

DNA Modification

International Edition: DOI: 10.1002/anie.201609007
German Edition: DOI: 10.1002/ange.201609007

2-Substituted dATP Derivatives as Building Blocks for Polymerase-Catalyzed Synthesis of DNA Modified in the Minor Groove

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Abstract: 2'-Deoxyadenosine triphosphate (dATP) derivatives bearing diverse substituents (Cl, NH₂, CH₃, vinyl, ethynyl, and phenyl) at position 2 were prepared and tested as substrates for DNA polymerases. The 2-phenyl-dATP was not a substrate for DNA polymerases, but the dATPs bearing smaller substituents were good substrates in primer-extension experiments, producing DNA substituted in the minor groove. The vinyl-modified DNA was applied in thiol-ene addition and the ethynyl-modified DNA was applied in a CuAAC click reaction to form DNA labelled with fluorescent dyes in the minor groove

Base-modified oligonucleotides (ONs) or DNA are widely used as tools in chemical biology, diagnostics, or materials science.^[1] The modification is mostly attached to position 5 of pyrimidines or position 7 of 7-deazapurines, not only because it then points out into the major groove of DNA and thus does not destabilize the duplex, but because in most cases, the corresponding substituted 2'-deoxyribonucleoside triphosphates (dNTPs) are good substrates for DNA polymerases and can be used in the polymerase-catalyzed synthesis of modified DNA.^[2,3] Diverse modifications, including fluorophores,^[4] redox^[5] or spin labels,^[6] reactive groups for conjugations,^[7] and biomolecules (e.g., oligonucleotides^[8] or proteins^[9]), have been introduced into the major groove through the enzymatic incorporation of modified nucleotides and applied in different fields. Modification or labelling of the minor groove has mostly been reported with 2'- and 4'-sugar-modified derivatives.^[10–13] 2-Chloroadenine^[14] and 2,6-diaminopurine^[15] dNTPs are the only minor-groove base-modified nucleotides that have been reported as substrates for DNA

polymerases, whereas 2-arylamino-dATP derivatives were found to act as polymerase inhibitors.^[16] The minor groove sites of the nucleobases are difficult to modify since they are crucial both for Watson–Crick base pairing and for key minor-groove interactions with DNA polymerase that are important for extension of the chain.^[17] On the other hand, 2-ethynylpyridone-C-nucleotide incorporated into DNA^[18] formed a stable base pair with adenine, and 2-(imidazolylalkylamino)purines in ONs also stabilized duplexes.^[19] Because the possibility of minor-groove base labelling would be attractive for many prospective applications, for example, the mapping of DNA–protein interactions, we envisaged that a small substituent at position 2 of a purine may not fully disturb the key H-bonding interactions with the opposite base and the polymerase, and we report herein the first enzymatic synthesis of minor-groove base-modified DNA.

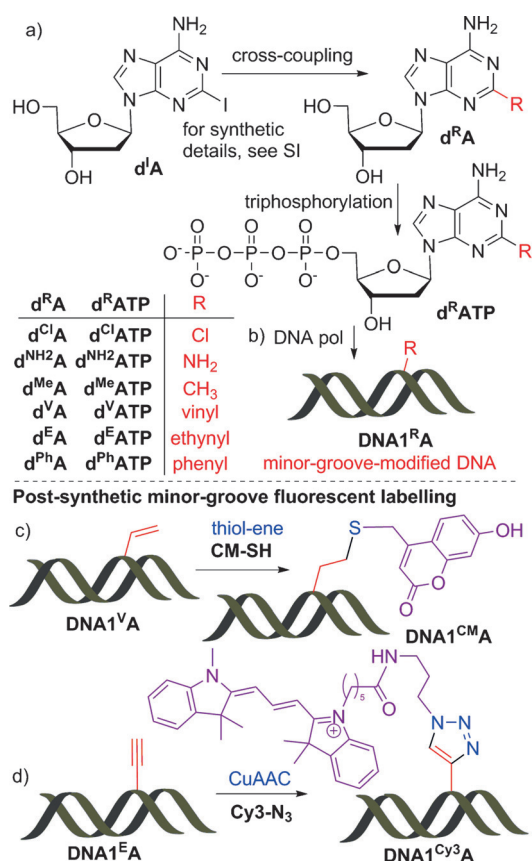
A series of six 2-substituted dATP derivatives bearing Cl, NH₂, CH₃, vinyl, ethynyl and phenyl substituents (**d^RATPs**) was designed to study the effect of substituents of different bulkiness at position 2 of adenine on polymerase-mediated incorporation. While **d^{Cl}ATP**^[14,20] and **d^{NH₂}ATP**^[15] were known, the others were prepared through triphosphorylation^[21] of the corresponding 2'-deoxy-ribonucleosides (**d^RAs**, Scheme 1), which were synthesized through cross-coupling reactions of the 2-iodo-2'-deoxyadenosine (for details of the synthesis, see the Supporting Information).

