Thermintor and also other DNA polymerases. Therefore it is concluded

that **d^{R}ITP**s are worse substrates for DNA polymerases than corresponding **d^{R}ATP**s. A slight destabilisation of the resulting DNA duplexes was observed, mostly assigned to a missing hydrogen bond with thymine and also to some possible structural changes to B-DNA duplex.

The direct enzymatic incorporation does not allow for direct introduction of bulky functional groups into the DNA minor groove. Post-synthetic transformations were therefore tested for all of the prepared and incorporated modified purine nucleobases. Vinyl- and ethynyl-linked adenine successfully participated in thiol-ene and CuAAC reaction respectively, leading to products of minor-groove fluorescent labelling. The thiol-ene reaction proceeded with ca. 60% yield (satisfactory for fluorescent labelling), while the CuAAC gave full-conversion of the starting ethynyl-modified DNAs.

The reactive allyl and propargyl groups were again used for the attachment of fluorescent labels, this time producing site-specifically labelled fluorescent DNA. In this case we observed full-conversion during thiol-ene reactions. When this approach was performed with biotinylated templates followed by magnetoseparation, single stranded oligonucleotides bearing one modification were prepared. They were then used for the preparation of FRET probes. The probes allowed for monitoring of the reversible process of DNA duplex denaturing and reannealing. It also provided a way of detecting specific oligonucleotide sequences.

Thiol-ene reaction with thiols was also used in the case of vinyl-substituted hypoxanthine nucleobase, affording fluorescently labelled DNA, again with ca. 60% conversion of the starting DNA. It was also possible to acquire a product of cross-linking between a cysteine-containing minor-groove binding peptide and vinyl-linked DNA. This reaction proceeded thanks to the proximity effect of vinyl and thiol groups upon binding of the peptide.

Finally, we explored the ability of different type II restriction endonucleases (REs) to cleave dsDNA bearing minor-groove modifications. We successfully prepared DNAs containing either 2-substituted adenines or hypoxanthines in the recognition site of REs and subjected them to RE cleavage. The cleavage depended on the type of modification and RE used in the experiment, but overall we found modifications that completely inhibited the RE cleavage and also modifications that were allowed. The outcome of the experiments suggests that although RE bind to dsDNA mainly thanks to

interactions in the DNA major groove, minor-groove modifications can also affect the recognitions and cleavage.

5 Experimental section

5.1 General remarks

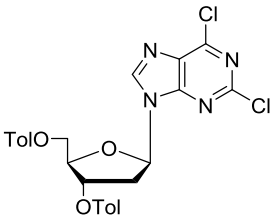
Reagents and solvents were purchased from Sigma–Aldrich and AlfaAesar. 3,5-Ditoluoyl-1-α-chloro-2-deoxy-D-ribose **16** was prepared as described previously.[185] 2'-Deoxyguanosine **24** was purchased from Berry&Associates, 2,6-dichloropurine **29** from Sumika Fine Chemicals. The reactions were monitored by thin-layer chromatography using Merck silica gel 60 F254 plates and visualized by UV (254 nm). Column chromatography was performed using silica gel (40–63 μm). Reversed-phase high-performance flash chromatography (HPFC) purifications were done on an ISCO CombiFlash Rf+ apparatus with C-18 columns or using a POROS HQ 50 μm packed column. Purification of nucleoside triphosphates was performed using HPLC (Waters modular HPLC system) on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å). NMR spectra were measured on a Bruker AVANCE 400 (^{1}H at 401.0 MHz, ^{13}C at 100.8 MHz and ^{31}P at 162.0 MHz), Bruker AVANCE 500 (^{1}H at 500.0 MHz, ^{13}C at 125.7 MHz and ^{31}P at 202.3 MHz) and Bruker AVANCE 600 (^{1}H at 600.1 MHz and ^{13}C at 150.9 MHz) NMR spectrometers in $CDCl_3$, DMSO-d_6 or D_2O solutions at 25 °C. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in ^{1}H spectra (δ($CHCl_3$) = 7.26 ppm, δ((CHD_2)SO(CD_3)) = 2.5 ppm) or to the solvent signal in ^{13}C spectra (δ($CDCl_3$) = 77.0 ppm, δ((CD_3)$_2$SO) = 39.7 ppm). 1,4-Dioxane was used as an internal standard for D_2O solutions (3.75 ppm for ^{1}H and 69.3 ppm for ^{13}C). Coupling constants (*J*) are given in Hz. The complete assignment of ^{1}H and ^{13}C signals was performed by an analysis of the correlated homonuclear H,H-COSY, and heteronuclear H,C-HSQC and H,C-HMBC spectra. Mass spectra were measured with a LCQ classic (Thermo-Finnigan) spectrometer using ESI or a Q-Tof Micro spectrometer (Waters, ESI source, internal calibration with lockspray). High resolution mass spectra were measured on a LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific). Oligonucleotides were purchased from Generi Biotech (Czech Republic) or Eurofins Genomics (Germany). Bst DNA polymerase Large Fragment, Vent(exo-) DNA polymerase, Therminator DNA polymerase, Phusion DNA polymerase and all of the restriction enzymes and corresponding reaction buffers, as well as natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP), were purchased from New England Biolabs. KOD XL DNA polymerase and corresponding

reaction buffer were obtained from Merck Millipore. Streptavidin magnetic beads were obtained from Roche. All solutions for biochemical reactions were prepared using Milli-Q water (18 MΩ.cm). The PAGE gels were visualized by a fluorescence scanner (Typhoon FLA 9500, GE Healthcare) and agarose gels were visualised using an electronic dual wave transilluminator equipped with GBox iChemi-XRQ Bio imaging system (Syngene, Life Technologies). GelRed was obtained from Biotium. Concentration of DNA solutions was calculated using A_{260} values measured on a Nanodrop and values obtained with OligoCalc.[225] Mass spectra of oligonucleotides were measured by MALDI-TOF, on UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser. The measurements were done in reflectron mode by droplet technique, with the mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9/1/1. The matrix (1 μl) was applied on the target (ground steel) and dried down at room temperature. The sample (1 μl) and matrix (1 μl) were mixed and added on the top of dried matrix preparation spot and dried down at room temperature. QIAquick nucleotide removal kit was purchased from QIAGEN. Fluorescent thiol (**CM-SH**) was prepared as described previously.[208] Fluorescent azides (**Cy3-N_3** and **Cy5-N_3**) were purchased from Jena Bioscience. Samples were concentrated on a CentriVap Vacuum Concentrator system (Labconco). Fluorescence spectra were measured on a Fluoromax 4 spectrofluorimeter (HORIBA Scientific). UV-visible spectra were measured on a Cary 100 UV-Vis spectrometer (Agilent Technologies). Conversion of biochemical reactions was determined using ImageJ software.[226] L-cysteine was obtained from Sigma-Aldrich. Dodecapeptides were purchased at IOCB Prague.

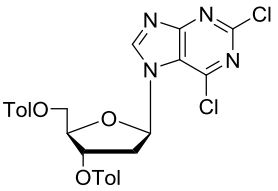
5.2 Synthesis of 2-substituted dATP derivatives and their use in enzymatic synthesis of minor-groove modified DNA

Synthetic procedures for the preparation of d^RATPs

2,6-Dichloro-3',5'-di-*O*-toluoyl-9-(2'-deoxy-β-D-ribofuranosyl)purine (21)

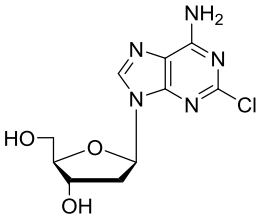


2,6-Dichloro-3',5'-di-*O*-toluoyl-7-(2'-deoxy-β-D-ribofuranosyl)purine (22)



The protected nucleoside was prepared following a literature procedure.[184] 2,6-Dichloropurine **20** (1.70 g, 9.0 mmol) and NaH (0.38 g, 9.4 mmol, 60% in mineral oil) were suspended in dry CH_3CN (60 ml) and the resulting mixture was stirred at rt (25 °C) for 30 min, after which halogenose **19** (3.50 g, 9.0 mmol) was added portionwise over the course of 20 min. Reaction mixture was stirred overnight at rt (25 °C). Then it was filtered through a pad of celite, celite was washed with DCM (50 ml) and collected organic phases were evaporated under reduced pressure. Column chromatography (0 → 6% acetone in toluene) afforded the desired N-9 isomer as a white solid (2.20 g, 45%) along with the N-7 isomer (white solid, 0.75 g, 15%). The characterisation data were in accordance with those reported in the literature.[184]

2-Chloro-2'-deoxyadenosine (23)



2-Chloro-2'-deoxyadenosine **23** was prepared according to a literature procedure[184] with minor modifications. Protected nucleoside **21** (700 mg, 1.29 mmol) was dissolved in MeOH saturated (at 0 °C) with ammonia (20 ml) and heated to 110 °C for 5 h in a sealed pressure tube. After cooling the solvent was evaporated. The residue was dissolved in MeOH, coevaporated with silica gel and purified by column chromatography (5% → 10% MeOH in CH_2Cl_2). The title compound was obtained as a

white solid (318 mg, 86%). The characterisation data were in accordance with those reported in the literature.[184]

R_f: 0.40 (20% MeOH in CH_2Cl_2).

^{1}H NMR (500.0 MHz, DMSO-d_6): 2.28 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.3, H-2′b); 2.65 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 7.6, $J_{2'a,3'}$ = 5.8, H-2′a); 3.51 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 6.0, $J_{5'b,4'}$ = 4.5, H-5′b); 3.60 (ddd, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = 5.1, $J_{5'a,4'}$ = 4.5, H-5′a); 3.86 (td, 1H, $J_{4',5'}$ = 4.5, 4.1, $J_{4',3'}$ = 2.8, H-4′); 4.39 (dddd, 1H, $J_{3',2'}$ = 5.8, 3.3, $J_{3',OH}$ = 4.2, $J_{3',4'}$ = 2.8, H-3′); 5.01 (dd, 1H, $J_{OH,5'}$ = 6.0, 5.1, OH-5′); 5.36 (d, 1H, $J_{OH,3'}$ = 4.2, OH-3′); 6.26 (dd, 1H, $J_{1',2'}$ = 7.6, 6.2, H-1′); 7.84 (bs, 2H, NH_2-6); 8.36 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.61 (CH_2-2′); 61.93 (CH_2-5′); 71.00 (CH-3′); 83.86 (CH-1′); 88.22 (CH-4′); 118.43 (C-5); 140.15 (CH-8); 150.33 (C-4); 153.25 (C-2); 157.06 (C-6).

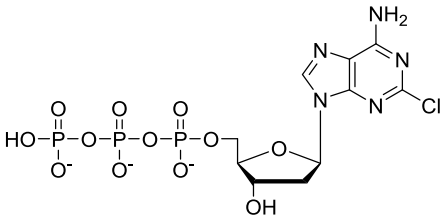
MS (ESI$^+$): *m/z* (%): 286.0 (4) [(^{35}Cl)M+H]$^+$, 288.0 (1) [(^{37}Cl)M+H]$^+$, 308.0 (100) [(^{35}Cl)M+Na]$^+$, 310.0 (34) [(^{37}Cl)M+Na]$^+$.

HRMS (ESI$^+$): calculated for $C_{10}H_{12}O_3N_5ClNa$: 308.05209; found: 308.05221.

General procedure A for the preparation of (modified) 2'-deoxynucleoside-5'-*O*-triphosphates

Nucleoside (1 equiv.) was dried in vacuo overnight. Then it was dissolved in anhydrous $PO(OMe)_3$ (100 equiv.) and cooled to 0 °C. Freshly distilled $POCl_3$ (1.2 equiv.) was added dropwise and the resulting mixture was stirred at 0 °C until complete disappearance of starting compound was observed by TLC (ca. 3-4 hours). Then an ice-cold solution of (*n*-$Bu_3NH)_2H_2P_2O_7$ (5.0 equiv.) and *n*-Bu_3N (4.0 equiv.) in anhydrous DMF [0.65 M solution of (*n*-$Bu_3NH)_2H_2P_2O_7$] was added and the reaction mixture was stirred at 0 °C for additional 1-1.5 h. The reaction was treated with 2 M TEAB solution (the same volume as volume of DMF). The mixture was concentrated on a rotavap and the residue was co-evaporated with distilled water three times. The crude product was dissolved in water (ca. 3-5 ml). The aqueous solution was purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotavap. The viscous oil was coevaporated with distilled water three times. The product was converted to Na^+ salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The desired nucleotides were isolated as lyophilisates.

2-Chloro-2'-deoxyadenosine-5'-*O*-triphosphate (10, $d^{Cl}ATP$)



The title nucleotide **10** was prepared following General procedure A starting from nucleoside **23** (35 mg, 0.123 mmol). It was isolated as a white lyophilisate (45 mg, 60%). NMR data were in good agreement with those reported previously.[227]

^{1}H NMR (500.0 MHz, D_2O): 2.63 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 3.6, H-2′b); 2.79 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.3, $J_{2'a,3'}$ = 6.1, H-2′a); 4.16 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 5.1, $J_{5'b,4'}$ = 3.6, H-5′b); 4.22 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 6.2, $J_{5'a,4'}$ = 3.6, H-5′a); 4.31 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 3.6, $J_{H,P}$ = 1.9, H-4′); 4.80 (dt, 1H, $J_{3',2'}$ = 6.1, 3.6, $J_{3',4'}$ = 3.6, H-3′); 6.36 (dd, 1H, $J_{1',2'}$ = 7.3, 6.3, H-1′); 8.40 (s, 1H, H-8).

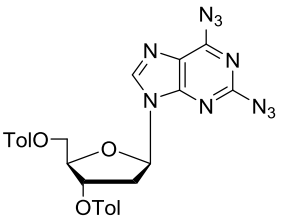
^{13}C NMR (125.7 MHz, D_2O): 41.98 (CH_2-2′); 68.16 (d, $J_{C,P}$ = 5.7, CH_2-5′); 73.80 (CH-3′); 86.41 (CH-1′); 88.53 (d, $J_{C,F}$ = 9.0, CH-4′); 120.03 (C-5); 142.70 (CH-8); 152.27 (C-4); 156.26 (C-2); 158.63 (C-6).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.86 (t, J = 19.3, P_β); -10.36 (d, J = 19.3, P_α); -9.35 (d, J = 19.3, P_γ).

MS (ESI^-): *m/z* (%): 364.0 (70) $[(^{35}Cl)M–H_3P_2O_6]^-$, 366.0 (27) $[(^{37}Cl)M–H_3P_2O_6]^-$, 444.0 (100) $[(^{35}Cl)M–H_2PO_3]^-$, 446.0 (42) $[(^{37}Cl)M–H_2PO_3]^-$, 466.0 (44) $[(^{35}Cl)M–H–H_2PO_3+Na]^-$, 468.0 (16) $[(^{37}Cl)M–H–H_2PO_3+Na]^-$.

HRMS (ESI^-): calculated for $C_{10}H_{13}O_{12}N_5ClP_3Na$: 545.93653; found: 545.93661.

2,6-Diazido-9-(2'-deoxy-3',5'-di-*O*-toluoyl-β-D-ribofuranosyl)purine (24)



Protected nucleoside **21** (409 mg, 0.74 mmol) was dissolved in EtOH/H_2O = 9/1 mixture (20 ml). To the resulting solution, NaN_3 (110 mg, 1.70 mmol) was added and the mixture was refluxed for 30 min. After cooling the solvent was evaporated and the residue was dissolved in $CHCl_3$ (30 ml) and washed with H_2O (3 × 30 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated on a rotavap. Obtained product (400 mg, 98%) was used in the next step without further purification.

R_f: 0.31 (30% EtOAc in n-hexane).

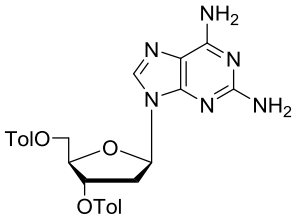
^{1}H NMR (500.0 MHz, $CDCl_3$): 2.40, 2.44 (2 × s, 2 × 3H, CH_3-Tol); 2.84 (ddd, 1H, J_{gem} = 14.3, $J_{2'b,1'}$ = 5.9, $J_{2'b,3'}$ = 2.3, H-2′b); 2.96 (ddd, 1H, J_{gem} = 14.3, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 6.1, H-2′a); 4.62 – 4.63 (m, 1H, H-4′); 4.65 (dd, 1H, J_{gem} = 11.7, $J_{5'b,4'}$ = 4.2, H-5′b); 4.74 (dd, 1H, J_{gem} = 11.7, $J_{5'a,4'}$ = 3.6, H-5′a); 5.78 (dt, 1H, $J_{3',2'}$ = 6.1, 2.3, $J_{3',4'}$ = 2.3, H-3′); 6.50 (dd, 1H, $J_{1',2'}$ = 8.1, 5.9, H-1′); 7.20 – 7.23 (m, 2H, H-*m*-Tol); 7.27 – 7.29 (m, 2H, H-*m*-Tol); 7.85 – 7.87 (m, 2H, H-*o*-Tol), 7.94 – 7.97 (m, 2H, H-*o*-Tol); 8.06 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, $CDCl_3$): 21.69, 21.75 (CH_3-Tol); 38.18 (CH_2-2′); 63.84 (CH_2-5′); 74.85 (CH-3′); 83.14 (CH-4′); 84.65 (CH-1′); 121.66 (C-5); 126.17, 126.40 (C-*i*-Tol); 129.28, 129.29 (CH-*m*-Tol); 129.51, 129.79 (CH-*o*-Tol); 141.44 (CH-8); 144.27, 144.59 (C-*p*-Tol); 153.18 (C-4); 153.82 (C-6); 156.07 (C-2); 165.89, 166.05 (CO-Tol).

MS (ESI$^+$): *m/z* (%): 577.2 (100) $[M+Na]^+$, 1131.5 (7) $[2M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{26}H_{22}O_5N_{10}Na$: 577.16668; found: 577.16675.

2,6-Diamino-9-(2'-deoxy-3',5'-di-*O*-toluoyl-β-D-ribofuranosyl)purine (25)



Protected purine nucleoside **24** (400 mg, 0.72 mmol) was dissolved in EtOH/DMA = 2/1 mixture (24 ml) and 10% palladium on charcoal was added (40 mg). The reaction mixture was then purge-and-refilled with H_2 4 times. Reaction mixture was stirred at room temperature (25 °C) for 24 h; then it was filtered through a pad of celite and evaporated to dryness. The residue was purified by column chromatography (0% → 3% MeOH in CH_2Cl_2) to give the desired product (348 mg, 96%) as a white solid.

R_f: 0.25 (2% MeOH in CH_2Cl_2).

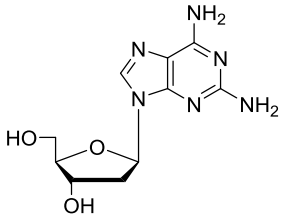
^{1}H NMR (500.0 MHz, $CDCl_3$): 2.39, 2.43 (2 × s, 2 × 3H, CH_3-Tol); 2.70 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 5.9, $J_{2'b,3'}$ = 2.0, H-2′b); 3.10 (ddd, 1H, J_{gem} = 14.2, $J_{2'a,1'}$ = 8.5, $J_{2'a,3'}$ = 6.2, H-2′a); 4.63 (ddd, 1H, $J_{4',5'}$ = 4.7, 4.4, $J_{4',3'}$ = 2.0, H-4′); 4.65 (dd, 1H, J_{gem} = 11.7, $J_{5'b,4'}$ = 4.7, H-5′b); 4.80 (dd, 1H, J_{gem} = 11.7, $J_{5'a,4'}$ = 4.4, H-5′a); 4.88 (bs, 2H, NH_2-2); 5.68 (bs, 2H, NH_2-6); 5.77 (dt, 1H, $J_{3',2'}$ = 6.2, 2.0, $J_{3',4'}$ = 2.0, H-3′); 6.37 (dd, 1H, $J_{1',2'}$ = 8.5, 5.9, H-1′); 7.21 – 7.23 (m, 2H, H-*m*-Tol); 7.25 – 7.27 (m, 2H, H-*m*-Tol); 7.66 (s, 1H, H-8); 7.89 –7.91 (m, 2H, H-*o*-Tol); 7.94 – 7.97 (m, 2H, H-*o*-Tol).

^{13}C NMR (125.7 MHz, $CDCl_3$): 21.67, 21.72 (CH_3-Tol); 37.08 (CH_2-2′); 64.08 (CH_2-5′); 75.24 (CH-3′); 82.50 (CH-4′); 84.22 (CH-1′); 114.75 (C-5); 126.40, 126.67 (C-*i*-Tol); 129.21, 129.24 (CH-*m*-Tol); 129.59, 129.74 (CH-*o*-Tol); 135.97 (CH-8); 144.07, 144.39 (C-*p*-Tol); 151.62 (C-4); 155.90 (C-6); 159.86 (C-2); 165.91, 166.26 (CO-Tol).

MS (ESI$^+$): *m/z* (%): 503.3 (100) $[M+H]^+$, 525.3 (29) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{26}H_{27}O_5N_6$: 503.20374; found: 503.20391.

2-Amino-2'-deoxyadenosine (26)



Protected nucleoside **25** (320 mg, 0.64 mmol) was dissolved in MeOH and K_2CO_3 (264 mg, 1.91 mmol) was added. Resulting solution was stirred at rt (25 °C) for 1 h. The reaction mixture was filtered and coevaporated with silica gel. The title compound, obtained after column chromatography (5% → 15% MeOH in CH_2Cl_2), was obtained as a white solid (162 mg, 96%). NMR data were in agreement with those reported previously.[186]

R_f: 0.15 (10% MeOH in CH_2Cl_2).

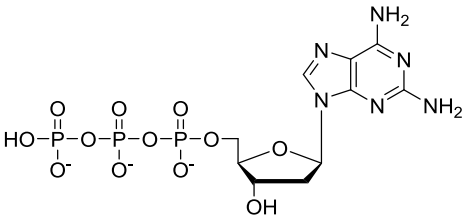
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.16 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 5.9, $J_{2'b,3'}$ = 2.6, H-2′b); 2.59 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.3, $J_{2'a,3'}$ = 5.6, H-2′a); 3.50 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 6.5, $J_{5'b,4'}$ = 4.1, H-5′b); 3.58 (dt, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = $J_{5'a,4'}$ = 4.6, H-5′a); 3.83 (ddd, 1H, $J_{4',5'}$ = 4.6, 4.1, $J_{4',3'}$ = 2.3, H-4′); 4.35 (dddd, 1H, $J_{3',2'}$ = 5.6, 2.6, $J_{3',OH}$ = 3.8, $J_{3',4'}$ = 2.3, H-3′); 5.29 (d, 1H, $J_{OH,3'}$ = 3.8, OH-3′); 5.32 (dd, 1H, $J_{OH,5'}$ = 6.5, 4.6, OH-5′); 5.78 (bs, 2H, NH_2-2); 6.16 (dd, 1H, $J_{1',2'}$ = 8.3, 5.9, H-1′); 6.80 (bs, 2H, NH_2-6); 7.92 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.62 (CH_2-2′); 62.27 (CH_2-5′); 71.32 (CH-3′); 83.38 (CH-1′); 87.93 (CH-4′); 113.67 (C-5); 136.09 (CH-8); 151.47 (C-4); 156.46 (C-6); 160.34 (C-2).

