Synthesis of 8-bromo-, 8-methyl- and 8-phenyl-dATP and their polymerase incorporation into DNA†

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Received 11th July 2008, Accepted 29th August 2008
First published as an Advance Article on the web 17th September 2008
DOI: 10.1039/b811935j

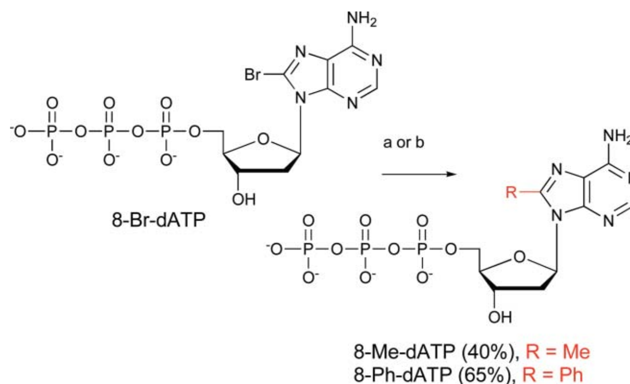
dATP derivatives bearing Br, Me or Ph groups in position 8 were prepared and tested as substrates for DNA polymerases to show that 8-Br-dATP and 8-Me-dATP were efficiently incorporated, while 8-Ph-dATP was a poor substrate due to its bulky Ph group.

DNA methylation plays crucial roles in a large variety of biological processes, *i.e.* regulation of gene expression, protection against endogenous restriction endonucleases, DNA repair and replication.[1] In higher eukaryotes, the methylation only occurs at C as 5-methylcytosine. However, in bacterial DNAs, N^6-methyladenine is observed as well. An even larger variety of methylated purine bases occurs in RNA. Apart from N^2 and *N*7-methylated G and *N*1 and N^6-methylated A, C-2 methylated A was also observed.[2] However, to the best of our knowledge, no natural occurrence of methylation in position 8 of purine has been reported.

8-Methyl-2′-deoxyadenosine (8-Me-dA) was prepared by the reaction of the corresponding protected 8-bromo-dA with methylmagnesium iodide,[3] trimethylaluminium[4] or tetramethylstannane[5] and it was chemically incorporated into oligonucleotides (ONs) forming stable quadruplexes with *syn*-conformation of the nucleobase.[6] 8-MeG[7] and 8-Me-7-deazaG[8] containing ONs were prepared by chemical synthesis to show that the presence of methyl groups facilitated the B–Z transition of DNA. 8-Me-A ribonucleoside showed antiviral activity against vaccinia virus[5] and 8-Me-ATP was studied as a substrate for actomyosine.[9]

Base-modified nucleic acids are of great current interest due to potential applications in chemical biology, bioanalysis or nanotechnology and material science.[10] Apart from chemical synthesis, base-modified DNA can be prepared enzymatically by incorporation of modified 2′-deoxyribonucleoside triphosphates (dNTPs) by DNA polymerases. This approach has been used[11,12] for construction of functionalized nucleic acids bearing diverse functional groups. Recently, we have combined aqueous-phase cross-coupling reactions of unprotected dNTPs with polymerase incorporation into a two-step methodology[13] of construction of modified nucleic acids containing amino acids,[14] ferrocene[15] or amino- and nitrophenyl groups.[16] It was shown by us[14] and others[12] that 8-substituted dATP derivatives are not good substrates for DNA polymerases, neither in primer extension (PEX) nor in PCR (presumably due to their preference for *syn*-conformation and steric hindrance between the substituent and DNA backbone). Therefore, 7-substituted 7-deazapurine dNTPs are used as surrogates of purines.[12–16] Most works dealing with enzymatic incorporation of 8-modified purine nucleotides into DNA have been focused on 8-oxo-dGTP (for studying oxidative damage of DNA) to show that it is a poor substrate for DNA polymerases and reverse transcriptases.[17] Enzymatic incorporation of 8-NH_2-dGTP was more feasible.[18] Perrin *et al.* reported incorporation of 8-[2-(4-imidazolyl)ethylamino]-deoxyriboadenosine-5′-triphosphate by Sequenase V2.0 or Dpo4 polymerases in construction of novel DNAzymes.[19] Even the Dpo4 polymerase, which is a Y-family enzyme capable of bypassing lesions, was unable to incorporate 8-substituted dATP linked *via* a C–C bond, but it successfully incorporated two examples of 8-[(imidazolylalkyl)amino]-dATPs.[19d]

We wanted to look into the polymerase incorporation of 8-substituted dATPs bearing either smaller Br or Me substituents or a bigger phenyl (Ph) substituent in order to define the scope and limitations of the polymerase incorporation of purine dNTPs, depending on the bulkiness of the substituent. We also wanted, if possible, to develop construction of DNA containing Br atoms for postsynthetic modifications (*e.g. via* cross-coupling) or containing 8-MeA for studying the influence of such unnatural methylation on DNA repair and gene expression.



Scheme 1 *Reagents and conditions:* methylboronic acid (a) or phenylboronic acid (b) and Cs_2CO_3, $Pd(OAc)_2$, $P(C_6H_4\text{-}3\text{-}SO_3Na)_3$, H_2O–CH_3CN (2 : 1).