The **d^RATPs** were then tested as substrates for DNA polymerases in primer extension (PEX) experiments. First, we performed PEX in presence of KOD XL, Vent(*exo*-), or Bst DNA polymerase, using a 15-nt primer (prim^{248short}) and 19-nt template (temp^{oligo1A}) designed for the incorporation of one modified nucleotide, and the outcome was analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). All three DNA polymerases (Figure 1a and Figure S1 in the Supporting Information) incorporated the 2-substituted deoxyadenosine nucleotides, giving clean full-length DNA products (**DNA1^RA**). The only exception was the 2-phenyl derivative **d^{Ph}ATP**, which apparently was not a substrate for DNA polymerases since almost no extension was observed. Then PEX was conducted using a longer 31-nt template (temp^{Prb4basII}, which is modified with TINA at 3'-end to prevent non-templated incorporation,^[22] Figure 1b and Figure S2) designed for the incorporation of 4 **^RA** modifications. Most of the modified **d^RATPs** were good substrates, giving full-length products (**DNA4^RA**). Only the PEX product from ethynylated **d^EATP** and KOD XL DNA polymerase was partially halted at the *n*–1 position (but Vent(*exo*-) and Bst DNA polymerases gave clean full-length products; see Figure S2), while **d^{Ph}ATP** did not give PEX with any of the tested DNA polymerases. All of the PEX experiments (with all of

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Scheme 1. a) Synthesis of 2-substituted dATP derivatives. b) PEX incorporation of the modified nucleotides into DNA. c, d) Post-synthetic minor-groove fluorescent labelling by thiol-ene (c) or CuAAC (d) reactions.

the d^RATPs and both templates) with KOD XL DNA polymerase were repeated using biotinylated templates, and the modified single-stranded oligonucleotides (ONs, ON1^RA or ON4^RA) were isolated by magnetoseparation^[23] and analyzed by MALDI-TOF, which confirmed their identity (Table S2 in the Supporting Information).

To further quantify the substrate activities of the modified d^RATPs, we conducted a simple kinetic analysis of single-nucleotide extension using KOD XL DNA polymerase and temp^{1A-term} (Figure 1c) and compared the conversion as a function of time to that observed with natural dATP. The rate of extensions when using the smaller derivatives d^{Cl}ATP, d^{NH₂}ATP, or d^{Me}ATP were comparable to the rate with natural dATP, whereas PEX with the bulkier d^VATP and d^EATP took approximately 2 min to reach completion. To study the influence of the 2-modifications on the base pairing and duplex stability, we measured the denaturing temperatures of all of the PEX products (Table 1). Except for 2,6-diaminopurine, which stabilized the dsDNA due to an additional H-bond with T, all of the other modifications destabilized the duplexes.