MS (ESI$^+$): *m/z* (%): 267.1 (35) $[M+H]^+$, 289.1 (100) $[M+Na]^+$, 555.1 (25) $[2M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{10}H_{14}O_3N_6Na$: 289.10196; found: 289.10210.

2-Amino-2'-deoxyadenosine-5'-*O*-triphosphate (11, $d^{NH_2}ATP$)



The title nucleotide **11** was prepared following General procedure A starting from nucleoside **26** (32 mg, 0.119 mmol). It was isolated as a white lyophilisate (13 mg, 18%).

^{1}H NMR (500.0 MHz, D_2O): 2.51 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 3.3, H-2′b); 2.77 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.7, $J_{2'a,3'}$ = 6.1, H-2′a); 4.14 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 5.2, $J_{5'b,4'}$ = 3.7, H-5′b); 4.20 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 6.2, $J_{5'a,4'}$ = 3.7, H-5′a); 4.26 (qd, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.7, $J_{H,P}$ = 1.8, H-4′); 4.78 (ddd, 1H, $J_{3',2'}$ = 6.1, 3.3, $J_{3',4'}$ = 3.7, H-3′); 6.33 (dd, 1H, $J_{1',2'}$ = 7.7, 6.3, H-1′); 8.16 (s, 1H, H-8).

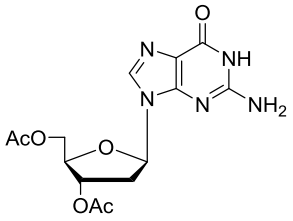
^{13}C NMR (125.7 MHz, D_2O): 41.52 (CH_2-2′); 68.20 (d, $J_{C,P}$ = 5.7, CH_2-5′); 73.96 (CH-3′); 86.02 (CH-1′); 88.37 (d, $J_{C,P}$ = 9.2, CH-4′); 115.58 (C-5); 140.48 (CH-8); 153.63 (C-4); 158.34 (C-6); 162.15 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.34 (t, J = 19.7, P_β); -10.56 (d, J = 19.7, P_α); -9.64 (d, J = 19.7, P_γ).

MS (ESI^-): *m/z* (%): 345.3 (34) $[M–H_3P_2O_6]^-$, 425.3 (100) $[M–H_2PO_3]^-$, 447.3 (42) $[M–H–H_2PO_3+Na]^-$.

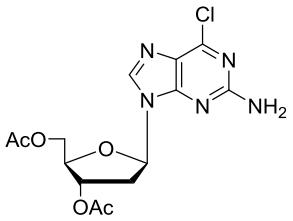
HRMS (ESI^-): calculated for $C_{10}H_{14}O_{12}N_6P_3Na_2$: 548.96834; found: 548.96863.

3',5'-Di-*O*-acetyl-2'-deoxyguanosine (28)



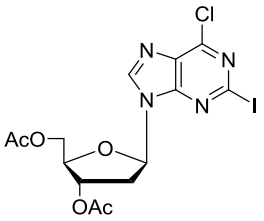
Acetylation of dG **27** was performed following a literature procedure.[187] dG **27** (3.5 g, 13.1 mmol), DMAP (0.1 g, 1.3 mmol) and Et_3N (5.5 ml, 39.3 mmol) were suspended in dry CH_3CN (65 ml). Ac_2O (3.7 ml, 39.3 mmol) was added and the resulting mixture was stirred at rt (25 °C) for 3 h. Reaction was stopped by addition of MeOH (3 ml) and the formed precipitate was filtered of. It was washed with cold EtOH (30 ml) and Et_2O (50 ml) and dried in vacuo. The title compound was isolated as a white solid (4.3 g, 93%). The analytical data were in accordance with the data published in the literature.[187]

2-Amino-6-chloro-9-(2'-deoxy-3',5'-di-*O*-acetyl-β-D-ribofuranosyl)purine (29)



Chlorination of protected nucleoside **28** was performed following a literature procedure.[187] Starting protected nucleoside **28** (500 mg, 1.4 mmol), benzyl(triethyl)ammonium chloride (485 mg, 2.1 mmol) and dimethylaniline (0.24 ml, 1.7 mmol) were dissolved in anhydrous CH_3CN (7 ml). Then $POCl_3$ (0.8 ml, 8.5 mmol) was added dropwise and the mixture was refluxed for 10 min (pre-heated oil bath 110 °C). The mixture was evaporated under reduced pressure, residue was dissolved in $CHCl_3$ (25 ml) and crushed ice was added. Vigorous stirring continued for 10 min (the ice mostly melted), after which aqueous ammonia was added to adjust the pH to 7. After all of the ice melted, phases were separated, the chloroform phase was washed with H_2O (30 ml). Water phase was then extracted with $CHCl_3$ (2 × 30 ml). Collected organic phases were washed with 1 M HCl (50 ml) and brine (50 ml), dried over anhydrous Na_2SO_4, filtered and concentrated on a rotavap. Column chromatography (0% → 60% EtOAc in CH_2Cl_2) afforded the desired product as a white solid (340 mg , 65%). The characterisation data were in accordance with the literature.[187]

6-chloro-2-iodo-9-(2'-deoxy-3',5'-di-*O*-acetyl-β-D-ribofuranosyl)purine (30)



Starting protected nucleoside **29** (3.30 g, 9.0 mmol), I_2 (2.27 g, 9.0 mmol), CuI (1.80 g, 9.4 mmol) and CH_2I_2 (7.4 ml, 92.0 mmol) were suspended in dry THF (90 ml) and isopentyl nitrite (3.7 ml, 27.5 mmol) was added. The resulting mixture was refluxed for 45 min. After cooling it was filtered and most of the volatiles were evaporated under reduced pressure. Residue was redissolved in $CHCl_3$ (150 ml) and poured into saturated $Na_2S_2O_3$ solution (100 ml). Mixture was stirred until change of color and then phases were separated. Water phase was extracted with $CHCl_3$ (2 x 100 ml), collected organic phases were washed with brine (100 ml), dried over anhydrous Na_2SO_4, filtered and evaporated. The title compound was isolated, after column chromatography (0 → 50% EtOAc in CH_2Cl_2), as a brown solid (2.86 g, 67%).

R_f = 0.32 (50% EtOAc in CH_2Cl_2).

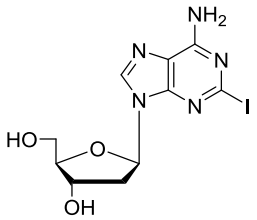
^{1}H NMR (400.1 MHz, $CDCl_3$): 2.09, 2.14 (2 × s, 2 × 3H, C**H**$_3$CO); 2.70 (ddd, 1H, *J* = 14.2, *J* = 6.0, *J* = 2.7, H-2′b); 2.83 (ddd, 1H, *J* = 14.2, *J* = 7.8, *J* = 6.3, H-2′a); 4.36 – 4.39 (m, 3H, H-4′, 5′); 5.38 – 5.43 (m, 1H, H-3′); 6.45 (dd, 1H, *J* = 7.8, *J* = 6.0, H-1′); 8.24 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, $CDCl_3$): 20.96, 21.04 (**C**H_3CO); 38.17 (CH_2-2′); 63.65 (CH_2-5′); 74.24 (CH-3′); 83.12 (CH-4′); 85.17 (CH-1′); 116.91 (C-2); 132.29 (C-5); 143.23 (CH-8); 150.89 (C-6); 151.88 (C-4); 170.36, 170.39 (CH_3**C**O).

MS (ESI$^+$): *m/z* (%): 503.0 (100) [(^{35}Cl)M+Na]$^+$, 505.0 (27) [(^{37}Cl)M+Na]$^+$.

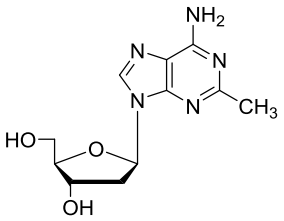
HRMS (ESI$^+$): calculated for $C_{14}H_{14}O_5N_4(^{35}Cl)INa$: 502.95896 found: 502.95864.

2-Iodo-2'-deoxyadenosine (31)



Reaction for the preparation of **31** was performed based on a published procedure.[187] Protected nucleoside **30** (2.5 g, 5.2 mmol) was suspended in methanolic ammonia (saturated at 0 °C, 65 ml) and the resulting mixture was heated at 100 °C for 5 h in a sealed pressure flask. After cooling, the volatiles were evaporated and the residue was redissolved in MeOH and coevaporated with silicagel. The desired product was isolated, after column chromatography (0 → 10% MeOH in CH_2Cl_2), as a white solid (1.72 g, 88%). The analytical data were in agreement with those published in the literature.[187]

2-Methyl-2'-deoxyadenosine (33)



2-Iodo-2'-deoxyadenosine **31** (100 mg, 0.27 mmol) and $(NH_4)_2SO_4$ (4 mg, 10 mol %) were dissolved in HMDS (2 ml) and the resulting solution was stirred at 40 °C for 20 minutes. Solvent was evaporated; the residue was dissolved in anhydrous THF (2 ml) and the flask was flushed with argon. To this $Pd(PPh_3)_4$ (31 mg, 10 mol %) dissolved in THF (0.5 ml) was added via syringe. Me_3Al (0.33 ml, 0.66 mmol, 2 M in toluene) was added dropwise. Reaction mixture was stirred at 60 °C for 2 h. After cooling, the

mixture was carefully quenched with MeOH (dropwise addition) and evaporated to dryness. The residue was dissolved in MeOH and coevaporated with silicagel. The desired product was purified by column chromatography (0% → 8% MeOH in CH_2Cl_2) and further purified by RP-HPFC on C-18 (0% → 100% MeOH in H_2O) affording a white solid (49 mg, 70%). ^{1}H NMR were in agreement with those reported previously.[189]

R_f: 0.18 (10% MeOH in CH_2Cl_2).

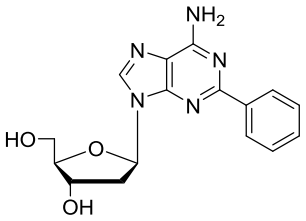
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.21 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 5.9, $J_{2'b,3'}$ = 2.4, H-2′b); 2.37 (s, 3H, CH_3); 2.69 (ddd, 1H, J_{gem} = 13.1, $J_{2'a,1'}$ = 8.4, $J_{2'a,3'}$ = 5.5, H-2′a); 3.53 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 7.5, $J_{5'b,4'}$ = 3.8, H-5′b); 3.63 (dt, 1H, J_{gem} = 11.8, $J_{5'a,4'}$ = $J_{5'a,OH}$ = 3.8, H-5′a); 3.89 (td, 1H, $J_{4',5'}$ = 3.8, $J_{4',3'}$ = 2.1, H-4′); 4.37 – 4.44 (bm, 1H, H-3′); 5.32 (bd, 1H, $J_{OH,3'}$ = 3.0, OH-3′); 5.59 (bdd, 1H, $J_{OH,5'}$ = 7.5, 3.8, OH-5′); 6.31 (dd, 1H, $J_{1',2'}$ = 8.4, 5.9, H-1′); 7.25 (bs, 2H, NH_2); 8.24 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 25.44 (CH_3); 39.70 (CH_2-2′); 62.38 (CH_2-5′); 71.49 (CH-3′); 84.51 (CH-1′); 88.43 (CH-4′); 117.76 (C-5); 139.40 (CH-8); 149.68 (C-4); 156.02 (C-6); 161.25 (C-2).

MS (ESI$^+$): *m/z* (%): 266.1 (44) $[M+H]^+$, 288.1 (100) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{11}H_{16}O_3N_5$: 266.12477; found: 266.12483.

2-Phenyl-2'-deoxyadenosine (34)



A flask containing 2-iodo-2'-deoxyadenosine **31** (100 mg, 0.27 mmol), $PhB(OH)_2$ (48 mg, 0.40 mmol), Cs_2CO_3 (259 mg, 0.80 mmol), $Pd(OAc)_2$ (3 mg, 5 mol %) and TPPTS (19 mg, 12.5 mol %) was filled with argon and H_2O/MeCN = 2/1 mixture (3.3 ml) was added. The reaction mixture was stirred at 80 °C until complete consumption of starting material was observed according to TLC (ca. 1.5 h). After cooling the solvent was evaporated. The residue was dissolved in MeOH, coevaporated with silicagel and purified by column chromatography (0% → 10% MeOH in CH_2Cl_2) to afford the desired nucleoside as a white solid (79 mg, 91%).

R_f: 0.27 (10% MeOH in CH_2Cl_2).

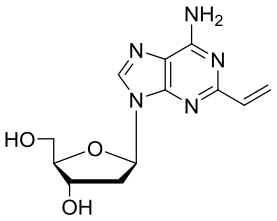
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.32 (ddd, 1H, J_{gem} = 13.3, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.1, H-2′b); 2.84 (ddd, 1H, J_{gem} = 13.3, $J_{2'a,1'}$ = 7.7, $J_{2'a,3'}$ = 5.8, H-2′a); 3.54 (ddd, 1H, J_{gem} = 11.6, $J_{5'b,OH}$ = 5.7, $J_{5'b,4'}$ = 4.8, H-5′b); 3.64 (dt, 1H, J_{gem} = 11.6, $J_{5'a,OH}$ = 5.4, $J_{5'a,4'}$ = 4.8, H-5′a); 3.89 (td, 1H, $J_{4',5'}$ = 4.8, $J_{4',3'}$ = 2.8, H-4′); 4.40 (dddd, 1H, $J_{3',2'}$ = 5.8, 3.1, $J_{3',OH}$ = 4.1, $J_{3',4'}$ = 2.8, H-3′); 4.95 (dd, 1H, $J_{OH,5'}$ = 5.7, 5.4, OH-5′); 5.36 (d, 1H, $J_{OH,3'}$ = 4.1, OH-3′); 6.44 (dd, 1H, $J_{1',2'}$ = 7.7, 6.2, H-1′); 7.32 (bs, 2H, NH_2); 7.40 – 7.50 (m, 3H, H-*m,p*-Ph); 8.33 – 8.35 (m, 2H, H-*o*-Ph); 8.36 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.32 (CH_2-2′); 62.07 (CH_2-5′); 71.17 (CH-3′); 83.54 (CH-1′); 87.98 (CH-4′); 118.47 (C-5); 127.87 (CH-*o*-Ph); 128.37 (CH-*m*-Ph); 129.74 (CH-*p*-Ph); 138.58 (C-*i*-Ph); 140.16 (CH-8); 150.34 (C-4); 156.00 (C-6); 158.05 (C-2).

MS (ESI$^+$): *m/z* (%): 328.1 (31) $[M+H]^+$, 350.1 (100) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{10}H_{14}O_3N_5Na$: 350.12236; found: 350.12243.

2-Vinyl-2'-deoxyadenosine (35)



A flask containing 2-iodo-2'-deoxyadenosine **31** (100 mg, 0.27 mmol), potassium vinyltrifluoroborate (53 mg, 0.40 mmol), Cs_2CO_3 (259 mg, 0.80 mmol), $Pd(OAc)_2$ (3 mg, 5 mol %) and TPPTS (19 mg, 12.5 mol %) was filled with argon and H_2O/MeCN = 2/1 mixture (3.3 ml) was added. The reaction mixture was stirred at 80 °C until complete consumption of starting material was observed according to TLC (ca 2.5 h). Solvent was evaporated and the residue was coevaporated with MeOH and silica gel. The title compound was obtained, after column chromatography purification (0% → 5% MeOH in CH_2Cl_2), as a white solid (59 mg, 80%). NMR data were in agreement with those reported previously.[227]

R_f: 0.25 (10% MeOH in CH_2Cl_2).

^{1}H NMR (500.0 MHz, DMSO-d_6): 2.25 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.8, H-2′b); 2.74 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 5.7, H-2′a); 3.53 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 6.8, $J_{5'b,4'}$ = 4.2, H-5′b); 3.63 (dt, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = $J_{5'a,4'}$ = 4.6, H-5′a); 3.89 (ddd, 1H, $J_{4',5'}$ = 4.6, 4.2, $J_{4',3'}$ = 2.4, H-4′); 4.42 (dddd, 1H, $J_{3',2'}$ = 5.7, 2.8, $J_{3',OH}$ = 4.0, $J_{3',4'}$ = 2.4, H-3′); 5.23 (dd, 1H, $J_{OH,5'}$ = 6.8, 4.6, OH-5′); 5.31 (d, 1H, $J_{OH,3'}$ =

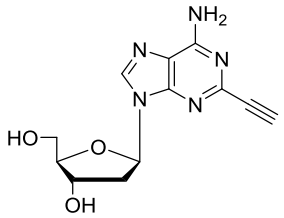
4.0, OH-3′); 5.54 (dd, 1H, J_{cis} = 10.5, J_{gem} = 2.3, $CH_a\mathbf{H_b}$=CH); 6.35 (dd, 1H, $J_{1',2'}$ = 8.1, 6.1, H-1′); 6.36 (dd, 1H, J_{trans} = 17.3, J_{gem} = 2.3, $C\mathbf{H_a}H_b$=CH); 6.60 (dd, 1H, J_{trans} = 17.3, J_{cis} = 10.5, C**H**=CH_2); 7.27 (bs, 2H, NH_2); 8.31 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.53 (CH_2-2′); 62.19 (CH_2-5′); 71.30 (CH-3′); 84.03 (CH-1′); 88.20 (CH-4′); 118.69 (C-5); 121.24 (**C**H_2=CH); 137.41 (**C**H=CH_2); 140.17 (CH-8); 149.71 (C-4); 155.88 (C-6); 157.94 (C-2).

MS (ESI$^+$): *m/z* (%): 278.1 (28) $[M+H]^+$, 300.1 (100) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{12}H_{15}O_3N_5Na$: 300.10671; found: 300.10683.

2-Ethynyl-2'-deoxyadenosine (36)



2-Ethynyl-2'-deoxyadenosine **36** was prepared according to the literature procedure with minor modifications.[187] Dry DMF (2.8 ml) was added to a flask containing 2-Iodo-2'-deoxyadenosine **31** (150 mg, 0.40 mmol), $PdCl_2(PPh_3)_2$ (28 mg, 10 mol %) and CuI (8 mg, 10 mol %) and the resulting mixture was purge-and-refilled with argon 3 times. Trimethylsilylacetylene (84 µl, 0.60 mmol) and Et_3N (110 µl, 0.80 mmol) were added via syringe and the mixture was stirred at rt (25 °C) until the complete consumption of the starting nucleoside was observed by TLC (ca. 3 hours). Then the reaction mixture was filtered through a pad of celite and evaporated under reduced pressure. The residue was dissolved in methanolic ammonia (saturated at 0 °C, 10 ml). The reaction mixture was stirred at room temperature for 1.5 h, silicagel was added and volatiles were evaporated. Purification by column chromatography (0% → 10% MeOH in CH_2Cl_2) and further purification by RP-HPFC on C-18 (0% → 100% MeOH in H_2O) provided the title compound as a white solid (86 mg, 79% over two steps).

R_f: 0.36 (10% MeOH in CH_2Cl_2).

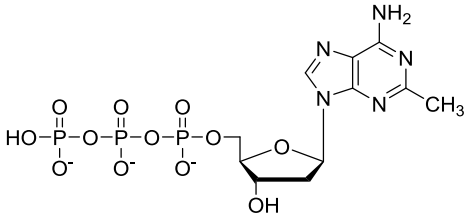
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.27 (ddd, 1H, J_{gem} = 13.2, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 3.1, H-2′b); 2.67 (ddd, 1H, J_{gem} = 13.2, $J_{2'a,1'}$ = 7.7, $J_{2'a,3'}$ = 5.8, H-2′a); 3.51 (ddd, 1H, J_{gem} = 11.8, $J_{5'b,OH}$ = 6.3, $J_{5'b,4'}$ = 4.4, H-5′b); 3.60 (ddd, 1H, J_{gem} = 11.8, $J_{5'a,OH}$ = 5.2, $J_{5'a,4'}$ = 4.4, H-5′a); 3.86 (td, 1H, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 2.7, H-4′); 4.02 (s, 1H, HC≡C-); 4.37 – 4.42 (bm, 1H, H-3′); 5.08 (bdd, 1H, $J_{OH,5'}$ = 6.3, 5.2, OH-5′); 5.34 (bd, 1H, $J_{OH,3'}$ = 4.0, OH-3′); 6.31 (dd, 1H, $J_{1',2'}$ = 7.7, 6.1, H-1′); 7.52 (bs, 2H, NH_2); 8.41 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.63 (CH_2-2′); 61.95 (CH_2-5′); 71.01 (CH-3′); 75.20 (H**C**≡C-); 83.51 (-**C**≡CH); 83.84 (CH-1′); 88.18 (CH-4′); 119.21 (C-5); 140.62 (CH-8); 144.48 (C-2); 149.09 (C-4); 156.06 (C-6).

MS (ESI$^+$): *m/z* (%): 276.1 (5) $[M+H]^+$, 298.1 (100) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{12}H_{13}O_3N_5Na$: 298.09106; found: 298.09123.

2-Methyl-2'-deoxyadenosine-5'-*O*-triphosphate (37, d^{Me}ATP)



The title nucleotide **37** was prepared following General procedure A starting from nucleoside **33** (10 mg, 0.038 mmol). It was isolated as a white lyophilisate (5.2 mg, 23%).

^{1}H NMR (500.0 MHz, D_2O): 2.52 (s, 3H, CH_3); 2.57 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 3.7, H-2′b); 2.79 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.3, $J_{2'a,3'}$ = 6.2, H-2′a); 4.14 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 5.0, $J_{5'b,4'}$ = 3.7, H-5′b); 4.23 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 6.2, $J_{5'a,4'}$ = 3.7, H-5′a); 4.28 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 3.7, $J_{H,P}$ = 1.9, H-4′); 4.80 (dt, 1H, $J_{3',2'}$ = 6.2, 3.7, $J_{3',4'}$ = 3.7, H-3′); 6.48 (dd, 1H, $J_{1',2'}$ = 7.3, 6.3, H-1′); 8.43 (s, 1H, H-8).

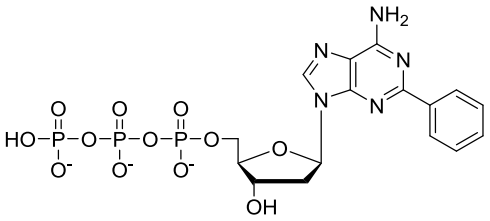
^{13}C NMR (125.7 MHz, D_2O): 26.76 (CH_3); 41.82 (CH_2-2′); 68.01 (d, $J_{C,P}$ = 5.7, CH_2-5′); 73.64 (CH-3′); 86.04 (CH-1′); 88.54 (d, $J_{C,P}$ = 9.1, CH-4′); 119.44 (C-5); 142.32 (CH-8); 152.34 (C-4); 158.05 (C-6); 165.66 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.82 (t, J = 19.7, P_β); -10.47 (d, J = 19.7, P_α); -7.41 (bs, P_γ).

MS (ESI$^-$): *m/z* (%): 344.1 (60) $[M-H_3P_2O_6]^-$, 424.1 (100) $[M-H_2PO_3]^-$, 446.1 (56) $[M-H-H_2PO_3+Na]^-$.

HRMS (ESI$^-$): calculated for $C_{11}H_{15}O_{12}N_5P_3Na$: 525.99115; found: 525.99033.