With the shorter (DNA1^VA or DNA1^EA) and longer (DNA4^VA or DNA4^EA) dsDNA containing one or four 2-vinyl- or 2-ethynyladenine modifications in hand, we tested whether they could be used for post-synthetic minor-groove

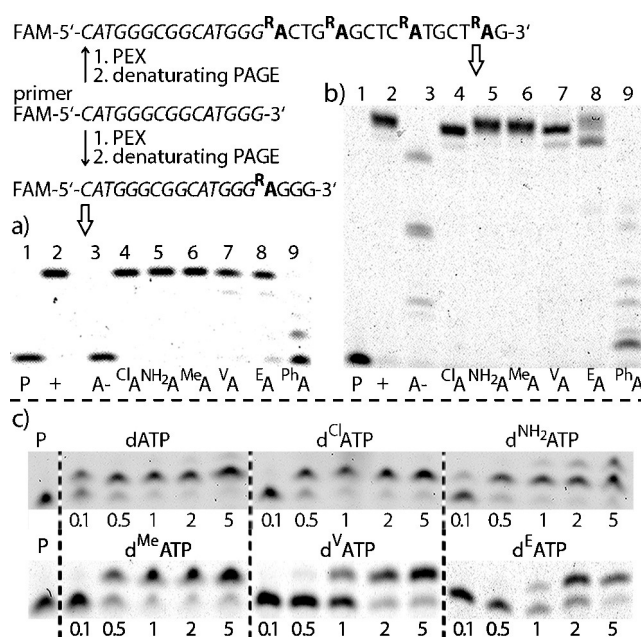


Figure 1. a, b) Denaturing PAGE of PEX experiments in presence of KOD XL with temp^{oligo1A} (a) or temp^{Prb4basII}-TINA (b). P: primer, +: products of PEX with natural dNTPs, A-: products of PEX with dTTP, dCTP, and dGTP; ^RA: products of PEX with dTTP, dCTP, dGTP, and functionalized d^RATP. c) PAGE analyses of the kinetics of single-nucleotide extension experiments with temp^{1A-term}, KOD XL, and d^RATP compared to natural dATP. Time intervals are given in minutes.

Table 1: Denaturing temperatures of modified DNA duplexes.

DNA	T _m [°C]	ΔT _m [°C] ^[a]	DNA	T _m [°C]	ΔT _m [°C] ^[a]
DNA1A	72.2	–	DNA4A	79.4	–
DNA1 ^{Cl} A	69.6	–2.6	DNA4 ^{Cl} A	71.0	–2.1
DNA1 ^{NH₂} A	72.5	+0.3	DNA4 ^{NH₂} A	81.4	+0.5
DNA1 ^{Me} A	70.5	–1.7	DNA4 ^{Me} A	72.3	–1.8
DNA1 ^V A	66.6	–5.6	DNA4 ^V A	72.6	–1.7
DNA1 ^E A	66.1	–6.1	DNA4 ^E A	71.1	–2.1
DNA1 ^{Cy₃} A	69.0	–3.2			

$$[a] \Delta T_m = (T_{m \text{ mod}} - T_{m \text{ natur}}) / r_{\text{mod}}$$

fluorescence labelling. The vinyl group was envisaged for use in the thiol-ene reaction (Scheme 1c),^[24] whereas the ethynyl group was envisaged for use in Cu-catalyzed alkyne-azide cycloaddition (CuAAC; Scheme 1d).^[25] We selected coumarinemethylthiol (CM-SH)^[26] and the commercially available azide-conjugated Cy3 (Cy3-N₃) as model reagents. The thiol-ene reactions of DNA1^VA or DNA4^VA with CM-SH proceeded in 3 days at 37°C without UV irradiation to give approximately 60% conversion (based on PAGE analysis, Figure 2d) to blue-fluorescent conjugates (DNA1^{CM}A or DNA4^{CM}A; Figure 2a,c). In this case, UV irradiation did not help, owing to bleaching of the fluorophore. The CuAAC reactions of DNA1^EA or DNA4^EA with Cy3-N₃ proceeded smoothly at 37°C in the presence of CuBr, sodium ascorbate, and tris(benzyltriazolylmethyl)amine (TBTA), quantitatively providing the red-fluorescent Cy3-triazole-modified DNA (DNA1^{Cy₃}A or DNA4^{Cy₃}A; Figure 2b,e,f). Interestingly, the