2-Phenyl-2'-deoxyadenosine-5'-*O*-triphosphate (38, d^{Ph}ATP)



The title nucleotide **38** was prepared following General procedure A starting from nucleoside **34** (28 mg, 0.086 mmol). It was isolated as a white lyophilisate (13.2 mg, 24%).

^{1}H NMR (600.0 MHz, D_2O): 2.60 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.5, $J_{2'b,3'}$ = 3.7, H-2′b); 2.86 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.3, $J_{2'a,3'}$ = 6.2, H-2′a); 4.19 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 5.2, $J_{5'b,4'}$ = 3.7, H-5′b); 4.25 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 3.7, H-5′a); 4.31 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 3.7, $J_{H,P}$ = 1.6, H-4′); 4.85 (dt, 1H, $J_{3',2'}$ = 6.2, 3.7, $J_{3',4'}$ = 3.7, H-3′); 6.63 (dd, 1H, $J_{1',2'}$ = 7.3, 6.5, H-1′); 7.51 – 7.59 (m, 3H, H-*m,p*-Ph); 8.09 – 8.15 (m, 2H, H-*o*-Ph); 8.49 (s, 1H, H-8).

^{13}C NMR (150.9 MHz, D_2O): 41.83 (CH_2-2′); 68.11 (d, $J_{C,P}$ = 5.8, CH_2-5′); 73.69 (CH-3′); 86.15 (CH-1′); 88.49 (d, $J_{C,P}$ = 9.0, CH-4′); 120.19 (C-5); 130.97 (CH-*o*-Ph); 131.40 (CH-*m*-Ph); 133.16 (CH-*p*-Ph); 140.19 (CH-*i*-Ph); 143.15 (CH-8); 152.94 (C-4); 158.49 (C-6); 163.42 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.75 (bt, J = 19.6, P_β); -10.42 (d, J = 19.6, P_α); -7.39 (bs, P_γ).

MS (ESI$^-$): *m/z* (%): 406.3 (50) $[M–H_3P_2O_6]^-$, 486.3 (100) $[M–H_2PO_3]^-$, 508.3 (22) $[M–H–H_2PO_3+Na]^-$.

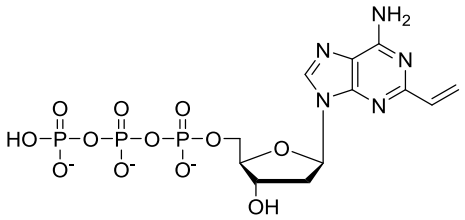
HRMS (ESI$^-$): calculated for $C_{16}H_{18}O_{12}N_5P_3Na$: 588.00680; found: 588.00623.

General procedure B for the preparation of (modified) 2'-deoxynucleoside-5'-*O*-triphosphates bearing unsaturated bonds

Nucleoside (1 equiv.) and proton sponge (1.2 equiv.) were dried in vacuo overnight. Then anhydrous $PO(OMe)_3$ (100 equiv.) was added and the resulting solution was cooled to 0 °C. Freshly distilled $POCl_3$ (1.2 equiv.) was added dropwise and the resulting mixture was stirred at 0 °C until complete disappearance of starting compound was observed by TLC (ca. 3-4 hours). Then an ice-cold solution of $(n\text{-}Bu_3NH)_2H_2P_2O_7$ (5.0 equiv.) and n-Bu_3N (4.0 equiv.) in anhydrous DMF [0.65 M solution of $(n\text{-}Bu_3NH)_2H_2P_2O_7$] was added and the reaction mixture was stirred at 0 °C for additional 1-1.5 h. The reaction was treated with 2 M TEAB solution (the same volume as volume

of DMF). The mixture was concentrated on a rotavap and the residue was co-evaporated with distilled water three times. The crude product was dissolved in water (ca. 3-5 ml). The aqueous solution was purified by semi-preparative HPLC using a linear gradient of methanol (5→100%) in 0.1 M TEAB buffer. The appropriate fractions were combined and evaporated on a rotavap. The viscous oil was coevaporated with distilled water three times. The product was converted to Na^+ salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The desired nucleotides were isolated as lyophilisates.

2-Vinyl-2'-deoxyadenosine-5'-*O*-triphosphate (39, d^VATP)



The title nucleotide **39** was prepared following General procedure B starting from nucleoside **35** (23.5 mg, 0.085 mmol). It was isolated as a white lyophilisate (11.5 mg, 22%).

^{1}H NMR (500.0 MHz, D_2O): 2.59 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.4, $J_{2'b,3'}$ = 3.7, H-2′b); 2.83 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.4, $J_{2'a,3'}$ = 6.0, H-2′a); 4.16 (ddd, 1H, J_{gem} = 11.8, $J_{H,P}$ = 5.2, $J_{5'b,4'}$ = 3.7, H-5′b); 4.24 (ddd, 1H, J_{gem} = 11.8, $J_{H,P}$ = 6.4, $J_{5'a,4'}$ = 3.7, H-5′a); 4.29 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 3.7, $J_{H,P}$ = 1.6, H-4′); 4.83 (dt, 1H, $J_{3',2'}$ = 6.0, 3.7, $J_{3',4'}$ = 3.7, H-3′); 5.72 (dd, 1H, J_{cis} = 10.8, J_{gem} = 1.4, $CH_a\mathbf{H_b}$=CH); 6.37 (dd, 1H, J_{trans} = 17.4, J_{gem} = 1.4, $C\mathbf{H_a}H_b$=CH); 6.53 (dd, 1H, $J_{1',2'}$ = 7.4, 6.4, H-1′); 6.72 (dd, 1H, J_{trans} = 17.4, J_{cis} = 10.8, CH_2=C**H**); 8.46 (s, 1H, H-8).

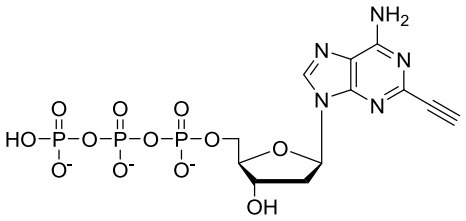
^{13}C NMR (125.7 MHz, D_2O): 41.83 (CH_2-2′); 68.08 (d, $J_{C,P}$ = 5.4, CH_2-5′); 73.71 (CH-3′); 86.09 (CH-1′); 88.50 (d, $J_{C,P}$ = 9.1, CH-4′); 120.34 (C-5); 126.27 (**C**H_2=CH); 137.66 (**C**H=CH_2); 143.02 (CH-8); 152.38 (C-4); 158.19 (C-6); 162.04 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.68 (t, J = 19.5, P_β); -10.39 (d, J = 19.5, P_α); -7.63 (d, J = 19.5, P_γ).

MS (ESI^-): *m/z* (%): 356.1 (30) $[M–H_3P_2O_6]^-$, 436.1 (100) $[M–H_2PO_3]^-$, 458.1 (45) $[M–H–H_2PO_3+Na]^-$.

HRMS (ESI^-): calculated for $C_{12}H_{17}O_{12}N_5P_3Na$: 516.00920; found: 516.00873.

2-Ethynyl-2'-deoxyadenosine-5'-*O*-triphosphate (40, d^{E}ATP)



The title nucleotide **40** was prepared following General procedure B starting from nucleoside **36** (33 mg, 0.101 mmol). It was isolated as a white lyophilisate (20.5 mg, 31%).

^{1}H NMR (600.0 MHz, D_2O): 2.60 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 3.7, H-2′b); 2.80 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.3, $J_{2'a,3'}$ = 6.1, H-2′a); 3.51 (s, 1H, HC≡C); 4.15 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 5.0, $J_{5'b,4'}$ = 3.7, H-5′b); 4.23 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 3.7, H-5′a); 4.30 (qd, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.7, $J_{H,P}$ = 2.0, H-4′); 4.81 (dt, 1H, $J_{3',2'}$ = 6.1, 3.7, $J_{3',4'}$ = 3.7, H-3′); 6.46 (dd, 1H, $J_{1',2'}$ = 7.3, 6.3, H-1′); 8.53 (s, 1H, H-8).

^{13}C NMR (150.9 MHz, D_2O): 41.98 (CH_2-2′); 68.07 (d, $J_{C,P}$ = 5.6, CH_2-5′); 73.73 (CH-3′); 78.74 (H**C**≡C-); 83.52 (-**C**≡CH); 86.38 (CH-1′); 88.62 (d, $J_{C,P}$ = 9.1, CH-4′); 121.29 (C-5); 143.54 (CH-8); 147.64 (C-2); 151.43 (C-4); 158.13.

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.00 (bt, J = 19.6, P_{β}); -10.52 (d, J = 19.6, P_{α}); -8.70 (bd, J = 19.6, P_{γ}).

MS (ESI$^-$): *m/z* (%): 354.1 (29) $[M–H_3P_2O_6]^-$, 434.0 (100) $[M–H_2PO_3]^-$, 456.0 (63) $[M–H–H_2PO_3+Na]^-$.

HRMS (ESI$^-$): calculated for $C_{12}H_{13}O_{12}N_5P_3Na_2$: 557.95744; found: 557.95752.

Enzymatic synthesis of minor-groove modified DNA using d^{R}ATPs

General remarks

Finished PEX reactions were stopped by the addition of PAGE stop solution (40 µl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 µl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gels were visualized by a fluorescent scanner.

Analytical PEX - Single incorporation of modified ***d^{R}ATPs***

Reaction mixture (20 μl) contained Prim15-FAM (0.150 μM), Temp19A (0.225 μM), either KOD XL (0.075 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** and 0.125 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**), Vent(exo-) (0.2 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** and 0.4 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**) or Bst LF (0.24 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** or 0.4 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**) DNA polymerase, natural dGTP (6 μM), either natural dATP (20 μM) or modified **d^{R}ATP** (10 μM **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** for and 20 μM for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 30 min.

Analytical PEX - Multiple incorporations of modified ***d^{R}ATPs***

Reaction mixture (20 μl) contained primer Prim15-FAM (0.150 μM), Temp31-TINA (0.225 μM), KOD XL (0.125 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** and 1.25 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**), Vent(exo-) (0.2 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** and 0.4 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**) or Bst LF (0.4 U) DNA polymerase, natural dNTPs (200 μM), either natural dATP (200 μM) or modified **d^{R}ATP** (200 μM for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** or 500 μM for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min.

Kinetic study of incorporation of modified ***d^{R}ATPs***

PEX reaction mixtures (30 μl) containing KOD XL DNA polymerase (0.5 U), Prim15-FAM (0.15 μM), Temp16A (0.225 μM), natural or modified dATPs (133 μM) and KOD XL reaction buffer (10×, 3 μl) were incubated at 60 °C for specific time intervals (0.1 – 5 minutes) and then the reaction was stopped by the addition of PAGE stop solution and immediate heating. Aliquots (5 μl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

Semi-preparative primer extension with magnetic separation

Reaction mixture (50 μl) contained *KOD XL* DNA polymerase (0.25 U for **d^{Cl}ATP**, **d^{NH_2}ATP** and **d^{Me}ATP** or 1.25 U for **d^{V}ATP**, **d^{E}ATP** and **d^{Ph}ATP**), Prim15 (3.2 μM), 5'-biotinylated template (Temp19A-bio or Temp31-bio; 3.2 μM), natural or modified dNTPs (dTTP, dCTP, dGTP and **d^{R}ATP**, 208 μM) and KOD XL reaction buffer (10×, 5

µl). The reaction mixture was incubated for 40 min at 60 °C in a thermal cycler and the reaction was stopped by cooling to 4 °C. Streptavidin magnetic particles (Roche, 50 µl) were washed with binding buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Then PEX solution (50 µl) and binding buffer (50 µl) were added to the magnetic beads. The mixture was incubated for 30 min at 15 °C and 1100 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (4 × 200 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 50 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS.

Semi-preparative preparation of modified DNA

Reaction mixture (250 µl) contained KOD XL DNA polymerase (2.5 U), Prim15 (4 µM), template (Temp19A or Temp31; 4 µM), natural dNTPs (dTTP, dGTP, dCTP, 80 µM), modified **d^RATPs** (80 µM) and reaction buffer for KOD XL DNA polymerase (10×, 25 µl). Reaction mixture was incubated at 60 °C for 40 min (70 °C for 60 min for Temp31) in a thermoblock and stopped by cooling to 4 °C. dsDNA was purified with a QIAquick nucleotide removal kit (QIAGEN); reaction mixture was divided into two columns. Product was eluted with 100 µl of H_2O and concentration was determined using NanoDrop and values obtained from OligoCalc.

Natural DNA for control experiments was prepared following the same procedure using natural dATP instead of modified **d^RATPs**.

PCR using ***d^RATPs***

d^{NH_2}ATP

Reaction mixture (10 µl) contained KOD XL DNA polymerase (0.5 U), Prim_FOR and Prim_REV (both 1 µM), Temp98 (25 nM), natural dNTPs (dCTP, dTTP, dGTP, 6 µM), either natural dATP or **d^{NH_2}ATP** (8 µM) and KOD XL reaction buffer (10×, 1 µl).

d^{Cl}ATP *and* ***d^{Me}ATP***

Reaction mixture (10 µl) contained KOD XL DNA polymerase (1.25 U), Prim_FOR and Prim_REV (both 2 µM), Temp98 (50 nM), natural dNTPs (dCTP, dTTP, dGTP, 300 µM), either natural dATP (8 µM) or **d^RATP** (1 mM) and KOD XL reaction buffer (10×, 1 µl).

d^VATP and d^EATP

Reaction mixture (10 μl) contained KOD XL DNA polymerase (1.25 U), Prim_FOR and Prim_REV (both 4 μM), Temp98 (50 nM), natural dNTPs (dCTP, dTTP, dGTP, 300 μM), either natural dATP (8 μM) or **d^RATP** (1 mM) and KOD XL reaction buffer (10×, 1 μl).

The PCR reactions were placed in a preheated PCR cycler (80 °C) and 35 (30 in the case of ***d^{NH_2}ATP)*** PCR cycles of the following conditions took place: 1. preheating at 94 °C for 3 min; 2. denaturation at 95 °C for 1 min; 3. primer annealing at 54 °C for 1 min; 4. extension at 72 °C for 1 min. Steps 2-4 were repeated 35 times and were followed by final extensions step at 75 °C for 5 min. Reactions were stopped by cooling to 4 °C. Products of the reactions were analysed on 2% agarose gel (containing 0.5x TBE buffer, pH 8) stained with GelRed (Biotium, 10 000× in H_2O). The gels were run at 118 V for ca. 60–90 min. The gels were visualised using an electronic dual wave transilluminator.

Post-synthetic modification of DNA

Thiol-ene reactions of vinyl modified DNA

Vinyl modified DNA (**DNA1^{V}A** or **DNA4^{V}A**; 0.2 nmol) was dissolved in H_2O (5 μl). To this thiol [**CM-SH**, 50 mM in TEAA buffer (pH = 8.0), 50 μl] was added and the reaction mixture was kept at 37 °C and 500 rpm for 3 days.

For steady-state fluorescence measurements the product of thiol-ene addition was purified with QIAquick nucleotide removal kit (QIAGEN). Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc, samples were concentrated. Concentration of DNA was adjusted to 1 μM in miliQ H_2O. Emission spectra were recorded using a 100 μl quartz cuvette in phosphate buffer (20 mM, pH = 7.0, 1 M NaCl) by adding 10 μl of DNA solution; solution was mixed carefully with a pipete and equilibrated for 2 min in a thermal holder (25 °C) before the fluorescence spectrum was recorded. Excitation wavelength for **DNAX^{CM}A** was 355 nm. Control experiments were performed using natural non-modified DNA following the same procedure.

To obtain single-stranded DNA for MALDI analysis, the reaction was performed as described above using DNA after PEX with biotinylated template. The reaction mixture was diluted to a total volume of 200 μl with binding buffer (10 mM Tris, 1 mM EDTA,

100 mM NaCl, pH 7.5). Streptavidin magnetic particles (Roche, 200 µl) were washed with binding buffer (3 × 250 µl). The diluted click reaction (200 µl) was then added to magnetic particles. Mixture was incubated at 15 °C and 1100 rpm for 30 min. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 250 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 250 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS.

To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp19A. The product of thiol-ene reaction was purified with QIAquick nucleotide removal kit (QIAGEN). Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc, concentration of DNA was adjusted to 150 nM with miliQ water: PAGE stop solution (1:2). Aliquots (5 µl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 75 min. The gel was visualized by a fluorescent scanner. Conversion of the reaction was determined using ImageJ software.

Click reaction of ethynyl modified DNA

Ethynyl modified DNA (**DNA1^{E}A** or **DNA4^{E}A**; 0.2 nmol) was dissolved in H_2O:DMSO:tBuOH mixture (9:3:1, 8 µl). The reaction mixture was prepared by adding azide (**Cy3-N_3**, 50 mM in DMSO, 5.6 µl), sodium ascorbate (5 mM in H_2O, 4 µl) and Cu^{I} solution, which was prepared just beforehand by mixing CuBr (100 mM in DMSO, 0.4 µl) and TBTA (100 mM in DMSO, 2 µl). The reaction mixture was incubated at 37 °C and 500 rpm for 5 h.

Steady-state fluorescence measurements were performed as described above. Excitation wavelength λ_{ex} for **DNAX^{Cy3}A** was 540 nm. Control experiments were performed using natural non-modified DNA following the same procedure.

To obtain single-stranded DNA for MALDI analysis, the reaction was performed as described above using DNA after PEX with biotinylated template. Sample for MALDI analysis was acquired following the magnetoseparation procedure mentioned above.

Product of CuAAC reaction was analyzed using PAGE as described for the analysis of thiol-ene reaction.

Thermal denaturation studies

The oligonucleotides for these experiments were prepared by PEX on a large scale with KOD XL DNA polymerase and templates Temp19A and Temp31 and Prim15. Alternatively the oligonucleotides were prepared by post-synthetic modification of **DNA1^{V}A** or **DNA1^{E}A** by fluorescent labels. Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc. Samples were concentrated and then redissolved in phosphate buffer (20 mM, pH = 7.0, 1 M NaCl) to have A_{260} approximately 0.1. Data of the thermal denaturation studies of DNA duplexes was collected from 6 independent heating-cooling cycles. Annealing temperatures (T_m / °C) were obtained by plotting temperature versus absorbance and applying a sigmodial curve fit.

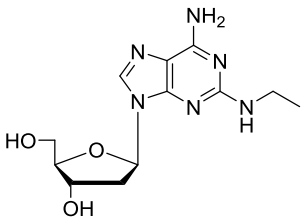
5.3 Synthesis of 2-alkylamino dATP derivatives and their use in site-specific minor-groove modified DNA synthesis

Synthetic procedures for the preparation of 2-alkylamino modified dATPs

General procedure C for the synthesis of 2-alkylamino modified 2'-deoxyadenosines (d^RAs)

2-Chloro-2'-deoxyadenosine **23** (1 equiv.) was dissolved in MeOH (0.08 M solution) and corresponding primary amine (30 equiv.) was added. Reaction mixture was heated at 130 °C in a sealed pressure flask for 24 h. After cooling, silicagel was added and the solvent was evaporated. The title compounds were obtained, after chromatographic purification, as solids.

2-Ethylamino-2'-deoxyadenosine (41)



2-Ethylamino-2'-deoxyadenosine **41** was prepared according to General procedure C starting from **23** (100 mg, 0.35 mmol) and ethylamine (2.0 M in methanol). The title compound was isolated, after RP-HPFC on C-18 column (0 → 100 % MeOH in H_2O), as a white solid (93 mg, 90%).

R_f: 0.31 (10% MeOH in $CHCl_3$).

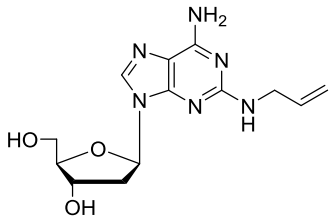
^{1}H NMR (500.0 MHz, DMSO-d_6): 1.09 (t, 3H, J_{vic} = 7.1, C**H$_3$**CH$_2$N); 2.16 (ddd, 1H, J_{gem} = 13.0, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.8, H-2′b); 2.70 (ddd, 1H, J_{gem} = 13.0, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 5.5, H-2′a); 3.25 (qd, 2H, J_{vic} = 7.1, 5.7, CH$_3$C**H$_2$**N); 3.49 (dd, 1H, J_{gem} = 11.6, $J_{5'b,4'}$ = 4.6, H-5′b); 3.58 (dd, 1H, J_{gem} = 11.6, $J_{5'a,4'}$ = 4.9, H-5′a); 3.82 (ddd, 1H, $J_{4',5'}$ = 4.9, 4.6, $J_{4',3'}$ = 2.5, H-4′); 4.37 (ddd, 1H, $J_{3',2'}$ = 5.5, 2.8, $J_{3',4'}$ = 2.5, H-3′); 5.03, 5.26 (2 × bs, 2 × 1H, OH-3′,5′); 6.15 (t, 1H, J_{vic} = 5.7, CH$_3$CH$_2$N**H**); 6.17 (dd, 1H, $J_{1',2'}$ = 8.1, 6.1, H-1′); 6.69 (bs, 2H, NH$_2$-6); 7.88 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 15.30 (**C**H$_3$CH$_2$N); 35.87 (CH$_3$**C**H$_2$N); 39.05 (CH$_2$-2′); 62.25 (CH$_2$-5′); 71.30 (CH-3′); 83.26 (CH-1′); 87.76 (CH-4′); 113.69 (C-5); 136.13 (CH-8); 151.45 (C-4); 156.19 (C-6); 159.49 (C-2).

MS (ESI$^+$): *m/z* (%): 295.1 (100) [M+H]$^+$, 317.1 (22) [M+Na]$^+$.

HRMS (ESI$^+$): calculated for $C_{12}H_{19}O_3N_6$: 295.15131; found: 295.15140.

2-Allylamino-2'-deoxyadenosine (42)



2-Allylamino-2'-deoxyadenosine **42** was prepared according to General procedure C starting from **23** (123 mg, 0.43 mmol) and allylamine. The title compound was isolated, after column chromatography (0 → 6 % MeOH in $CHCl_3$), as a yellowish solid (99 mg, 75%).

R_f: 0.34 (10% MeOH in $CHCl_3$).

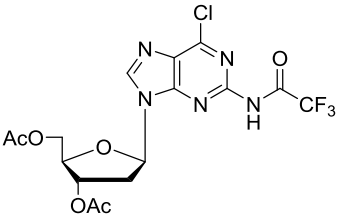
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.16 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.8, H-2′b); 2.70 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 8.1, $J_{2'b,3'}$ = 5.7, H-2′b); 3.49, 3.58 (2 × dt, 2 × 1H, J_{gem} = 11.7, $J_{5',4'}$ = $J_{5',OH}$ = 4.8, H-5′); 3.81 (td, 1H, $J_{4',5'}$ = 4.8, $J_{4',3'}$ = 2.8, H-4′); 3.84 – 3.95 (m, 2H, -CH_2CH=CH_2); 4.36 (ddt, 1H, $J_{3',2'}$ = 5.7, 2.8, $J_{3',OH}$ = 3.8, $J_{3',4'}$ = 2.8, H-3′); 4.99 (bs, 1H, OH-5′); 5.01 (ddt, 1H, J_{cis} = 10.3, J_{gem} = 1.9, ^4J = 1.6, -CH_2CH=CH_aH_b); 5.14 (ddt, 1H, J_{trans} = 17.2, J_{gem} = 1.9, ^4J = 1.7, -CH_2CH=CH_aH_b); 5.25 (bd, 1H, $J_{OH,3'}$ = 3.8, OH-3′); 5.91 (ddt, 1H, J_{trans} = 17.2, J_{cis} = 10.3, J_{vic} = 5.2, -CH_2CH=CH_2); 6.17 (dd, 1H, $J_{1',2'}$ = 8.1, 6.1, H-1′); 6.34 (t, 1H, J = 6.0, NH); 6.72 (bs, 2H, NH_2); 7.90 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 39.03 (CH_2-2′); 43.63 (-CH_2CH=CH_2); 62.23 (CH_2-5′); 71.28 (CH-3′); 83.20 (CH-1′); 87.75 (CH-4′); 113.82 (C-5); 114.54 (-CH_2CH=CH_2); 136.24 (CH-8); 137.07 (-CH_2CH=CH_2); 151.38 (C-4); 156.19 (C-6); 159.35 (C-2).

MS (ESI$^+$): *m/z* (%): 307.1 (100) $[M+H]^+$, 329.1 (38) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{13}H_{18}O_3N_6Na$: 329.13326; found: 329.13331.

6-Chloro-2-trifluoroacetamido-9-(2'-deoxy-3',5'-di-*O*-acetyl-β-D-ribofuranosyl)purine (43)



To a solution of **29** (500 mg, 1.35 mmol) in anhydrous CH_2Cl_2 (11 ml), DIPEA (0.7 ml, 4.06 mmol) was added. Then TFA_2O (0.4 ml, 2.70 mmol) was added dropwise and the

reaction mixture was stirred at rt (25 °C) for 1 h. Then saturated NH_4Cl solution (30 ml) was added and the phases were separated. Water phase was extracted with CH_2Cl_2 (2 × 30 ml). Collected organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography (0% → 50% EtOAc in PE). The title compound was obtained as yellowish foam (587 mg, 93%). The compound decomposes slowly on the bench, long-term storage at -20 °C.

R_f: 0.27 (50% EtOAc in PE).

^{1}H NMR (500.0 MHz, $CDCl_3$): 2.06, 2.15 (2 × s, 2 × 3H, CH_3CO); 2.67 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 6.3, $J_{2'b,3'}$ = 2.8, H-2′b); 3.15 (ddd, 1H, J_{gem} = 14.2, $J_{2'b,1'}$ = 7.5, $J_{2'b,3'}$ = 6.5, H-2′b); 4.37 – 4.47 (m, 3H, H-4′,5′); 5.53 (dt, 1H, $J_{3',2'}$ = 6.5, 2.8, $J_{3',4'}$ = 2.8, H-3′); 6.42 (dd, 1H, $J_{1',2'}$ = 7.5, 6.2, H-1′); 8.25 (s, 1H, H-8); 8.85 (bs, 1H, NH).

^{13}C NMR (125.7 MHz, $CDCl_3$): 20.75, 20.92 (**C**H_3CO); 37.02 (CH_2-2′); 63.63 (CH_2-5′); 74.25 (CH-3′); 83.06 (CH-4′); 85.64 (CH-1′); 115.18 (q, $J_{C,F}$ = 289.3, **C**F_3CO); 130.22 (C-5); 144.21 (CH-8); 149.62 (C-6); 151.63 (C-4); 151.88 (C-2); 153.62 (q, $J_{C,F}$ = 38.7, CF_3**C**O); 170.22, 170.43 (CH_3**C**O).

^{19}F NMR (470.4 MHz, $CDCl_3$): -72.48.

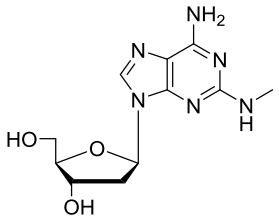
MS (ESI^+): *m/z* (%): 488.0 (100) $[^{35}(Cl)M+Na]^+$, 490.0 (38) $[^{37}(Cl)M+Na]^+$.

HRMS (ESI^+): calculated for $C_{16}H_{15}O_6N_5ClF_3Na$: 488.05552; found: 488.05556.

General procedure D for the synthesis of 2-alkylamino modified 2'-deoxyadenosines (d^RAs)

Protected nucleoside (1 equiv.) and K_2CO_3 (2 equiv.) were suspended in anhydrous DMF (0.1 M). To this, corresponding alkyl halide (1.25 equiv.) was added dropwise and the reaction mixture was stirred at rt (25 °C) for 24 h. Mixture was evaporated to dryness and the residue was dissolved in methanolic ammonia (saturated at 0 °C; 0.13 M). The reaction mixture was stirred in a sealed pressure flask at 100 °C overnight. After cooling the solvent was evaporated and the residue was coevaporated with silicagel. The desired nucleosides were obtained, after chromatographic purification, as solids.

2-Methylamino-2'-deoxyadenosine (46)



2-Methylamino-2'-deoxyadenosine **46** was prepared according to General procedure D starting from **43** (250 mg, 0.54 mmol) and methyl iodide. The title compound was isolated, after RP-HPFC on C-18 column (0 → 100 % MeOH in H_2O), as a white solid (105 mg, 70% over two steps).

R_f: 0.27 (10% MeOH in $CHCl_3$).

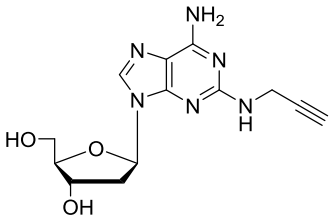
^{1}H NMR (500.0 MHz, DMSO-d_6): 2.16 (ddd, 1H, J_{gem} = 13.0, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.8, H-2′b); 2.71 (ddd, 1H, J_{gem} = 13.0, $J_{2'a,1'}$ = 8.1, $J_{2'a,3'}$ = 5.5, H-2′a); 2.75 (d, 3H, J = 4.8, C**H$_3$**NH); 3.50 (dd, 1H, J_{gem} = 11.7, $J_{5'b,4'}$ = 4.5, H-5′b); 3.59 (dd, 1H, J_{gem} = 11.7, $J_{5'a,4'}$ = 4.8, H-5′a); 3.82 (ddd, 1H, $J_{4',5'}$ = 4.8, 4.5, $J_{4',3'}$ = 2.5, H-4′); 4.38 (ddd, 1H, $J_{3',2'}$ = 5.5, 2.8, $J_{3',4'}$ = 2.5, H-3′); 4.92 – 5.45 (bm, 2H, OH-3′,5′); 6.16 (q, 1H, J = 4.8, CH_3N**H**); 6.19 (dd, 1H, $J_{1',2'}$ = 8.1, 6.1, H-1′); 6.71 (bs, 2H, NH_2-6); 7.89 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 28.58 (CH_3NH); 39.08 (CH_2-2′); 62.25 (CH_2-5′); 71.33 (CH-3′); 83.29 (CH-1′); 87.79 (CH-4′); 113.68 (C-5); 136.15 (CH-8); 151.44 (C-4); 156.15 (C-6); 160.19 (C-2).

MS (ESI$^+$): *m/z* (%): 281.1 (100) $[M+H]^+$, 303.1 (25) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{11}H_{17}O_3N_6$: 281.13566; found: 281.13570.

2-Propargylamino-2'-deoxyadenosine (47)



2-Propargylamino-2'-deoxyadenosine **47** was prepared according to General procedure D starting from **43** (300 mg, 0.64 mmol) and propargyl bromide (80 wt. % in toluene). The title compound was isolated, after column chromatography (0% → 10% MeOH in CH_2Cl_2), as a yellowish solid (146 mg, 74% over two steps).

R_f: 0.20 (10% MeOH in $CHCl_3$).

^{1}H NMR (500.0 MHz, DMSO-d_6): 2.17 (ddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 6.1, $J_{2'b,3'}$ = 2.8, H-2′b); 2.72 (bddd, 1H, J_{gem} = 13.1, $J_{2'b,1'}$ = 8.0, $J_{2'b,3'}$ = 5.7, H-2′b); 2.95 (t, 1H, ^4J = 2.4, **H**C≡C-); 3.50, 3.59 (2 × dt, 2 × 1H, J_{gem} = 11.6, $J_{5',4'}$ = $J_{5',OH}$ = 4.9, H-5′); 3.82 (td, 1H,

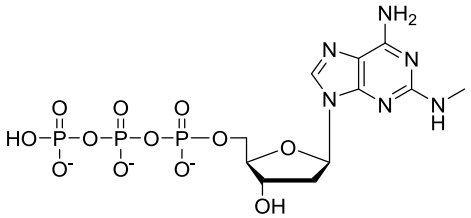
$J_{4',5'}$ = 4.9, $J_{4',3'}$ = 2.6, H-4′); 3.99, 4.04 (2 × ddd, 2 × 1H, J_{gem} = 17.5, J_{vic} = 6.1, ^4J = 2.4, -C**H**$_2$C≡CH); 4.37 (dddd, 1H, $J_{3',2'}$ = 5.7, 2.8, $J_{3',OH}$ = 3.8, $J_{3',4'}$ = 2.6, H-3′); 4.97 (bs, 1H, OH-5′); 5.26 (bd, 1H, $J_{OH,3'}$ = 3.8, OH-3′); 6.19 (dd, 1H, $J_{1',2'}$ = 8.0, 6.1, H-1′); 6.54 (t, 1H, J_{vic} = 6.1, NH); 6.83 (bs, 2H, NH_2); 7.94 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, DMSO-d_6): 30.73 (-**C**H$_2$C≡CH); 39.10 (CH$_2$-2′); 62.22 (CH$_2$-5′); 71.28 (CH-3′); 72.02 (H**C**≡C-); 83.22 (CH-1′); 83.30 (-**C**≡CH); 87.79 (CH-4′); 114.07 (C-5); 136.51 (CH-8); 151.21 (C-4); 156.23 (C-6); 158.81 (C-2).

MS (ESI$^+$): *m/z* (%): 305.1 (82) [M+H]$^+$, 327.1 (100) [M+Na]$^+$.

HRMS (ESI$^+$): calculated for $C_{13}H_{16}O_3N_6Na$: 327.11761; found: 327.11769.

2-Methylamino-2'-deoxyadenosine-5'-*O*-triphosphate (48, d^{MA}ATP)



2-Methylamino-2'-deoxyadenosine-5'-*O*-triphosphate **48** was prepared according to General procedure A starting from **46** (25.2 mg, 0.09 mmol). The title compound was isolated as a white lyophilizate (10.5 mg, 20%).

^{1}H NMR (500.0 MHz, D$_2$O, ref(dioxane) = 3.75 ppm): 2.51 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.4, $J_{2'b,3'}$ = 3.5, H-2′b); 2.79 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.6, $J_{2'a,3'}$ = 6.3, H-2′a); 2.90 (s, 3H, CH$_3$); 4.16 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 5.3, $J_{5'b,4'}$ = 3.9, H-5′a); 4.21 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 3.9, H-5′a); 4.25 (qd, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 3.9, $J_{H,P}$ = 1.7, H-4′); 4.81 (m, 1H, H-3′, overlapped with HDO signal); 6.39 (dd, 1H, $J_{1',2'}$ = 7.6, 6.3, H-1′); 8.12 (s, 1H, H-8).

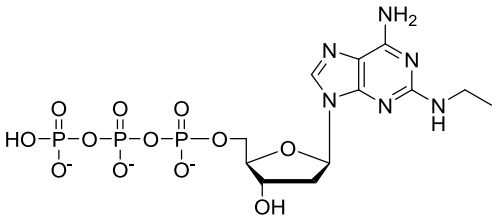
^{13}C NMR (125.7 MHz, D$_2$O, ref(dioxane) = 69.3 ppm): 30.70 (CH$_3$); 41.42 (CH$_2$-2′); 68.20 (d, $J_{C,P}$ = 5.8, CH$_2$-5′); 73.75 (CH-3′); 85.84 (CH-1′); 88.27 (d, $J_{C,F}$ = 9.0, CH-4′); 115.13 (C-5); 140.19 (CH-8); 154.07 (C-4); 158.44 (C-6); 162.82 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D$_2$O): -21.89 (t, J = 19.6, P$_\beta$); -10.38 (d, J = 19.6, P$_\alpha$); -8.22 (d, J = 19.6, P$_\gamma$).

MS (ESI$^-$): *m/z* (%): 359.3 (40) [M–H$_3$P$_2$O$_6$]$^-$, 439.3 (100) [M–H$_2$PO$_3$]$^-$, 461.1 (39) [M–H–H$_2$PO$_3$+Na$^+$]$^-$.

HRMS (ESI$^-$): calculated for $C_{11}H_{18}O_{12}N_6P_3$: 519.02010; found: 519.02075.

2-Ethylamino-2'-deoxyadenosine-5'-*O*-triphosphate (49, $d^{EA}ATP$)



2-Ethylamino-2'-deoxyadenosine-5'-*O*-triphosphate **49** was prepared according to General procedure A starting from **41** (27 mg, 0.09 mmol). The title compound was isolated as a white lyophilizate (10.8 mg, 20%).

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane - external) = 3.75 ppm): 1.19 (t, 3H, J_{vic} = 7.2, C**H$_3$**CH$_2$); 2.51 (m, 1H, H-2′b); 2.80 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.6, $J_{2'a,3'}$ = 6.3, H-2′a); 3.37 (q, 2H, J_{vic} = 7.2, CH$_3$C**H$_2$**); 4.16 (bm, 1H, H-5′b); 4.21 (ddd, 1H, J_{gem} = 11.5, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 4.0, H-5′a); 4.26 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 4.0, $J_{H,P}$ = 1.6, H-4′); 4.71 (m, 1H, H-3′); 6.39 (dd, 1H, $J_{1',2'}$ = 7.6, 6.3, H-1′); 8.12 (s, 1H, H-8).

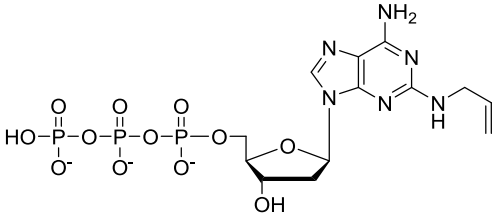
^{13}C NMR (125.7 MHz, D_2O, ref(dioxane - external) = 69.3 ppm): 16.71 (**C**H$_3$CH$_2$); 39.15 (CH$_3$**C**H$_2$); 41.37 (CH$_2$-2′); 68.17 (d, $J_{C,P}$ = 5.4, CH$_2$-5′); 73.78 (CH-3′); 85.78 (CH-1′); 88.22 (d, $J_{C,F}$ = 8.7, CH-4′); 115.19 (C-5); 140.19 (CH-8); 154.05 (C-4); 158.47 (C-6); 162.18 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.88 (bm, P_β); -10.37 (bm, P_α); -7.75 (bm, P_γ).

MS (ESI$^-$): *m/z* (%): 373.1 (100) [M–H$_3$P$_2$O$_6$]$^-$, 453.1 (76) [M–H–H$_2$PO$_3$]$^-$.

HRMS (ESI$^-$): calculated for $C_{12}H_{20}O_{12}N_6P_3$: 533.03575; found: 533.03589.

2-Allylamino-2'-deoxyadenosine-5'-*O*-triphosphate (50, $d^{AA}ATP$)



2-Allylamino-2'-deoxyadenosine-5'-*O*-triphosphate **50** was prepared according to General procedure B starting from **42** (25 mg, 0.08 mmol). The title compound was isolated as a white lyophilizate (17 mg, 34%).

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.50 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.4, $J_{2'b,3'}$ = 3.6, H-2′b); 2.76 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.4, $J_{2'a,3'}$ = 6.3, H-2′a); 4.00 (dt, 1H, J_{vic} = 5.0, ^4J = 1.7, NHC**H$_2$**CH=CH$_2$); 4.17 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 5.6, $J_{5'b,4'}$ = 4.0, H-5′b); 4.22 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 6.2, $J_{5'a,4'}$ = 4.0, H-5′a); 4.25 (qd, 1H, $J_{4',3'} = J_{4',5'}$ = 4.0, $J_{H,P}$ = 1.7, H-4′); 4.80 (ddd, 1H, $J_{3',2'}$ = 6.3, 3.6, $J_{3',4'}$ = 4.0, H-3′); 5.15 (dq, 1H, J_{cis} = 10.3, $J_{gem} = {}^4J$ = 1.7, NHCH$_2$CH=CH$_a$**H$_b$**); 5.25 (dq, 1H, J_{trans} =

17.3, J_{gem} = ^4J = 1.7, NHCH$_2$CH=C**H$_a$**H$_b$); 5.99 (ddt, 1H, J_{trans} = 17.3, J_{cis} = 10.3, J_{vic} = 5.0, NHCH$_2$C**H**=CH$_2$); 6.36 (dd, 1H, $J_{1',2'}$ = 7.4, 6.4, H-1′); 8.11 (s, 1H, H-8).

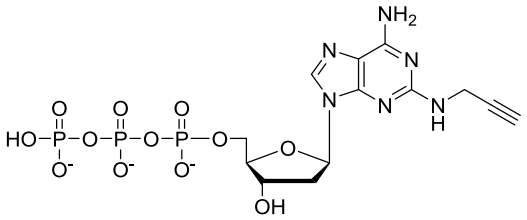
^{13}C NMR (125.7 MHz, D$_2$O, ref(dioxane) = 69.3 ppm): 41.49 (CH$_2$-2′); 46.19 (NH**C**H$_2$CH=CH$_2$); 68.31 (d, $J_{C,P}$ = 5.7, CH$_2$-5′); 73.87 (CH-3′); 86.03 (CH-1′); 88.21 (d, $J_{C,P}$ = 8.8, CH-4′); 114.97 (C-5); 117.91 (NHCH$_2$CH=**C**H$_2$); 137.93 (NHCH$_2$**C**H=CH$_2$); 140.63 (CH-8); 153.91 (C-4); 157.52 (C-6); 160.70 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D$_2$O): -22.07 (t, J = 19.5, P$_\beta$); -10.39 (d, J = 19.5, P$_\alpha$); -9.26 (d, J = 19.5, P$_\gamma$).

MS (ESI$^-$): *m/z* (%): 385.1 (34) [M–H$_3$P$_2$O$_6$]$^-$, 465.1 (100) [M–H$_2$PO$_3$]$^-$, 487.1 (18) [M–H–H$_2$PO$_3$+Na$^+$]$^-$.

HRMS (ESI$^-$): calculated for C$_{13}$H$_{19}$O$_{12}$N$_6$P$_3$Na: 567.01770; found: 567.01746.

2-Propargylamino-2'-deoxyadenosine-5'-*O*-triphosphate (51, d^{PA}ATP)



2-Propargylamino-2'-deoxyadenosine-5'-*O*-triphosphate **51** was prepared according to General procedure B starting from **47** (24.5 mg, 0.08 mmol). The title compound was isolated as a white lyophilizate (11.7 mg, 24%).

^{1}H NMR (600.0 MHz, D$_2$O, ref(dioxane) = 3.75 ppm): 2.51 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.6, $J_{2'b,3'}$ = 3.9, H-2′b); 2.58 (t, 1H, ^4J = 2.3, **H**C≡C-); 2.87 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.2, $J_{2'a,3'}$ = 6.5, H-2′a); 4.15 (d, 2H, ^4J = 2.3, NHC**H$_2$**C≡CH); 4.18 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 5.8, $J_{5'b,4'}$ = 4.0, H-5′b); 4.22 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 6.4, $J_{5'a,4'}$ = 4.0, H-5′a); 4.26 (qd, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 4.0, $J_{H,P}$ = 1.5, H-4′); 4.83 (ddd, 1H, $J_{3',2'}$ = 6.5, 3.9, $J_{3',4'}$ = 4.0, H-3′); 6.41 (dd, 1H, $J_{1',2'}$ = 7.2, 6.6, H-1′); 8.14 (s, 1H, H-8).

^{13}C NMR (150.9 MHz, D$_2$O, ref(dioxane) = 69.3 ppm): 33.74 (NH**C**H$_2$C≡CH); 41.15 (CH$_2$-2′); 68.26 (d, $J_{C,P}$ = 5.5, CH$_2$-5′); 73.72 (H**C**≡C-); 73.74 (CH-3′); 84.35 (-**C**≡CH); 86.01 (CH-1′); 88.22 (d, $J_{C,P}$ = 8.7, CH-4′); 116.01 (C-5); 140.71 (CH-8); 153.86 (C-4); 158.86 (C-6); 161.92 (C-2).

^{31}P{^{1}H} NMR (202.3 MHz, D$_2$O): -22.49 (bdd, J = 19.4, 16.3, P$_\beta$); -11.13 (d, J = 19.4, P$_\alpha$); -8.05 (bd, J = 16.3, P$_\gamma$).

MS (ESI$^-$): *m/z* (%): 383.3 (33) [M–H$_3$P$_2$O$_6$]$^-$, 463.3 (100) [M–H$_2$PO$_3$]$^-$, 485.3 (28) [M–H–H$_2$PO$_3$+Na$^+$]$^-$, 543.3 (28) [M+H$^+$]$^-$, 565.3 (17) [M+H+Na$^+$]$^-$.

HRMS (ESI$^-$): calculated for $C_{13}H_{18}O_{12}N_6P_3Na$: 565.00205; found: 565.00125.

Enzymatic synthesis of minor-groove modified DNA using 2-alkylamino modified dATPs

General remarks

Finished PEX reactions were stopped by the addition of PAGE stop solution (40 µl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 µl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gels were visualized by a fluorescent scanner.

*Example of incorporation of all **d^{R}ATP**s using Temp19A*

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp19A (0.225 µM), KOD XL DNA polymerase (0.125 U), natural dGTP (6 µM), either natural or modified dATP (20 µM) and KOD XL reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 40 min.

*Incorporation of all **d^{R}ATP**s using Temp16A*

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp16A (0.225 µM), Therminator DNA polymerase (0.1 U), either natural or modified dATP (20 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 20 min.

*Comparison of incorporation of modified **d^{R}ATP**s using Temp16A and different DNA polymerases*

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp16 (0.225 µM), either Therminator DNA polymerase (0.1 U), KOD XL DNA polymerase (0.125 U) or Vent(exo-) DNA polymerase (0.1 U), either natural or modified dATP (20 µM) and corresponding reaction buffer (10×, 2 µl). Reactions were incubated at 60 °C for 20 min.

SNE followed by PEX with Temp31_AC-TINA

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp31_AC-TINA (0.225 µM), Therminator DNA polymerase (0.1 U), modified **d^{R}ATP**s (7.5 µM, 50 equiv. towards primer) and Thermopol reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 30 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.375 µl) was added and the reaction mixture was incubated at 60 °C for additional 15 min.

SNE followed by PEX with Temp31_AT

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp31_AT (0.225 µMl), Therminator DNA polymerase (0.1 U), modified **d^{R}ATP**s (15 µM, 100 equiv. towards primer) and Thermopol reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 60 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.75 µl) was added and the reaction mixture was incubated at 60 °C for additional 5 min.

SNE followed by PEX with Temp31_AG

Reaction mixture (20 µl) contained primer Prim15-FAM (0.150 µM), template Temp31_AG (0.225 µM), Therminator DNA polymerase (0.1 U), modified **d^{R}ATP**s (7.5 µM, 50 equiv. towards primer) and Thermopol reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 30 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.375 µl) was added and the reaction mixture was incubated at 60 °C for additional 5 min.