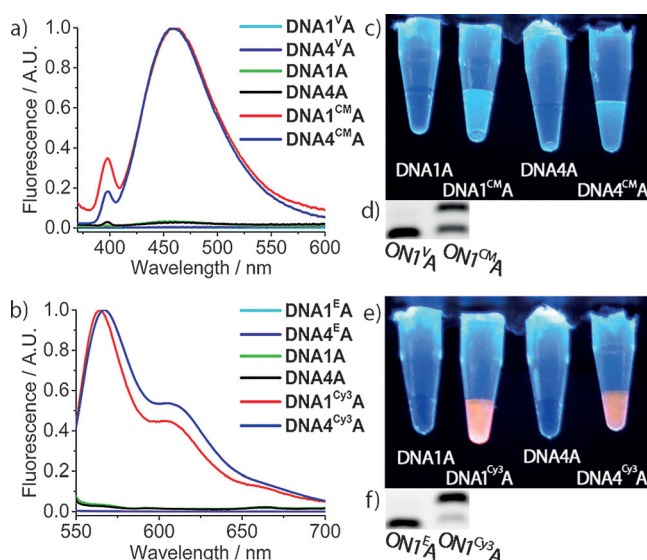


Figure 2. a) Normalized emission spectra of **DNA1^{VA}** and **DNA4^{VA}** compared to non-modified **DNA1A** or **DNA4A** before and after thiol-ene reaction with CM-SH. b) Normalized emission spectra of **DNA1^{FA}** and **DNA4^{FA}** compared to non-modified **DNA1A** or **DNA4A** before and after CuAAC reaction with Cy3-N₃. c) A photograph of vials containing CM-linked DNA under UV irradiation (365 nm) compared to non-modified DNA treated with the same reagent. d) PAGE analysis of PEX product **ON1^{VA}** and the product of subsequent thiol-ene reaction, **ON1^{CM A}**. e) A photograph of vials containing Cy3-linked DNA (in H₂O/glycerol) under UV irradiation (365 nm) compared to non-modified DNA treated with the same reagent. f) PAGE analysis of PEX product **ON1^{FA}** and the product of subsequent CuAAC reaction, **ON1^{Cy3 A}**.

triazole-linked duplex was more stable than the starting ethynyl-modified DNA (Table 1).

In conclusion, we found that not only 2-chloro-^[14] and 2-aminoadenine^[15] dNTPs, but also dATP derivatives bearing smaller C substituents at position 2 (CH₃, vinyl, and ethynyl) are good substrates for DNA polymerases and can be used for the enzymatic synthesis of base-modified DNA bearing substituents in the minor groove. Conversely, the phenyl group is too bulky because the corresponding **d^{Ph}ATP** was not a substrate for any tested DNA polymerase. The minor-groove vinyl- or ethynyl-modified DNAs can be post-synthetically labelled through thiol-ene or CuAAC reactions with thiols or azides, which was exemplified by fluorescent labelling with coumarine or Cy3. To the best of our knowledge, this is the first example of polymerase-catalyzed synthesis of DNA modified at the minor groove sites of nucleobases, and it paves the way for other minor-groove nucleobase modifications and conjugations,^[27] which could be useful in applications in chemical biology or imaging. Studies along these lines are underway.

Acknowledgements

This work was supported by the Czech Academy of Sciences (RVO: 61388963 and the Praemium Academiae award to M. H.), by the Czech Science Foundation (14-04289S to M.H.) by

the Marie Skłodowska-Curie Innovative Training Network (ITN) Click Gene (H2020-MSCA-ITN-2014-642023 to J.M.).

Keywords: bioconjugation · DNA modification · DNA polymerase · nucleotides · fluorescent labelling

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 15856–15859
Angew. Chem. **2016**, *128*, 16088–16091

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Manuscript received: September 14, 2016

Final Article published: November 23, 2016