SNE followed by PEX with Temp31_AA

For monoincorporation:

Reaction mixture (10 µl) contained primer Prim15-FAM (0.30 µM), template Temp31_AA (0.45 µM), Therminator DNA polymerase (0.1 U), modified **d^{R}ATP**s (3.00 µM, 10 equiv. towards primer) and Thermopol reaction buffer (10×, 1 µl). The reaction was incubated at 60 °C for 30 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.375 µl) was added and the reaction mixture was incubated at 60 °C for additional 5 min.

For incorporation of two modified d^RATPs followed by extension with natural dNTPs:
Reaction mixture (20 µl) contained primer Prim15-FAM (0.30 µM), template Temp31_AA (0.45 µM), Therminator DNA polymerase (0.1 U), modified **d^RATP**s (22.50 µM, 75 equiv. towards primer) and Thermopol reaction buffer (10×, 1 µl). The reaction was incubated at 60 °C for 60 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.375 µl) was added and the reaction mixture was incubated at 60 °C for additional 10 min.

SNE followed by PEX with revTemp31_AT
Reaction mixture (20 µl) contained primer revPrim19-FAM (0.150 µM), template revTemp31_AT (0.225 µM), Therminator DNA polymerase (0.4 U), modified **d^RATP**s (15 µM, 100 equiv. towards primer) and Thermopol reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 90 min. For further extension, a mixture of natural dNTPs (only dGTP, dCTP and dTTP in the case of negative controls) (0.4 mM, 0.375 µl) was added and the reaction mixture was incubated at 60 °C for additional 5 min.

Preparation of site-specifically labeled oligonucleotides by PEX on a semi-preparative scale followed by magnetoseparation
General remarks
Streptavidine magnetic particles (Roche, 50 µl) were washed with binding buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Finished PEX reactions were stopped by cooling to 4 °C. Then the PEX solution (20-30 µl) and binding buffer (50 µl) were added to the magnetic beads. The mixture was incubated for 30 min at 15 °C and 1100 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS.

SNE followed by PEX with Temp31_AC-(bio)TINA
Reaction mixture (20 µl) contained Therminator DNA polymerase (1.07 U), primer Prim15 (15 µM), template Temp31_AC-(bio)TINA (17.5 µM), modified **d^RATP**s [45

μM for **d^{AA}ATP** (3 equiv. towards primer) and 75 μM for **d^{PA}ATP** (5 equiv. towards primer)] and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 2.25 μl) was added and the reaction mixture was incubated at 60 °C for additional 30 min.

SNE followed by PEX with Temp31_AT-bio

Reaction mixture (20 μl) contained Therminator DNA polymerase (1.07 U), primer Prim15 (15 μM), template Temp31_AT-bio (17.5 μM), modified **d^{R}ATP**s [150 μM for both modified dATPs (10 equiv. towards primer)] and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 5.63 μl) was added and the reaction mixture was incubated at 60 °C for additional 60 min.

SNE followed by PEX with Temp31_AG-bio

Reaction mixture (20 μl) contained Therminator DNA polymerase (1.07 U), primer Prim15 (15 μM), template Temp31_AG-bio (17.5 μM), modified **d^{R}ATP**s [45 μM for **d^{AA}ATP** (3 equiv. towards primer) and 75 μM for **d^{PA}ATP** (5 equiv. towards primer)] and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 5.63 μl) was added and the reaction mixture was incubated at 60 °C for additional 60 min.

SNE followed by PEX with Temp31_AA-bio

For monoincorporation:

Reaction mixture (20 μl) contained Therminator DNA polymerase (1.07 U), primer Prim15 (15 μM), template Temp31_AA-bio (17.5 μM), modified **d^{R}ATP**s [45 μM for **d^{AA}ATP** (3 equiv. towards primer) and 75 μM for **d^{PA}ATP** (5 equiv. towards primer)] and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 3.75 μl) was added and the reaction mixture was incubated at 60 °C for additional 60 min.

For incorporation of two modified d^RATPs followed by extension with natural dNTPs:

Reaction mixture (20 μl) contained Therminator DNA polymerase (1.07 U), primer Prim15 (15 μM), template Temp31_AA-bio (17.5 μM), modified **d^RATP**s [150 μM for **d^{AA}ATP** (10 equiv. towards primer) and 300 μM for **d^{PA}ATP** (20 equiv. towards primer)] and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 7.5 μl) and additional Therminator DNA polymerase (0.2 U, only for the reaction mixture containing **d^{PA}ATP**) were added and the reaction mixture was incubated at 60 °C for additional 60 min.

SNE followed by PEX with revTemp31_AT-bio

Reaction mixture (20 μl) contained Therminator DNA polymerase (2.0 U), primer revPrim19 (15 μM), template revTemp31_AT-bio (17.5 μM), modified **d^{PA}ATP** (375 μM; 25 equiv. towards primer) and Thermopol reaction buffer (10×, 2 μl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler. For subsequent extension a mixture of natural dNTPs (4 mM, 2.25 μl) was added and the reaction mixture was incubated at 60 °C for additional 30 min.

Semi-preparative preparation of modified DNA

PEX reactions were performed as described above for preparation of modified DNA followed by magnetoseparation, with the exception of using non-biotinylated templates. Reactions were stopped by cooling to 4 °C. Prepared modified dsDNA was purified with a QIAquick nucleotide removal kit (QIAGEN) following the provided procedure. Products were eluted with 50 μl of H_2O and concentration was determined using NanoDrop and values obtained from OligoCalc.

Natural DNA for control experiments was prepared by preparing a reaction mixture (20 μl) containing Therminator DNA polymerase (0.3 U), primer Prim15 (15 μM), template (17.5 μM), natural dNTPs (120 μM) and Thermopol reaction buffer (10×, 2 μl). Reaction mixtures were incubated at 60 °C for 40 min. Prepared dsDNA was purified as described above.

Post-synthetic modification of DNA

Thiol additions on allyl-modified DNA

Reaction mixture was prepared by dissolving **DNA31_1^{AA}AC** (1.5 nmol) in H_2O (10.9 µl). To this, thiol [**CM-SH**, (500 mM in 0.5 M TEAA buffer, pH = 8.0), 32.7 µl] was added and the reaction mixture was kept at 37 °C and 500 rpm for 3 days.

For steady-state fluorescence measurements the product of thiol-ene reaction was purified with QIAquick nucleotide removal kit (QIAGEN). Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc, samples were concentrated. Emission spectra were recorded using a 0.5 µM solution of DNA in phosphate buffer (20 mM, pH = 7.0, 1 M NaCl) using 100 µl quartz cuvette. Solution was equilibrated for 2 min in a thermal holder (25 °C) before the fluorescence spectrum was recorded. Excitation wavelength for **DNA31_1^{CM}AC** was 370 nm and the range of the emission spectra was 380-650 nm. Control experiments were performed using natural non-modified DNA following the same procedure.

To obtain single-stranded DNA for MALDI analysis, the thiol-ene reaction was performed as described above using DNA after PEX with biotinylated template. Reaction mixture was first purified using QIAquick nucleotide removal kit (QIAGEN). Modified DNA was eluted using 50 µl of miliQ H_2O and diluted by another 50 µl of binding buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Streptavidine magnetic particles (Roche, 100 µl) were washed with binding buffer (3 × 200 µl). The diluted click reaction product (100 µl) was then added to the magnetic particles. Mixture was incubated at 15 °C and 1100 rpm for 30 min. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF MS.

To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp31_AC. The product of thiol-ene reaction was purified with QIAquick nucleotide removal kit (QIAGEN). Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc, an aliquot was taken and diluted with miliQ H_2O to a total

volume of 20 µl of approximately 500 nM concentration. PAGE stop solution (40 µl) was added and sample was denatured at 95 °C for 5 min. Aliquot (3.5 µl) was subjected to vertical electrophoresis in 20% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 120 min. The gel was visualized by a fluorescent scanner.

CuAAC reaction of propargyl-modified DNAs

General procedure: DNA31_1^{PA}AC **(**1.5 nmol) was dissolved in H_2O:DMSO:*t*BuOH mixture (9:3:1, 10.93 µl). The reaction mixture was prepared by adding azide (**Cy5-N_3**; 50 mM in DMSO, 3.0 µl), and Cu^I solution, which was freshly prepared by mixing CuBr (50 mM in DMSO, 1.5 µl) and TBTA (50 mM in DMSO, 6.0 µl). The reaction mixture was incubated at 37 °C and for 24 h.

Steady-state fluorescence measurements were performed as described above for the product of thiol-ene reaction. Excitation wavelength λ_{ex} for DNA31_1^{Cy5}AC was 620 nm and the range of the emission spectra was 630-750 nm. Control experiments were performed using natural non-modified DNA following the same procedure.

To obtain single-stranded DNA for MALDI analysis or fluorescence measurements, the reaction was performed as described above using DNA after PEX with biotinylated template. Single-stranded DNA was obtained following the same procedure, as for the preparation of ssDNA after thiol-ene reaction.

Product of CuAAC reaction was analyzed using PAGE as described for the analysis of the product of thiol-ene reaction.

Thermal denaturation studies

Thermal denaturation studies were performed following the same procedure as was described above.

FRET measurements

Study of reversible melting and annealing process of dsDNA

Site-specifically minor-groove labelled DNAs (revDNA31_1^{PA}AT and DNA31_1^{PA}AC) were prepared as described above for the preparation of DNA on a semi-preparative scale, using biotinylated templates. Both of these DNAs were subjected to CuAAC

reaction, either with **Cy3-N_3** or **Cy5-N_3**, as described above, followed by purification and magnetoseparation to generate ssDNAs, revON31_1^{Cy3}AT and ON31_1^{Cy5}AC. Concentration of ONs was approximately 15 μM in H_2O. Fluorescence spectra were recorded using a 100 μl quartz cuvette. A sample for fluorescence measurement was prepared by mixing 100 μl of phosphate buffer (20 mM, pH = 7.0; 1 M NaCl) and 2 μl of revON31_1^{Cy3}AT solution and 2 μl of H_2O (final concentration of ssDNA ~ 0.3 μM). Sample was equilibrated for 2 min in a thermal holder (25 °C) before the fluorescence spectrum was recorded. Fluorescence spectrum was recorded with the excitation wavelength λ_{ex} = 535 nm and the range of the emission spectra was 540-750 nm.

Another sample was prepared the same way, but using 2 μl of ON31_1^{Cy5}AC solution instead of H_2O. This sample was heated at 95 °C for 3 min and then left to cool down at room temperature. Fluorescence spectrum was then recorded as described above, showing successful FRET. This sample was then heated at 80 °C at the thermal holder of the spectrometer and the fluorescence spectrum was recorded again. After cooling of the sample to 25 °C the fluorescence spectrum was recorded again. These measurements were duplicated.

Absorption spectra were recorded for both of these samples in order to confirm the same concentrations and to prove that the absorption of donor fluorescence label is not affected by the addition of the acceptor labelled DNA.

Hybridization experiment

Cy3-labelled ssDNA (revON31_1^{Cy3}AT) used in this study was prepared as described above. ON16_1^{Cy5}A was prepared by performing PEX on a semi-preparative scale with primer Prim15 and template Temp31_AC-bio and **d^{PA}ATP**, using conditions for SNE only (without the addition of natural dNTPs; the final extension of the primer was not completed). This short dsDNA was then subjected to CuAAC reaction with **Cy5-N_3**, purified and ssDNA (ON16_1^{Cy5}A) was generated by magnetoseparation as described above. For fluorescence measurements, samples containing only the donor (revON31_1^{Cy3}AT) and with both donor and acceptor (revON31_1^{Cy3}AT and ON16_1^{Cy5}A) fluorescent labels were prepared as described above. Fluorescence spectrum was recorded for the sample containing revON31_1^{Cy3}AC as described above.

The second sample (containing both donor and acceptor) was heated at 95 °C for 3 min and the left to cool down at room temperature. Fluorescence spectrum was recorded as

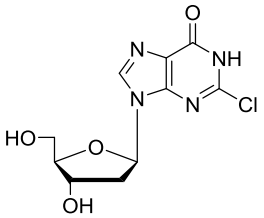
mentioned above, showing successful FRET. Then, 0.5 equiv. of revTemp31_AT (0.312 µl, 50 µM) was added, sample was heated at 95 °C for 3 min, left to cool down at room temperature and fluorescence spectrum was recorded again, showing a partial loss of FRET. This process was repeated after the addition of another 0.5 equiv. of revTemp31_AT, showing a disappearance of FRET. These measurements were duplicated.

Absorption spectra were recorded for both of these samples in order to confirm the same concentrations and to prove that the absorption of donor fluorescence label is not affected by the addition of the acceptor labelled DNA.

5.4 Synthesis of 2-modified dITP derivatives and their use in enzymatic synthesis of minor-groove modified DNA; study of the effect of minor-groove modifications on cleavage of DNA by REs

Synthetic procedures for the preparation of 2-modified dITPs

2-Chloro-2'-deoxyinosine (52)



Starting protected nucleoside **21** (200 mg, 0.40 mmol), DABCO (4 mg, 0.04 mmol) and CsOAc (213 mg, 1.10 mmol) were suspended in dry DMF (3.7 ml). Et_3N (0.16 ml, 1.10 mmol) was added this mixture was stirred at rt (25 °C) overnight. H_2O (2 ml) was added and stirring continued for another 30 min, after which $CHCl_3$ (25 ml) and H_2O (20 ml) were added. Phases were separated, water phase was extracted with $CHCl_3$ (2 x 25 ml). Collected organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated. Residue was dissolved in MeOH (3.7 ml) and K_2CO_3 (128 mg, 0.90 mmol) was added. This mixture was stirred at rt (25 °C) for 1 h, silica gel was added and volatiles were evaporated. Purification by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O) afforded the desired product as a white solid (76 mg, 72% over two steps).

R_f = 0.12 (10% MeOH in CH_2Cl_2).

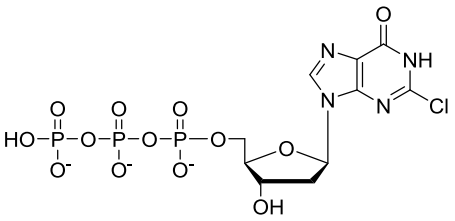
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.15 (ddd, 1H, J = 13.0, J = 6.0, J = 2.5, H-2′b); 2.62 (ddd, 1H, J = 13.0, J = 8.4, J = 5.6, H-2′a); 3.49 (dd, 1H, J = 11.9, J = 4.0, H-5′a); 3.59 (dd, 1H, J = 11.9, J = 4.0, H-5′b); 3.84 (td, 1H, J = 4.0, J = 2.1, H-4′); 4.35 (td, 1H, J = 5.6, J = 2.1, H-3′); 5.29 (bs, 2H, OH-3′, OH-5′); 6.15 (dd, 1H, J = 8.4, J = 6.0, H-1′); 7.85 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, DMSO-d_6): 39.38 (CH_2-2′); 62.15 (CH_2-5′); 71.18 (CH-3′); 83.82 (CH-1′); 87.89 (CH-4′); 123.94 (C-5); 135.70 (CH-8); 149.76 (C-4); 153.74 (C-2); 166.17 (C-6).

MS (ESI$^+$): *m/z* (%): 309.1 (100) [(^{35}Cl)M+Na]$^+$, 311.1 (31) [(^{37}Cl)M+Na]$^+$, 325.1 (32) [(^{35}Cl)M+K]$^+$, 327.1 (10) [(^{37}Cl)M+K]$^+$.

HRMS (ESI$^+$): calculated for $C_{10}H_{11}O_4N_4(^{35}Cl)Na$: 309.03610 found: 309.03621.

2-Chloro-2'-deoxyinosine-5'-*O*-triphosphate (53, $d^{Cl}ITP$)



The title nucleotide **53** was prepared following General procedure A starting from nucleoside **52** (28 mg, 0.086 mmol). It was isolated as a white lyophilisate (8 mg, 21%).

^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.55 (ddd, 1H, *J* = 14.0, *J* = 6.3, *J* = 3.8, H-2′b); 2.77 (ddd, 1H, *J* = 14.0, *J* = 7.3, *J* = 6.2, H-2′a); 4.15 (ddd, 1H, *J* = 11.5, *J* = 5.3, *J* = 3.8, H-5′b); 4.23 (ddd, 1H, *J* = 11.5, *J* = 6.3, *J* = 3.8, H-5′a); 4.28 (qd, 1H, *J* = 3.8, *J* = 2.4, H-4′); 4.80 (dt, 1H, *J* = 6.2, *J* = 3.8, *J* = 3.7, H-3′); 6.38 (dd, 1H, *J* = 7.3, 6.3, H-1′); 8.28 (s, 1H, H-8).

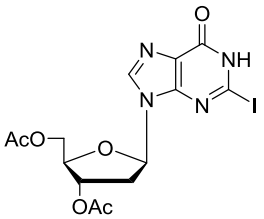
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.75 (CH_2-2′); 67.99 (d, *J* = 5.5, CH_2-5′); 73.53 (CH-3′); 86.04 (CH-1′); 88.41 (d, *J* = 8.8, CH-4′); 124.96 (C-5); 140.80 (CH-8); 153.18 (C-4); 156.75 (C-2); 170.16 (C-6).

^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -18.86 (t, *J* = 19.2, P_β); -8.21 (d, *J* = 19.2, P_α); -3.51 (d, *J* = 19.2, P_γ).

MS (ESI$^-$): *m/z* (%): 365.0 (100) [(^{35}Cl)M–$H_3P_2O_6$]$^-$, 367.0 (24) [(^{37}Cl)M–$H_3P_2O_6$]$^-$, 445.0 (38) [(^{35}Cl)M–H_2PO_3–H]$^-$, 467.0 (33) [(^{35}Cl)M–H–H_2PO_3+Na]$^-$, 524.9 (67) [(^{35}Cl)M–H]$^-$, 526.9 (14) [(^{37}Cl)M–H]$^-$.

HRMS (ESI$^-$): calculated for $C_{10}H_{13}O_{13}N_4(^{35}Cl)P_3$: 524.93860; found: 524.93829; calculated for $C_{10}H_{12}O_{13}N_4(^{35}Cl)P_3Na$: 546.92054; found: 546.92024.

2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine (54)



To starting nucleoside **30** (2.78 g, 5.8 mmol) and CsOAc (3.33 g, 17.4 mmol) in dry DMF (45 ml), DABCO (0.07 g, 0.6 mmol) and Et_3N (2.4 ml, 17.4 mmol) were added. This mixture was stirred at room temperature (25 °C) overnight, then H_2O (30 ml) was added and stirring continued for another 30 min. Volatiles were evaporated, residue was dissolved in MeOH and coevaporated with silica gel. Purification by column chromatography (0 → 10% MeOH in CH_2Cl_2) afforded the desired product (1.85 g, 69%).

R_f = 0.26 (10% MeOH in CH_2Cl_2).

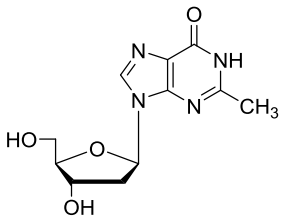
^{1}H NMR (400.1 MHz, $CDCl_3$): 2.11, 2.15 (2 × s, 2 × 3H, C**H**$_3$CO); 2.65 (ddd, 1H, *J* = 14.2, *J* = 6.1, *J* = 2.6, H-2′b); 2.79 (ddd, 1H, *J* = 14.2, *J* = 7.7, *J* = 6.4, H-2′a); 4.32 – 4.41 (m, 3H, H-4′,5′); 5.39 (dt, 1H, *J* = 6.4, *J* = 2.6, H-3′); 6.36 (dd, 1H, *J* = 7.7, *J* = 6.1, H-1′); 7.93 (s, 1H, H-8); 12.06 (bs, 1H, H-1).

^{13}C NMR (100.8 MHz, $CDCl_3$): 20.86, 20.96 (**C**H_3CO); 38.23 (CH_2-2′); 63.64 (CH_2-5′); 74.30 (CH-3′); 82.81 (CH-4′); 84.69 (CH-1′); 104.99 (C-2); 124.77 (C-5); 137.74 (CH-8); 148.33 (C-4); 158.48 (C-6); 170.28, 170.37 (CH_3**C**O).

MS (ESI$^+$): *m/z* (%): 263.1 (64) [nucleobase+H]$^+$, 485.4 (100) [M+Na]$^+$.

HRMS (ESI$^+$): calculated for $C_{14}H_{15}O_6N_4INa$: 484.99285; found: 484.99216.

2-Methyl-2’-deoxyinosine (55)



2-Iodo-3’,5’-di-*O*-acetyl-2’-deoxyinosine **54** (250 mg, 0.54 mmol) and $Pd(PPh_3)_4$ (63 mg, 0.05 mmol) were dissolved in anhydrous THF (4.2 ml). Me_3Al (1.1 ml, 2.17 mmol, 2 M in toluene) was then added dropwise and this mixture was stirred at 60 °C for 3.5 h. Reaction was carefully stopped by dropwise addition of MeOH (2 ml) and evaporated to dryness. Residue was dissolved in MeOH (5 ml) and MeONa (0.5 ml, 2.19 mmol, 25 wt.% in MeOH) was added. Mixture was stirred at rt (25°C) overnight. Silica gel was added and volatiles were evaporated. Desired product was isolated after RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O) as a white solid (140 mg, 97% over two steps).

R_f = 0.14 (10% MeOH in CH_2Cl_2).

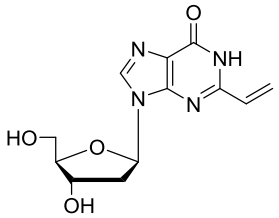
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.17 (ddd, 1H, *J* = 13.0, *J* = 5.9, *J* = 2.2, H-2′b); 2.26 (s, 3H, CH_3); 2.69 (ddd, 1H, *J* = 13.0, *J* = 8.5, *J* = 5.4, H-2′a); 3.52 (dd, 1H, *J* = 12.1, *J* = 3.3, H-5′a); 3.62 (dd, 1H, *J* = 12.1, *J* = 3.3, H-5′b); 3.90 (td, 1H, *J* = 3.3, *J* = 2.2, H-4′); 4.39 (td, 1H, *J* = 5.4, *J* = 2.2, H-3′); 6.23 (dd, 1H, *J* = 8.5, *J* = 5.9, H-1′); 7.97 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, DMSO-d_6): 23.33 (CH_3); 39.62 (CH_2-2′); 62.30 (CH_2-5′); 71.40 (CH-3′); 84.66 (CH-1′); 88.35 (CH-4′); 122.78 (C-5); 136.88 (CH-8); 148.70 (C-4); 158.64 (C-2); 162.93 (C-6).

MS (ESI$^+$): *m/z* (%): 289.1 (100) $[M+Na]^+$, 555.3 (54) $[2M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{11}H_{14}O_4N_4Na$: 289.09073; found: 289.09049.

2-Vinyl-2'-deoxyinosine (56)



A flask containing 2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **54** (180 mg, 0.39 mmol), potassium vinyltrifluoroborate (78 mg, 0.58 mmol), Cs_2CO_3 (380 mg, 1.17 mmol), $Pd(OAc)_2$ (4 mg, 0.05 mmol) and TPPTS [3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt, 28 mg, 0.125 mmol] was put under argon and a mixture of CH_3CN/H_2O (2/1, 5 ml) was added. Mixture was stirred at 80°C for 2.5 h. After cooling, volatiles were evaporated and residue was dissolved in MeOH (5 ml) and MeONa (0.3 ml, 1.31 mmol, 25 wt.% in MeOH) was added. Resulting solution was stirred at rt (25°C) overnight. Silica gel was added and after evaporation the product was purified by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O). Product was isolated as a white solid (80 mg, 74% over two steps).

R_f = 0.12 (10% MeOH in CH_2Cl_2).

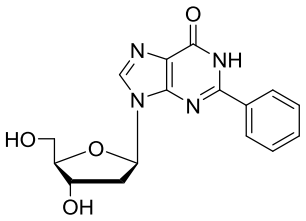
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.11 (ddd, 1H, *J* = 12.9, *J* = 5.8, *J* = 1.7, H-2′b); 2.78 (ddd, 1H, *J* = 12.9, *J* = 9.0, *J* = 5.3, H-2′a); 3.48 – 3.57 (bm, 1H, H-5′a); 3.64 (dd, 1H, *J* = 12.0, *J* = 2.9, H-5′b); 3.90 (td, 1H, *J* = 3.0, *J* = 1.4, H-4′); 4.40 (d, 1H, *J* = 5.2, H-3′); 5.24 (bs, 1H, OH-5′); 5.30 (dd, 1H, *J* = 10.4, *J* = 2.7, CH=C**H**$_a$H$_b$); 6.19 (dd, 1H, *J* = 17.3, *J* = 2.7, CH=CH$_a$**H**$_b$); 6.23 (dd, 1H, *J* = 9.0, *J* = 5.8, H-1′); 6.33 (bs, 1H, OH-3′); 6.44 (dd, 1H, *J* = 17.3, *J* = 10.4, C**H**=CH$_2$); 7.83 (s, 1H, H-8).

^{13}C NMR (100.8 MHz, DMSO-d_6): 39.42 (CH_2-2′); 62.62 (CH_2-5′); 71.77 (CH-3′); 85.05 (CH-1′); 88.30 (CH-4′); 118.70 (CH=**C**H$_2$); 124.50 (C-5); 136.49 (CH-8); 138.07 (**C**H=CH$_2$); 149.04 (C-4); 155.84 (C-6); 158.13 (C-2).

MS (ESI$^+$): *m/z* (%): 301.0 (100) $[M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{12}H_{14}O_4N_4Na$: 301.09073; found: 301.09024.

2-Phenyl-2'-deoxyinosine (57)



To a flask containing 2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **54** (200 mg, 0.43 mmol), $PhB(OH)_2$ (79 mg, 0.65 mmol), Cs_2CO_3 (423 mg, 1.30 mmol), $Pd(OAc)_2$ (5 mg, 0.02 mmol) and TPPTS [3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt, 31 mg, 0.05 mmol] a mixture of CH_3CN/H_2O (2/1, 5.4 ml) was added under argon. This mixture was heated at 80 °C for 1.5 h. After cooling, volatiles were evaporated and residue was dissolved in MeOH (5 ml) and MeONa (0.4 ml, 1.75 mmol, 25 wt.% in MeOH) was added. This mixture was stirred at rt (25 °C) for 1 h. Silica gel was added, volatiles were evaporated and the title compound was obtained, after purification by RP-FPLC on a C-18 column (0 → 100 % MeOH in H_2O), as a white solid (130 mg, 92% over two steps). NMR data were in good agreement with those published previously.[223]

R_f = 0.14 (10% MeOH in CH_2Cl_2).

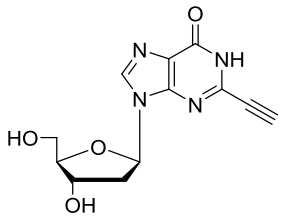
^{1}H NMR (401.0 MHz, DMSO-d_6): 2.33 (ddd, 1H, *J* = 13.2, *J* = 6.3, *J* = 3.3, H-2′b); 2.71 (ddd, 1H, *J* = 13.2, *J* = 7.9, *J* = 5.9, H-2′a); 3.53 (dd, 1H, *J* = 11.7, *J* = 4.7, H-5′a); 3.60 (dd, 1H, *J* = 11.7, *J* = 4.7, H-5′b); 3.88 (td, 1H, *J* = 4.7, *J* = 2.8, H-4′); 4.43 (td, 1H, *J* = 5.9, *J* = 3.3, H-3′); 4.94 (bs, 1H, OH-5′); 5.39 (bs, 1H, OH-3′); 6.40 (dd, 1H, *J* = 7.9, *J* = 6.3, H-1′); 7.50-7.60 (m, 3H, H-*m,p*-Ph); 8.12 (dd, 2H, *J* = 8.1, *J* = 1.7, H-*o*-Ph); 8.32 (s, 1H, H-8); 12.53 (bs, 1H, H-1).

^{13}C NMR (100.8 MHz, DMSO-d_6): 39.59 (CH_2-2′); 61.67 (CH_2-5′); 70.76 (CH-3′); 83.41 (CH-1′); 87.92 (CH-4′); 122.99 (C-5); 127.85 (CH-*o*-Ph); 128.64 (CH-*m*-Ph); 131.25 (CH-*p*-Ph); 132.48 (C-*i*-Ph); 139.06 (CH-8); 148.36 (C-4); 153.55 (C-2); 157.72 (C-6).

MS (ESI^+): *m/z* (%): 351.1 (100) $[M+Na]^+$.

HRMS (ESI^+): calculated for $C_{16}H_{16}O_4N_4Na$: 351.10638; found: 351.10638.

2-Ethynyl-2'-deoxyinosine (58)



2-Iodo-3',5'-di-*O*-acetyl-2'-deoxyinosine **54** (183 mg, 0.40 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol) and CuI (8 mg, 0.04 mmol) were placed in a flask and dry DMF (5.0 ml) was added. The flask was purge-and-refilled with argon six times, trimethylsilylacetylene (0.45 ml, 3.17 mmol) and Et_3N (0.55 ml, 4.0 mmol) were added and the mixture was stirred at rt (25 °C) for 3 h. Volatiles were evaporated, residue was dissolved in MeOH and silica gel was added. After evaporation of MeOH, the 2-trimethylsilylethynyl-3',5'-di-*O*-acetyl-2'-deoxyinosine was partially purified by column chromatography (0 → 10% MeOH in CH_2Cl_2). The crude product was dissolved in MeOH saturated (at 0 °C) with ammonia (5.0 ml) and this mixture was stirred at rt (25 °C) overnight. Silica gel was added and, after evaporation of all volatiles, the desired product was purified by RP-FPLC on a C-18 column (0 → 100% MeOH in H_2O). The title compound was isolated as a white solid (80 mg, 73% over two steps). The compound slowly decomposes on the bench, therefore long term storage at -20 °C is necessary.

R_f = 0.11 (10% MeOH in CH_2Cl_2).

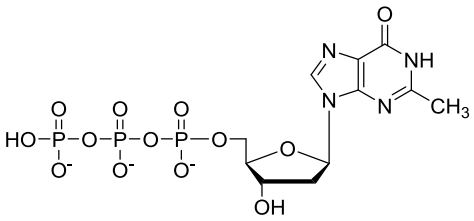
^{1}H NMR (600.1 MHz, DMSO-d_6): 2.13 (ddd, 1H, *J* = 12.9, *J* = 5.9, *J* = 2.2, H-2′b); 2.67 (ddd, 1H, *J* = 12.9, *J* = 8.5, *J* = 5.5, H-2′a); 3.50 (ddd, 1H, *J* = 11.9, *J* = 8.0, *J* = 3.6, H-5′b); 3.56 – 3.63 (m, 1H, H-5′a); 3.59 (s, 1H, HC≡C-); 3.86 (td, 1H, *J* = 3.6, *J* = 2.2, H-4′); 4.35 – 4.38 (m, 1H, H-3′); 5.25 (bd, 1H, *J* = 3.0, OH-5′); 5.66 (dd, 1H, *J* = 7.4, *J* = 4.1, OH-3′); 6.19 (dd, 1H, *J* = 8.5, *J* = 5.9, H-1′); 7.90 (s, 1H, H-8).

^{13}C NMR (150.9 MHz, DMSO-d_6): 39.63 (CH_2-2′); 62.48 (CH_2-5′); 71.53 (CH-3′); 72.33 (H**C**≡C-); 84.41 (CH-1′); 85.03 (-**C**≡CH); 88.22 (CH-4′); 125.63 (C-5); 136.54 (CH-8); 145.98 (C-2); 148.81 (C-4); 166.36 (C-6).

MS (ESI$^+$): *m/z* (%): 299.1 (100) $[M+Na]^+$, 575.2 (33) $[2M+Na]^+$.

HRMS (ESI$^+$): calculated for $C_{12}H_{12}O_4N_4Na$: 299.07508; found: 299.07495.

2-Methyl-2'-deoxyinosine-5'-*O*-triphosphate (59, $d^{Me}ITP$)



The title nucleotide **59** was prepared following General procedure A starting from nucleoside **55** (25 mg, 0.10 mmol). It was isolated as a white lyophilisate (27 mg, 56%).

^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.53 (s, 3H, CH_3); 2.60 (ddd, 1H, *J* = 14.0, *J* = 6.5, *J* = 4.3, H-2′b); 2.78 (ddd, 1H, *J* = 14.0, *J* = 7.1, *J* = 5.7, H-2′a); 4.14 – 4.24 (m, 2H, H-5′a, H-5′b); 4.31 (qd, 1H, *J* = 3.4, *J* = 1.2, H-4′); 4.76 – 4.80 (m, 1H, H-3′); 6.45 (dd, 1H, *J* = 7.1, *J* = 6.5, H-1′); 8.36 (s, 1H, H-8).

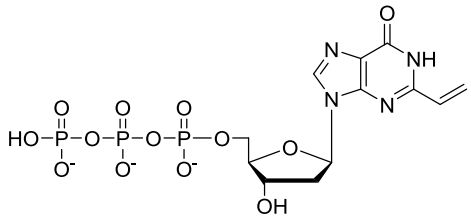
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 23.33 (CH_3); 41.79 (CH_2-2′); 67.89 (d, *J* = 5.4, CH_2-5′); 73.34 (CH-3′); 86.42 (CH-1′); 88.58 (d, *J* = 8.2, CH-4′); 124.16 (C-5); 142.05 (CH-8); 151.83 (C-4); 159.95 (C-2); 162.09 (C-6).

^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -17.78 (t, *J* = 18.5, P_β); -7.85 (d, *J* = 18.2, P_α); -2.29 (d, *J* = 19.0, P_γ).

MS (ESI^-): *m/z* (%): 365.0 (75) $[M–H_3P_2O_6]^-$, 425.0 (100) $[M–H_2PO_3]^-$, 447.0 (53) $[M–H–H_2PO_3+Na]^-$, 469.0 (31) $[M–2H–H_2PO_3+2Na]^-$, 549.0 (28) $[M–3H+2Na]^-$.

HRMS (ESI^-): calculated for $C_{11}H_{16}O_{13}N_4P_3$: 504.99322; found: 504.99313; calculated for $C_{11}H_{15}O_{13}N_4P_3Na$: 526.97516; found: 526.97504.

2-Vinyl-2'-deoxyinosine-5'-*O*-triphosphate (60, $d^{V}ITP$)



The title nucleotide **60** was prepared following General procedure A starting from nucleoside **56** (20 mg, 0.07 mmol). It was isolated as a white lyophilisate (11.4 mg, 31%).

^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.57 (ddd, 1H, *J* = 13.9, *J* = 6.3, *J* = 3.9, H-2′b); 2.83 (ddd, 1H, *J* = 13.9, *J* = 7.6, *J* = 5.9, H-2′a); 4.14 – 4.26 (m, 2H, H-5′a, H-5′b); 4.28 (qd, 1H, *J* = *J* = 4.0, *J* = 1.7, H-4′); 4.76 – 4.79 (m, 1H, H-3′); 5.68 (dd, 1H, *J* = 10.7, *J* = 1.3, $CH_a\mathbf{H_b}$=CH); 6.33 (dd, 1H, *J* = 17.4, *J* = 1.3, $C\mathbf{H_a}H_b$=CH); 6.51 (dd, 1H, *J* = 7.6, 6.3, H-1′); 6.68 (dd, 1H, *J* = 17.4, *J* = 10.7, CH_2=C**H**); 8.32 (s, 1H, H-8).

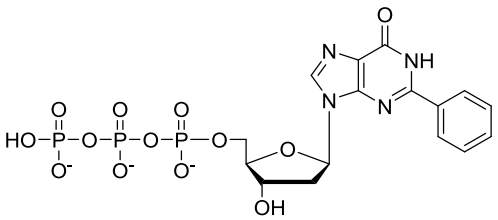
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.83 (CH_2-2′); 68.10 (d, J = 5.9, CH_2-5′); 73.63 (CH-3′); 85.91 (CH-1′); 88.27 (d, J = 8.3, CH-4′); 124.87 (C-5); 125.54 (**C**H_2=CH); 137.70 (**C**H=CH_2); 141.22 (CH-8); 153.00 (C-4); 162.53 (C-2); 167.88 (C-6).

^{31}P{^{1}H} NMR (162.0 MHz, D_2O): -20.29 (t, J = 20.6, P_β); -8.97 (d, J = 20.0, P_α); -4.06 (d, J = 21.0, P_γ).

MS (ESI$^-$): *m/z* (%): 357.1 (73) [M–$H_3P_2O_6$]$^-$, 437.0 (100) [M–H_2PO_3]$^-$, 517.0 (57) [M–H]$^-$, 539.0 (39) [M–2H+Na]$^-$.

HRMS (ESI$^-$): calculated for $C_{12}H_{16}O_{13}N_4P_3$: 516.99322; found: 516.99285; calculated for $C_{12}H_{15}O_{13}N_4P_3Na$: 538.97516; found: 538.97488.

2-Phenyl-2'-deoxyinosine-5'-*O*-triphosphate (61, d^{Ph}ITP)



The title nucleotide **61** was prepared following General procedure A starting from nucleoside **57** (24 mg, 0.07 mmol). It was isolated as a white lyophilisate (16 mg, 39%).

^{1}H NMR (401.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.60 (ddd, 1H, J = 13.9, J = 6.4, J = 3.9, H-2′b); 2.84 (ddd, 1H, J = 13.9, J = 7.6, J = 7.0, H-2′a); 4.17 – 4.29 (m, 2H, H-5′a, H-5′b); 4.31 (qd, 1H, J = 3.7, J = 1.5, H-4′); 4.83 (dt, 1H, J = 7.0, J = 3.8, H-3′); 6.60 (dd, 1H, J = 7.6, J = 6.4, H-1′); 7.50 – 7.53 (m, 3H, H-*m,p*-Ph); 8.06 – 8.09 (m, 2H, H-*o*-Ph); 8.34 (s, 1H, H-8).

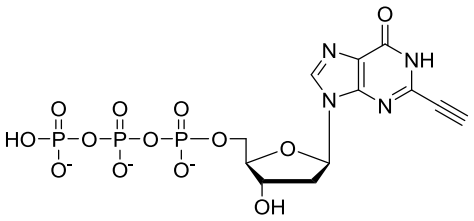
^{13}C NMR (100.8 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.84 (CH_2-2′); 68.14 (d, J = 5.6, CH_2-5′); 73.65 (CH-3′); 85.92 (CH-1′); 88.25 (d, J = 8.6, CH-4′); 124.54 (C-5); 130.90 (CH-*o*-Ph); 131.22 (CH-*m*-Ph); 132.69 (CH-*p*-Ph); 140.65 (CH-*i*-Ph); 141.15 (CH-8); 153.52 (C-4); 164.24 (C-6); 170.27 (C-2).

^{31}P{^{1}H} NMR (162.3 MHz, D_2O): -18.23 (t, J = 18.9, P_β); -8.03 (d, J = 18.9, P_α); -2.77 (d, P_γ).

MS (ESI$^-$): *m/z* (%): 407.1 (57) [M–$H_3P_2O_6$]$^-$, 487.0 (100) [M–H_2PO_3]$^-$, 567.0 (50) [M–H]$^-$, 589.0 (30) [M–2H+Na]$^-$.

HRMS (ESI$^-$): calculated for $C_{16}H_{18}O_{13}N_4P_3$: 567.00887; found: 567.00867; calculated for $C_{16}H_{17}O_{13}N_4P_3Na$: 588.99081; found: 588.99048.

2-Ethynyl-2'-deoxyinosine-5'-*O*-triphosphate (62, d^EITP)



2-Ethynyl-2'-deoxyinosine **58** (19 mg, 0.07 mmol) and proton sponge (17 mg, 0.08 mmol) were dried in vacuo overnight. Dry $PO(OMe)_3$ (0.8 ml) was added and resulting mixture was cooled down to 0 °C. Freshly distilled $POCl_3$ (8 µl, 0.09 mmol) was added dropwise and this mixture was stirred at 0 °C until no more starting material could be observed by TLC (ca. 3 hours). Then an ice-cold solution of $(n\text{-}Bu_3NH)_2H_2P_2O_7$ (185 mg, 0.34 mmol) and $n\text{-}Bu_3N$ (70 µl, 0.27 mmol) in dry DMF (0.5 ml) was added dropwise. This mixture was stirred for another 1 hour at 0 ºC and then stopped by the addition of cold 1 M NH_4HCO_3 (1 ml). The mixture was concentrated on a rotavap; the residue was co-evaporated with distilled water three times. The crude product was dissolved in water (ca. 3 ml); the aqueous solution was purified by semi-preparative HPLC using a linear gradient of methanol (5 → 100%) in 0.1 M NH_4HCO_3 buffer and further purified by ion-exchange HPLC on POROS HQ with linear gradient of 0.4 M NH_4HCO_3 (0 → 100%) in H_2O. The appropriate fractions were combined and evaporated on a rotavap. The viscous oil was coevaporated with distilled water three times. The product was converted to sodium salt on an ion-exchange column (Dowex 50WX8 in Na^+ cycle) and freeze-dried. The title compound was obtained as a white lyophilizate (6 mg, 17%).

^{1}H NMR (600.1 MHz, DMSO-d_6): 2.60 (ddd, 1H, J = 13.6, J = 6.0, J = 3.7, H-2′b); 2.78 (ddd, 1H, J = 13.6, J = 7.1, J = 5.7, H-2′a); 4.15 – 4.24 (m, 2H, H-5′); 3.56 – 3.63 (m, 1H, H-5′a); 4.27 – 4.31 (m, 1H, H-4′); 4.74 – 4.78 (m, 1H, H-3′); 6.46 (dd, 1H, J = 7.1, J = 6.0, H-1′); 8.49 (bs, 1H, H-8).

^{13}C NMR (150.9 MHz, DMSO-d_6): 42.02 (CH_2-2′); 68.23 (d, J = 4.4, CH_2-5′); 73.70 (CH-3′); 77.71 (H**C**≡C-); 83.70 (-**C**≡CH); 86.40 (CH-1′); 88.26 (d, J = 8.4, CH-4′). Multiple aromatic signals missing in the spectra, most probably due to tautomerism.

^{31}P{^{1}H} NMR (202.4 MHz, D_2O): -19.09 (bs, P_β); -10.34 (s, P_α); -5.45 (bs, P_γ).

MS (ESI$^-$): *m/z* (%): 355.0 (100) $[M–H_3P_2O_6]^-$, 457.0 (33) $[M–H–H_2PO_3+Na]^-$.

HRMS (ESI$^-$): calculated for $C_{12}H_{14}O_{13}N_4P_3$: 514.97757; found: 514.97760; calculated for $C_{12}H_{13}O_{13}N_4P_3Na$: 536.95951; found: 536.95996.

Enzymatic synthesis of minor-groove modified DNA using $d^{R}ITPs$

Analytical PEX - General remarks

Finished PEX reactions were stopped by the addition of PAGE stop solution (40 μl; 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (3.5 μl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gels were visualized by a fluorescent scanner.

Analytical PEX - Incorporation of one modified ***$d^{R}ITP$***

Reaction mixture (20 μl) was prepared by mixing primer Prim15-FAM (0.150 μM), template Temp19G (0.225 μM), Therminator DNA polymerase (0.1 U), natural dTTP (1.5 μM), either natural dGTP or modified **$d^{R}ITP$** (8 μM for **$d^{Cl}ITP$** and **$d^{Me}ITP$** or 20 μM for **$d^{V}ITP$**, **$d^{E}ITP$** and **$d^{Ph}ITP$**) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min (30 min in case of the reaction with natural dGTP).

Analytical PEX - Incorporation of four modified ***$d^{R}ITP$***

Reaction mixture (20 μl) was prepared by mixing primer Prim15-FAM (0.150 μM), template Temp31-TINA (0.225 μM), Therminator DNA polymerase (0.2 U), natural dNTPs (dTTP, dATP, dCTP, 20 μM), either natural dGTP or modified **$d^{R}ITP$** (100 μM) and reaction buffer (10×, 2 μl). The reaction was incubated at 60 °C for 60 min.

Kinetic study of incorporation of ***$d^{Me}ITP$*** *and* ***$d^{V}ITP$***

Multiple PEX reaction mixtures (10 μl, 6 samples for each nucleotide used in the reactions, 18 in total) containing Therminator DNA polymerase (0.2 U), primer Prim15-FAM (0.150 μM), template Temp16G (0.225 μM), natural dGTP or modified dITPs (200 μM) and reaction buffer (10×, 1 μl) were incubated at 60 °C for specific time intervals (0.5, 1, 2, 5, 10 and 30 minutes) and then the reaction was stopped by the addition of PAGE stop solution (10 μl) and immediate heating to 95 °C for 5 minutes. Aliquots (3.5 μl) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1 × TBE buffer (pH 8.0) and 7 M urea at 42 mA for 1 hour. The gel was visualized by a fluorescent scanner.

*Incorporation of one **d^{V}ITP** into the 26-mer DNA used for reaction between peptide and DNA*

Reaction mixture (20 µl) was prepared by mixing primer Prim16-FAM (0.150 µM), template Temp26 (0.225 µM), Therminator DNA polymerase (0.05 U), natural dNTPs (dATP, dCTP, dTTP, 10 µM), either natural dGTP or modified **d^{V}ITP** (20 µM) and reaction buffer (10×, 2 µl). The reaction was incubated at 60 °C for 60 min (30 min in case of the reaction with natural dGTP).

Semipreparative scale - General remarks

Streptavidine magnetic particles (Roche, 50 µl) were washed with binding buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Finished PEX reactions were stopped by cooling to 4 °C. Then the PEX solution (20 µl) and binding buffer (50 µl) were added to the magnetic beads. The mixture was incubated for 30 min at 15 °C and 1100 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF.

*Reaction with **d^{Me}ITP** and template Temp19-bio*

Reaction mixture (20 µl) contained Therminator DNA polymerase (0.6 U), primer Prim15 (8 µM), template Temp19-bio (10 µM), modified **d^{Me}ITP** (40 µM), natural dTTP (72 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler.

*Reaction with **d^{V}ITP** and template Temp19-bio*

Reaction mixture (20 µl) contained Therminator DNA polymerase (0.6 U), primer Prim15 (8 µM), template Temp19-bio (10 µM), modified **d^{V}ITP** (100 µM), natural dTTP (150 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction mixture was incubated for 90 min at 60 °C in a thermal cycler.

*Reaction with **d^{Me}ITP** and template Temp31-(bio)TINA*

Reaction mixture (20 µl) contained Therminator DNA polymerase (0.6 U), primer Prim15 (8 µM), template Temp31-(bio)TINA (10 µM), modified **d^{Me}ITP** (200 µM), natural dNTPs (dATP, dCTP, dTTP, 200 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler.

*Reaction with **d^{V}ITP** and template Temp31-(bio)TINA*

Reaction mixture (20 µl) contained Therminator DNA polymerase (1 U), primer Prim15 (8 µM), template Temp31-(bio)TINA (10 µM), modified **d^{V}ITP** (1000 µM), natural dNTPs (dATP, dCTP, dTTP, 600 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction mixture was incubated for 120 min at 60 °C in a thermal cycler.

*Reaction with **d^{V}ITP** and template Temp26-bio*

Reaction mixture (20 µl) contained Therminator DNA polymerase (0.75 U), primer Prim16-FAM (8 µM), template Temp26-bio (10 µM), modified d^{V}ITP (300 µM), natural dNTPs (dATP, dCTP, dTTP, 200 µM) and Thermopol reaction buffer (10×, 2 µl). The reaction mixture was incubated for 60 min at 60 °C in a thermal cycler.

Semi-preparative preparation of modified DNA

PEX reactions were performed as described above for preparation of modified DNA followed by magnetoseparation, with the exception of using non-biotinylated templates. Reactions were stopped by cooling to 4 °C. Prepared modified dsDNA was purified with a QIAquick nucleotide removal kit (QIAGEN) following the provided procedure. Products were eluted with 50 µl of H_2O and concentration was determined using NanoDrop and values obtained from OligoCalc.

Natural DNA for control experiments was prepared by preparing a reaction mixture (20 µl) containing Therminator DNA polymerase (0.3 U), primer Prim-15 (8 µM), template (either Temp19G or Temp31-TINA, 10 µM), natural dNTPs (120 µM) and Thermopol reaction buffer (10×, 2 µl). Reaction mixtures were incubated at 60 °C for 40 min. Prepared dsDNA was purified as described above.

PCR using ***d^{R}ITPs***

Reaction mixture (10 μl) contained Therminator DNA polymerase (1.25 U), Prim_FOR and Prim_REV (both 4 μM), Temp98 (50 nM), natural dNTPs (dCTP, dTTP, dGTP, 300 μM), either natural dATP (8 μM) or **d^{R}ITP** (1 mM) and Thermopol reaction buffer (10×, 1 μl).

The PCR reactions were placed in a preheated PCR cycler (80 °C) and 35 PCR cycles of the following conditions took place: 1. preheating at 94 °C for 3 min; 2. denaturation at 95 °C for 1 min; 3. primer annealing at 54 °C for 1 min; 4. extension at 72 °C for 1 min. Steps 2-4 were repeated 35 times and were followed by final extensions step at 75 °C for 5 min. Reactions were stopped by cooling to 4 °C. Products of the reactions were analysed on 2% agarose gel (containing 0.5x TBE buffer, pH 8) stained with GelRed (Biotium, 10 000× in H_2O). The gels were run at 118 V for ca. 60–90 min. The gels were visualised using an electronic dual wave transilluminator.

Primer extension experiments for studies of cleavage by restriction endonucleases

Reaction mixtures (20 μl) were prepared by mixing primer (0.150 μM), template (0.225 μM), Therminator or KOD XL DNA polymerases, natural or modified dNTPs and corresponding reaction buffer (10×, 2 μl). The reactions were incubated at 60 °C for 30-100 min. The reaction mixtures were then divided into two portions. The first portion (11.5 μl) was used directly for PAGE analysis and the second portion (8.5 μl) was used for DNA cleavage studies.

Incorporation of modified ***d^{R}ATPs*** *within the recognition sequence of AfeI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.05 U), natural dNTPs (dGTP, dCTP, dTTP, 10 μM), natural dATP or modified **d^{R}ATPs** (40 μM), primer Prim15-FAM and template Temp_AfA. Reaction mixtures were incubated at 60 °C for 60 min. Restriction endonuclease AfeI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ITPs** within the recognition sequence of AfeI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_AfG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease AfeI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ATPs** within the recognition sequence of EcoRI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.1 U), natural dNTPs (dGTP, dCTP, dTTP, 140 μM), natural dATP or modified **d^{R}ATPs** (200 μM), primer Prim15-FAM and template Temp_EcA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease EcoRI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ITPs** within the recognition sequence of EcoRI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_EcG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease EcoRI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ATPs** within the recognition sequence of KpnI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.05 U), natural dNTPs (dGTP, dCTP, dTTP, 10 μM), natural dATP or modified **d^{R}ATPs** (40 μM), primer Prim15-FAM and template Temp_KpA. Reaction mixtures were incubated at 60 °C for 60 min. Restriction endonuclease KpnI (5 U) and NEB 1.1 reaction buffer (10×, 1 μl) were

added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ITPs*** *within the recognition sequence of KpnI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim_KpG-FAM and template Temp_KpG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease KpnI (5 U) and NEB 1.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ATPs*** *within the recognition sequence of PstI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified **d^{R}ATPs** (20 μM), primer Prim15-FAM and template Temp_PsA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease PstI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ITPs*** *within the recognition sequence of PstI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.14 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_PsG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease PstI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ATPs** within the recognition sequence of PvuII restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.063 U), natural dNTPs (dGTP, dCTP, dTTP, 100 μM), natural dATP or modified **d^{R}ATPs** (100 μM), primer Prim15-FAM and template Temp_PvA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease PvuII (5 U) and NEB 3.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ITPs** within the recognition sequence of PvuII restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_PvG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease PvuII (5 U) and NEB 3.1 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ATPs** within the recognition sequence of RsaI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 100 μM), natural dATP or modified **d^{R}ATPs** (200 μM), primer Prim15-FAM and template Temp_RsA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease RsaI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ITPs** within the recognition sequence of RsaI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_RsG. Reaction mixtures were incubated at 60 °C for 90 min. Restriction endonuclease RsaI (5 U) and NEB 4 reaction buffer (10×, 1 μl) were

added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ATPs*** *within the recognition sequence of SacI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.025 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified **d^{R}ATPs** (20 μM), primer Prim15-FAM and template Temp_SaA. Reaction mixtures were incubated at 60 °C for 30 min. Restriction endonuclease SacI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ITPs*** *within the recognition sequence of SacI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.16 U), natural dNTPs (dATP, dCTP, dTTP, 16 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_SaG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease SacI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Incorporation of modified ***d^{R}ATPs*** *within the recognition sequence of ScaI restriction endonuclease*

PEX was performed using KOD XL DNA polymerase (0.075 U), natural dNTPs (dGTP, dCTP, dTTP, 20 μM), natural dATP or modified **d^{R}ATPs** (20 μM), primer Prim15-FAM and template Temp_ScA. Reaction mixtures were incubated at 60 °C for 40 min. Restriction endonuclease ScaI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

*Incorporation of modified **d^{R}ITPs** within the recognition sequence of ScaI restriction endonuclease*

PEX was performed using Therminator DNA polymerase (0.04 U), natural dNTPs (dATP, dCTP, dTTP, 10 μM), natural dGTP or modified **d^{R}ITPs** (40 μM), primer Prim15-FAM and template Temp_ScG. Reaction mixtures were incubated at 60 °C for 100 min. Restriction endonuclease ScaI-HF (10 U) and CutSmart reaction buffer (10×, 1 μl) were added to the second portion of the PEX reaction and the mixture was incubated for 1 h at 37 °C.

Before loading of the PAGE gel, PAGE stop solution (either 11.5 or 8.5 μl, depending on the original portion of the PEX reaction) was added and samples were denatured by heating at 95 °C for 5 min. Aliquots (3.5 μl) were analyzed by denaturing PAGE (12.5%). The gels were visualized by a fluorescent scanner. Conversion of the cleavage was determined using ImageJ software by comparison of the ratios of the intensity of the cleavage product and sum of intensities of the cleavage product and the rest of the products.

Post-synthetic modification of DNA

Fluorescent labelling of vinyl modified DNAs

Reaction mixture was prepared by dissolving vinyl modified DNA (DNA19_1^{V}H or DNA31_4^{V}H) (0.5 nmol) in H_2O (12.5 μl). To this, thiol [**CM-SH**, (500 mM in 0.5 M TEAA buffer, pH = 8.0), 12.5 μl] was added and the reaction mixture was kept at 37 °C and 500 rpm for 3 days.

Steady-state fluorescence measurements of the products of thiol-ene reaction were performed with DNA after thiol-ene reaction and purification with QIAquick nucleotide removal kit (QIAGEN). DNA concentration was determined on a Nanodrop and recalculated using values from OligoCalc. Emission spectra were recorded using a 0.5 μM solution of DNA in phosphate buffer (20 mM, pH = 7.0, 1 M NaCl) using 100 μl quartz cuvette. Solution was equilibrated for 2 min in a thermal holder (25 °C) before the fluorescence spectrum was recorded. Excitation wavelength was 390 nm and the range of the emission spectra was 410-650 nm. Control experiments were performed

using natural non-modified DNA following the same procedure (thiol-ene reaction, purification and emission spectra measurement).

To obtain single-stranded DNA for MALDI analysis, the thiol-ene reaction was performed as described above using DNA after PEX with biotinylated template. Reaction mixture was first purified using QIAquick nucleotide removal kit (QIAGEN) and subsequently ssDNAs were generated by magnetoseparation. Thus a solution of modified DNA obtained after elution from the spin column (50 µl of miliQ H_2O) was diluted by 50 µl of binding buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Streptavidine magnetic particles (Roche, 100 µl) were washed with binding buffer (3 × 200 µl). The diluted modified DNA sample (100 µl) was then added to the magnetic particles. Mixture was incubated at 15 °C and 1100 rpm for 30 min. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen) and washed with washing buffer (3 × 200 µl, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 µl). Then water (50 µl) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial and analyzed by MALDI-TOF.

To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp19G. The product of thiol-ene reaction was purified with QIAquick nucleotide removal kit (QIAGEN) two times. Concentration of DNA was determined on a Nanodrop and recalculated using values from OligoCalc. An aliquot was taken and diluted with miliQ H_2O to a total volume of 20 µl of approximately 500 nM concentration. PAGE stop solution (20 µl) was added and sample was denaturated at 95 °C for 5 min. Aliquot (3.5 µl) was subjected to vertical electrophoresis in 20% denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 120 min. The gel was visualized by a fluorescent scanner.

Reaction of vinyl modified DNAs and L-cysteine

Reaction mixture was prepared by dissolving vinyl modified DNA (DNA19_1^{V}H or DNA31_4^{V}H) (0.5 nmol) in H_2O (12.5 µl). To this, L-cysteine (500 mM in 0.5 M TEAA buffer, pH = 8.0, 12.5 µl) was added and the reaction mixture was kept at 50 °C and 500 rpm overnight.

To obtain single-stranded DNA for MALDI analysis, reaction mixture was first purified using QIAquick nucleotide removal kit (QIAGEN) and subsequently ssDNAs were generated by magnetoseparation as described above.

To analyze the product of thiol-ene reaction using PAGE, the reaction was performed as described above using DNA after PEX with Prim15-FAM and Temp19. The PAGE analysis was performed as described above.

Reaction of vinyl modified DNA and dodecapeptides

DNA for these reactions was prepared using primer Prim16-FAM and template Temp26-bio. Reaction mixture was prepared as follows: in a PCR eppendorf, 2 µl of vinyl modified DNA solution (DNA26_1^{V}H-FAM, 26 µM in TEAA buffer, pH = 8.0) was mixed with the corresponding amount of peptide (1-100 eq., solutions in TEAA buffer). The final concentration was of DNA was adjusted to 5 µM with TEAA buffer. Reactions were kept at 20 °C for 1 h and then the temperature was increased over the course of 2 h to 37 °C. Reactions were kept at this temperature for 1 day (in one case reaction was run for 2 days). Stop solution (3 µl) and reaction mixtures (3 µl) were then combined and this mixture was denatured at 95 °C for 12 min. Residues were subjected to vertical electrophoresis in 12.5 % denaturing polyacrylamide gel containing 1×TBE buffer (pH 8.0) and 7 M urea at 42 mA for 80 min. The gel was visualized by a fluorescent scanner.

To obtain DNA suitable for MALDI analysis, reaction was performed on a bigger scale using 30 µl of the aforementioned DNA solution and 10 eq. of peptide_pos. After completion of the reaction, ssDNA was obtained by magnetoseparation as described above using 100 µl of magnetic beads.

Thermal denaturation studies

Thermal denaturation studies were performed as described above.

6 List of publications of the author

Publications related to the thesis

[1] J. Matyašovský, P. Perlíková, V. Malnuit, R. Pohl, M. Hocek: 2-Substituted dATP Derivatives as Building Blocks for Polymerase-Catalyzed Synthesis of DNA Modified in the Minor Groove. *Angew. Chem. Int. Ed.* **2016**, *55*, 15856–15859.

[2] J. Matyašovský, R. Pohl, M. Hocek: 2-Allyl- and -propargylamino-dATPs for site-specific enzymatic introduction of a single modification in minor groove of DNA. *Chem. Eur. J.* **2018**, *24*, 14938–14941.

[3] J. Matyašovský, M. Hocek: 2-Substituted 2'-Deoxyinosine 5'-Triphosphates as Substrates for Polymerase Synthesis of Minor-Groove-Modified DNA and Effects on Restriction Endonuclease Cleavage. *Org. Biomol. Chem.* **2019**, *Manuscript accepted*, doi: 10.1039/C9OB02502B.

Other publications of the author

[4] N. Sabat, P. Nauš, J. Matyašovský, D. Dziuba, L. Poštová Slavětínská, M. Hocek: Synthesis of Fluorescent 2-Substituted 6-(Het)aryl-7-deazapurine Bases {4-(Het)aryl-pyrrolo[2,3-d]pyrimidines} by Aqueous Suzuki–Miyaura Cross-Coupling Reactions. *Synthesis* **2016**, *48*, 1029–1045.

[5] D. Dziuba, P. Pospíšil, J. Matyašovský, J. Brynda, D. Nachtigallová, L. Rulíšek, R. Pohl, M. Hof, M. Hocek: Solvatochromic fluorene-linked nucleoside and DNA as color-changing fluorescent probes for sensing interactions. *Chem. Sci.* **2016**, *7*, 5775–5785.

[6] P. Güixens-Gallardo, Z. Zawada, J. Matyašovský, D. Dziuba, R. Pohl, T. Kraus, M. Hocek: Brightly Fluorescent 2'-Deoxyribonucleoside Triphosphates Bearing Methylated Bodipy Fluorophore for in cellulo Incorporation to DNA, Imaging and Flow Cytometry. *Bioconjug. Chem.* **2018**, *29*, 3906–3912.

7 Appendices

Appendix 1. Copy of MALDI spectra of the thiol-ene reaction between **CM-SH** and DNA4^{V}A

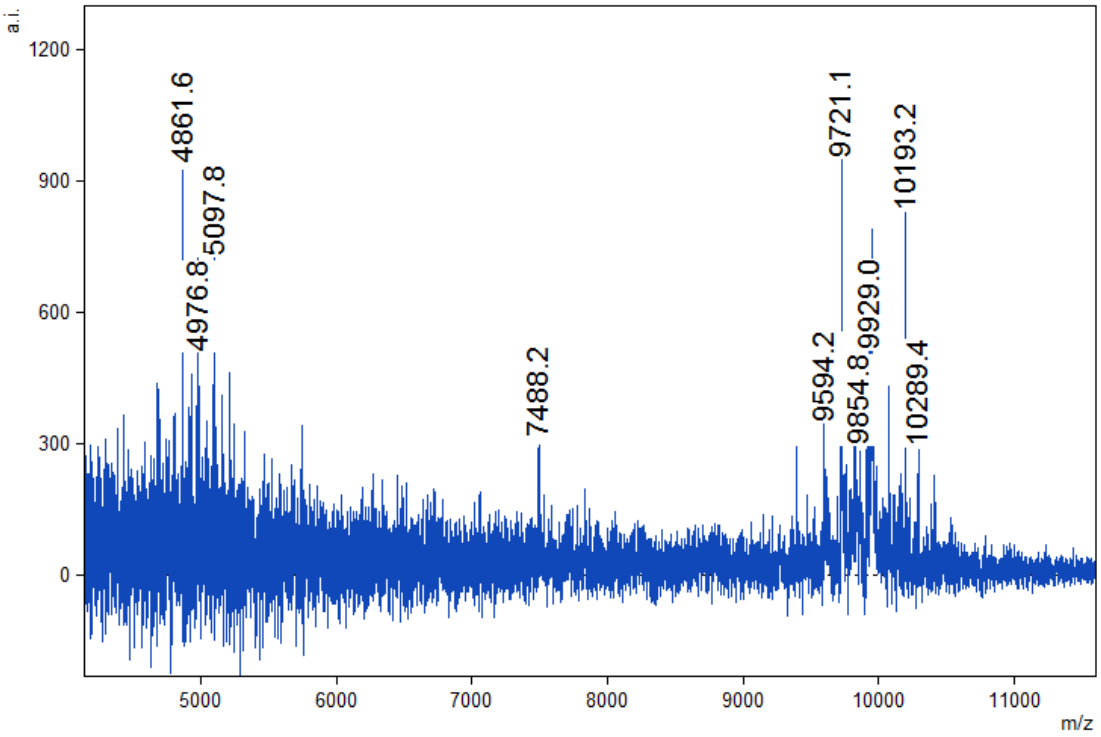


Figure 52: MALDI-TOF MS spectrum of **ON4^{CM}A** after thiol-ene reaction of **DNA4^{V}A** with **CM-SH**, calculated for $[M+H]^+$: 10555.0 Da; found: 10558.4 Da; visible peak at 9721.1 Da indicates presence of the starting material. Peaks at 9929.0 Da and 10136.2 Da can be assigned to products of thiol-ene reaction of one and two vinyl groups respectively.

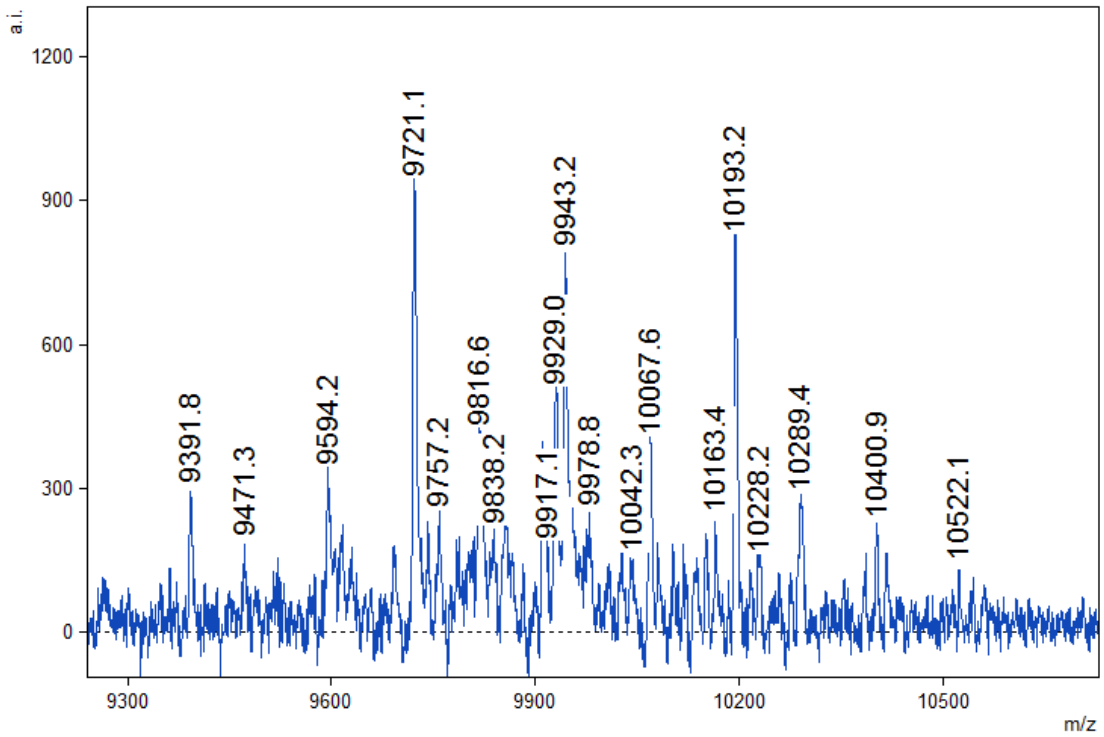


Figure 53: MALDI-TOF MS spectrum of **ON4^{CM}A** after thiol-ene reaction of **DNA4^{V}A** with **CM-SH** zoomed in.

Appendix 2. Copies of MALDI spectra resulting from the experiment described in **Figure 31**, without the click reaction taking place (products of the PEX experiment).

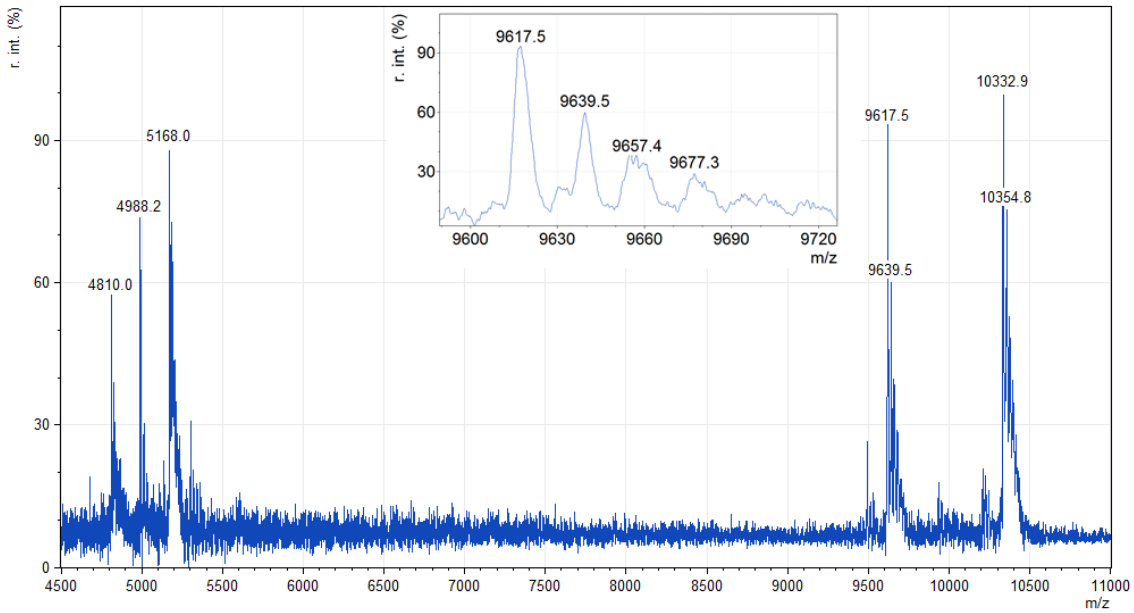


Figure 54: MALDI-TOF MS spectrum of DNA31_X^{AA}AC, natural DNA: calculated for $[M+H]^+$: 9617.3 Da; found: 9617.5; the peak at *m/z* = 10332.9 can be assigned to the biotinylated template. Missing peak at 9672.3 indicates that in the presence of natural dATP, **d^{AA}ATP** in not being incorporated.

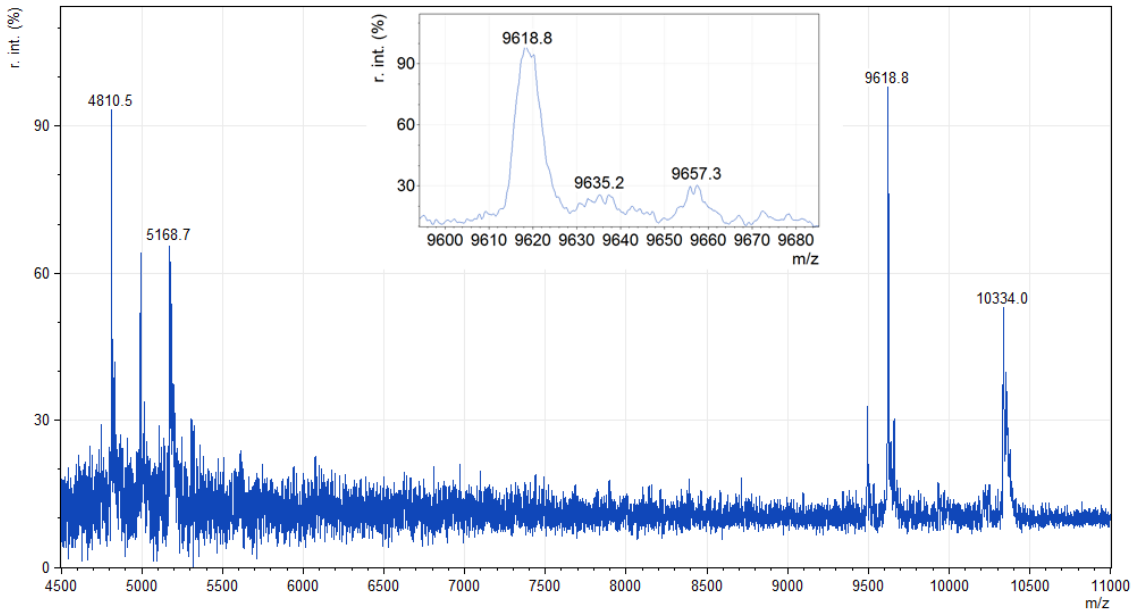


Figure 55: MALDI-TOF MS spectrum of DNA31_X^{PA}AC, natural DNA: calculated for $[M+H]^+$: 9617.3 Da; found: 9618.8; the peak at *m/z* = 10334.0 can be assigned to the biotinylated template. Missing peak at 9670.3 indicates that in the presence of natural dATP, **d^{PA}ATP** in not being incorporated.

Appendix 3. UV/Vis absorption spectra of samples used for measurement of FRET

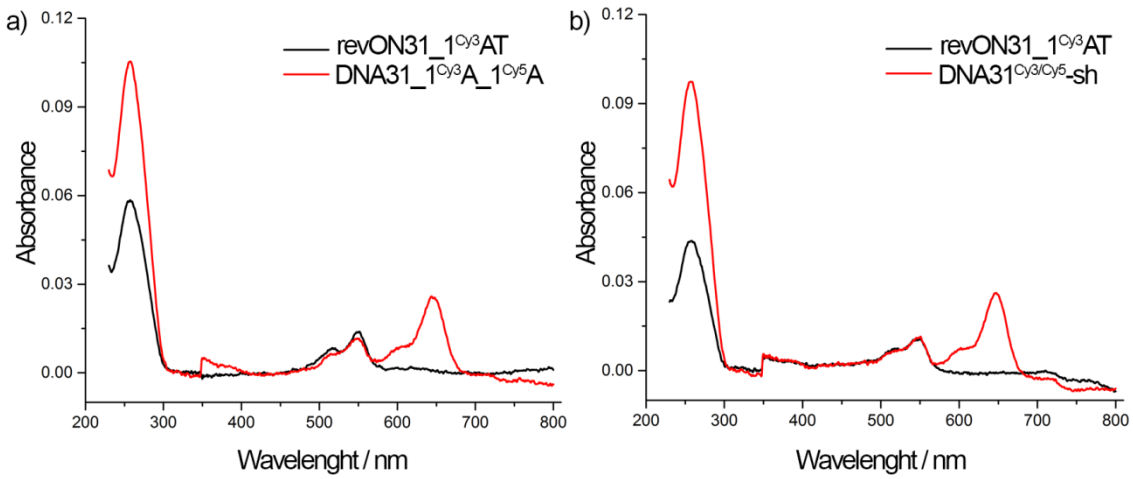


Figure 56: Absorption spectra of oligonucleotide samples used for FRET measurements; a) melting/annealing experiment; b) hybridization experiment. Note that the absorbance at the region responsible for absorption of Cy3 (ca. 550 nm) is approximalty the same for both samples, indicating approximately same concentration of the samples. Note that the addition of Cy5 containing ONs does not significantly affect the absorbance at the range characteristic for Cy3.

Appendix 4. ^{1}H, ^{13}C and ^{31}P NMR spectra of **d^{E}ITP**

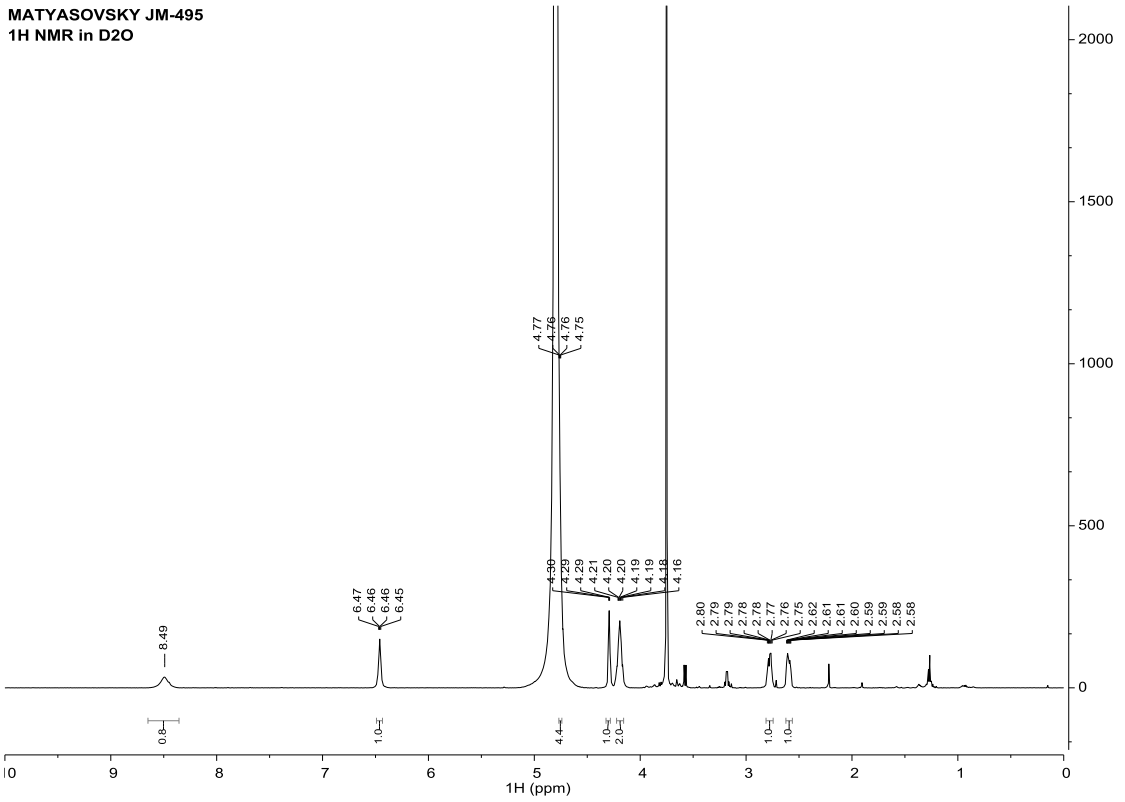


Figure 57: ^{1}H NMR spectrum of **d^{E}ITP**.

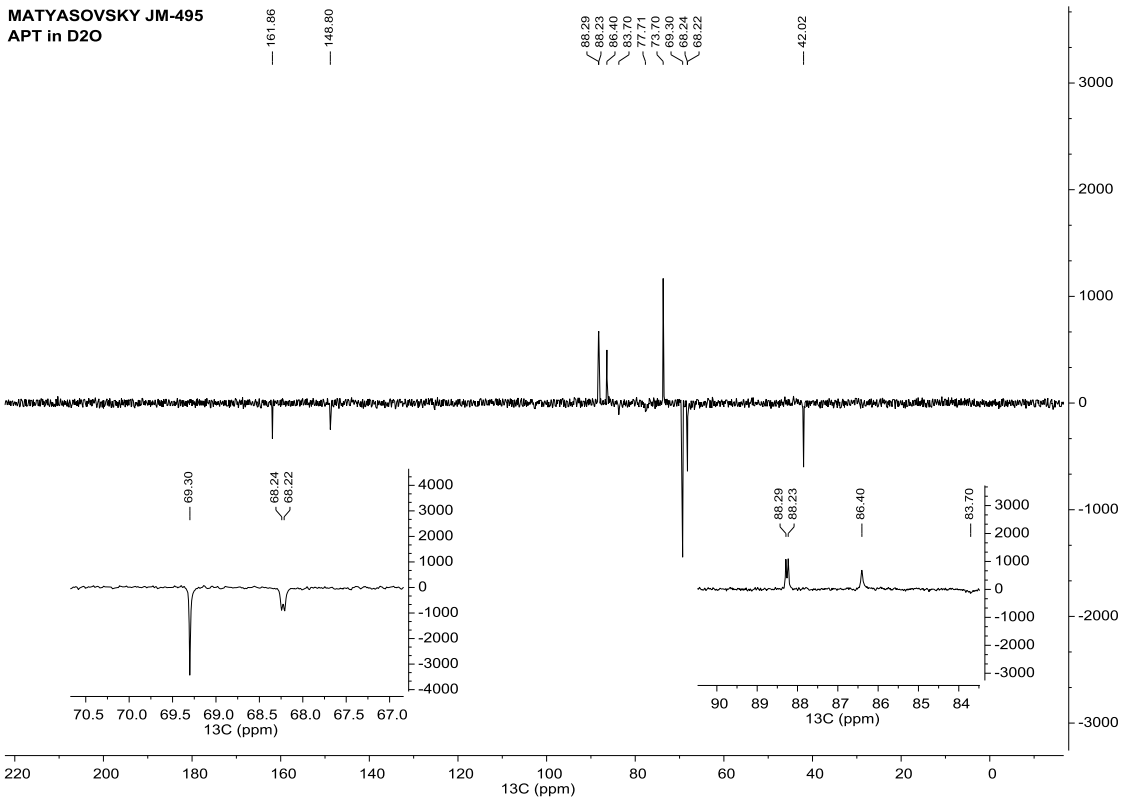


Figure 58: ^{13}C (APT) NMR spectrum of **d^{E}ITP**.

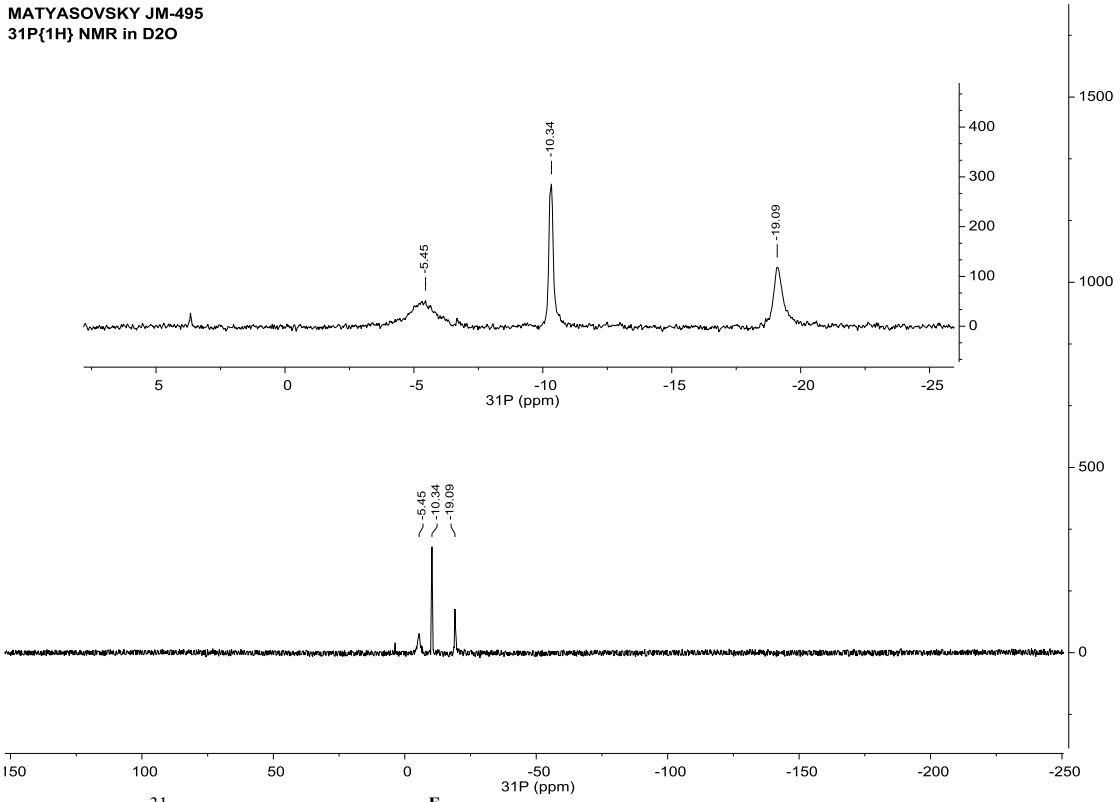


Figure 59: ^{31}P NMR spectrum of **d^{E}ITP**.

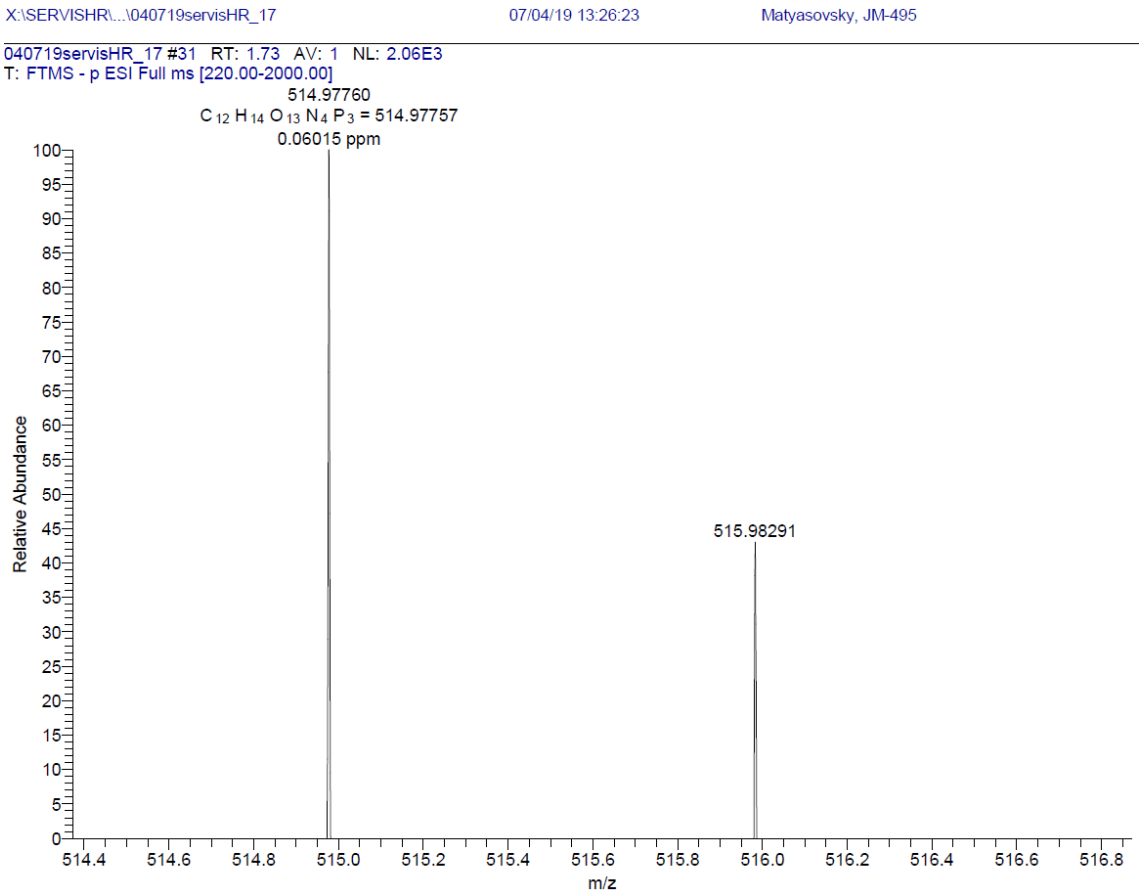


Figure 60: HRMS spectrum of **d^{E}ITP**.

Appendix 5. Copies of MALDI spectra of the thiol-ene reaction between **CM-SH** or L-cysteine and DNA31_4^{V}H.

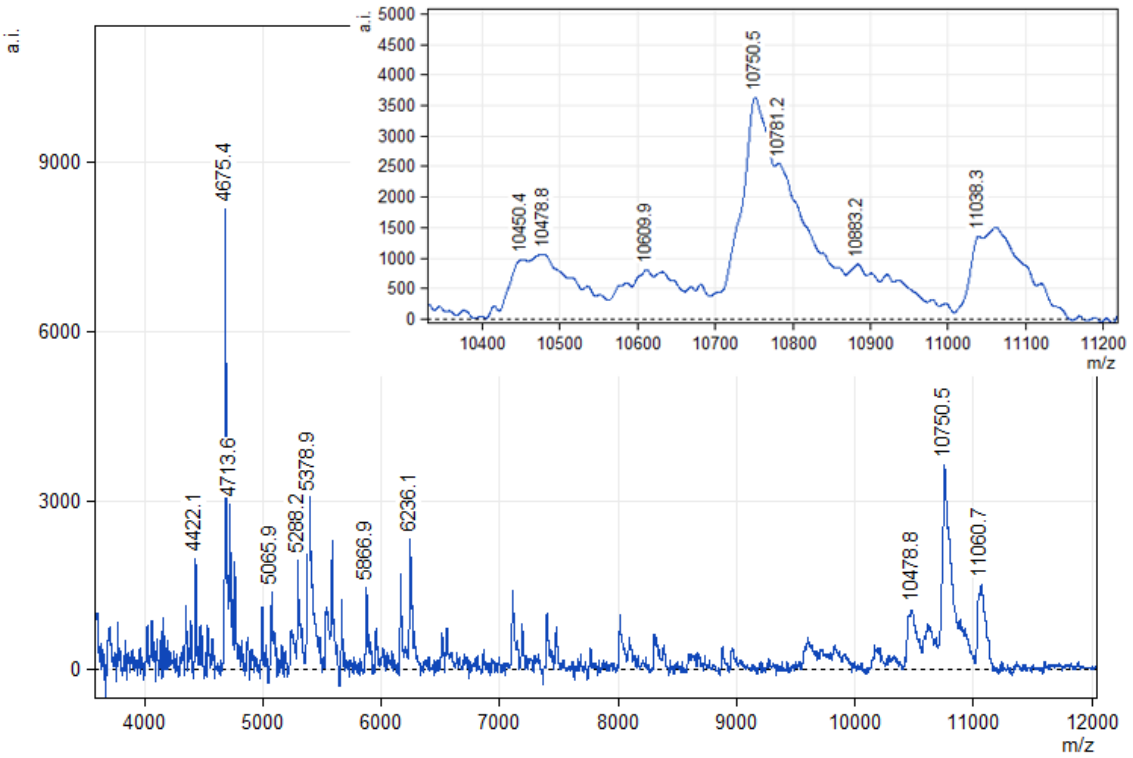


Figure 61: MALDI-TOF MS spectrum of ON31_4^{CM}H after thiol-ene reaction of DNA31_4^{V}H and **CM-SH**. Starting vinyl modified DNA was extended by multiple As at the 3' end due to non-templated extension. Products of thiol-ene reaction: $[M+2As+2CM+K]^{+}$: 10756.4, found 10750.5 – product of reaction of two vinyl groups, $[M+3As+2CM+Na]^{+}$: 11037.6, found 11038.3, etc.

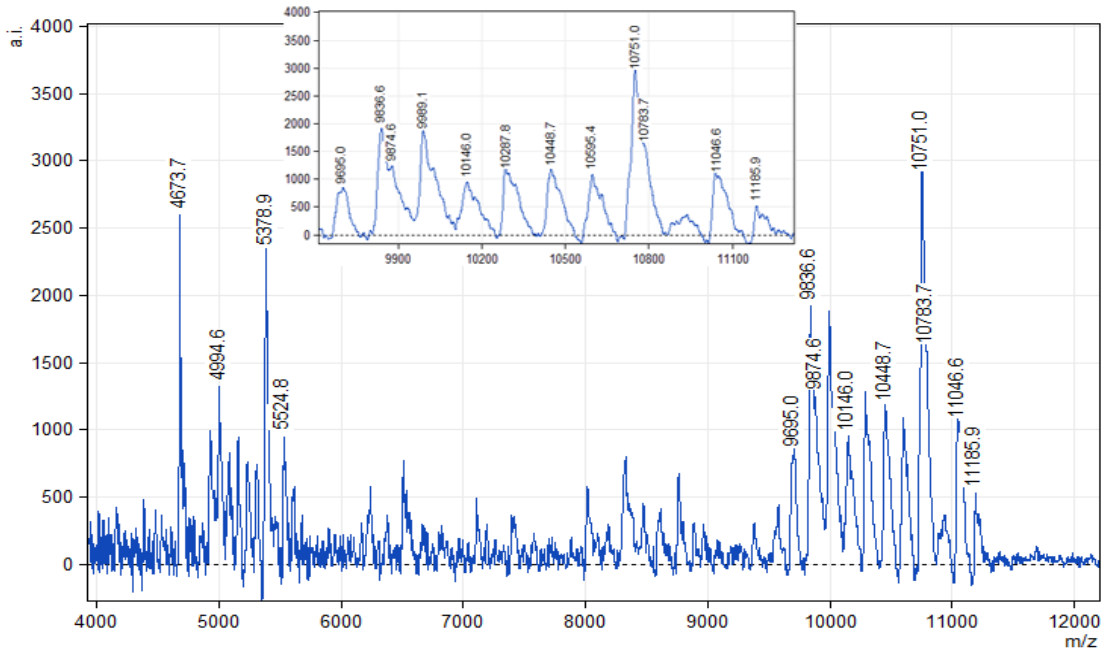


Figure 62: MALDI-TOF MS spectrum of ON31_4^{Cys}H after thiol-ene reaction of DNA31_4^{V}H and L-cysteine. Starting vinyl modified DNA was extended by multiple additional As at the 3' end due to non-templated extension (calculated for $[M+2As+H]^+$: 10287.4 Da; found: 10287.8; $[M+3As+H]^+$ 10600.6; found 10595.4). Products of thiol-ene reaction: $[M+2As+cys+K]^+$: 10448.4, found 10448.7 – product of reaction of one vinyl group; $[M+3As+cys+Na]^+$: 10746.4, found 10751.0 – product of reaction of one vinyl group, etc.

8 References

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