Bromination of dATP was used for the synthesis of 8-bromo-2′-deoxyadenosine 5′-O-triphosphate (8-Br-dATP).[20] 8-Methyl-2′-deoxyadenosine 5′-O-triphosphate (8-Me-dATP) and 8-phenyl-2′-deoxyadenosine 5′-O-triphosphate (8-Ph-dATP) were prepared by the single-step aqueous-phase cross-coupling reactions of 8-Br-dATP (Scheme 1),‡ in analogy to our previously developed

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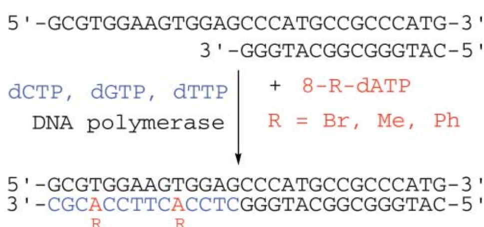
† Electronic supplementary information (ESI) available: Complete experimental part, characterization data, additional PEX results, procedures for PEX, CD spectra, T_m determination and MALDI, copies of NMR spectra. See DOI: 10.1039/b811935j

procedures.[14–16] The Suzuki–Miyaura cross-coupling reaction of 8-Br-dATP with methylboronic or phenylboronic acid in the presence of $Pd(OAc)_2$ and $P(C_6H_4\text{-}3\text{-}SO_3Na)_3$ in a mixture of water and CH_3CN for 30 min gave the corresponding 8-modified dATP in acceptable yields after purification by HPLC (40% for 8-Me-dATP and 65% for 8-Ph-dATP). The former reaction was the first example of direct attachment of an sp^3 hybridized alkyl group to nucleoside triphosphate by aqueous cross-coupling. The yield of the reaction with $MeB(OH)_2$ was somewhat lower than that for the $PhB(OH)_2$, but it is still a practical method for introduction of the methyl group.

Four types of polymerases—*E. coli* polA (Klenow fragment (3′→5′ exo-)), *Thermus brockianus* (Dynazyme II), *Thermococcus litoralis* (Vent(exo-)) and *Pyrococcus woesei* (Pwo)—have been tested for incorporation of 8-Br-dATP, 8-Me-dATP and 8-Ph-dATP into different sequences of DNA by PEX (Chart 1, Scheme 2).§



Scheme 2 PEX with template temp^{2A} and 8-modified dATP.

Primer	5'-CATGGGCGGCATGGG-3'
temp^{A}	5'-GAAA**T**CCCATGCCGCCCATG-3'
temp^{2A}	5'-GCG**T**GGAAG**T**GGAGCCCATGCCGCCCATG-3'
temp^{4A}	5'-C**T**AGCA**T**GAGC**T**CAG**T**CCCATGCCGCCCATG-3'
temp^{ATr}	5'-**T**A**T**A**T**A**T**A**T**A**T**CCCATGCCGCCCATG-3'

Chart 1 Templates and primer used in this study.

8-Br-dATP was successfully incorporated into all four tested sequences of DNA and was a good substrate for all four enzymes. 8-Me-dATP was still successfully incorporated into sequences bearing one, two or four modifications in one molecule DNA (temp^{A}, temp^{2A}, temp ^{4A}). However, using a more complex repetitive sequence with high density of modifications (temp^{ATr}), the full extension was not achieved. Synthesis of ONs containing 8-PhA was the most difficult. It was shown that 8-Ph-dATP is a poor substrate for all the tested polymerases. Fig. 1 shows the incorporation of 8-modified dATP by PEX using temp^{4A} and all four tested polymerases. 8-Br-dATP (lines 4, 9, 14, 19) as well as 8-Me-dATP (lines 5, 10, 15, 20) were accepted by all polymerases as good substrates instead of natural dATP. Only partial incorporations of 8-Ph-dATP were observed in reactions with polymerases from Family B (Vent(exo-) and Pwo).

Melting temperatures and CD spectra of our modified DNAs containing 8-BrA or 8-MeA were also studied. The ONs for these measurements were prepared by PEX on a large scale using Vent(exo-) as polymerase, templates temp^{A}, temp^{2A} and temp^{4A}, dCTP, dGTP, dTTP and dATP (positive control) or 8-Br-dATP and 8-Me-dATP as surrogates of natural dATP. Higher concentrations of the template and primers have been used to get cleaner products. The melting temperatures of the natural and

Table 1 Melting temperatures of DNA duplexes[a]

	temp^{A}		temp^{2A}		temp^{4A}	
	T_m/°C	ΔT_m[b]	T_m/°C	ΔT_m[b]	T_m/°C	ΔT_m[b]
dATP	76	0	86	0	86	0
8-Br-dATP	76	0	84	−1	81	−1.25
8-Me-dATP	71	−5	79	−3.5	74	−3

[a] Prepared by PEX with temp^{A}, temp^{2A}, temp^{4A}, Vent(exo-), dCTP, dGTP, dTTP, dATP or 8-Br-dATP, 8-Me-dATP. [b] $\Delta T_m = (T_{mmod.} - T_{mnat.})/n_{mod}$.

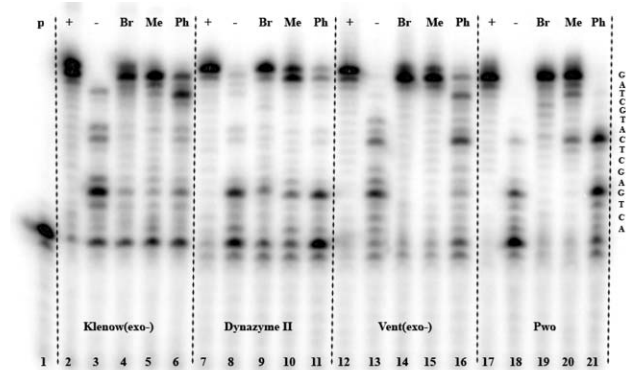


Fig. 1 PEX with temp^{4A}, dCTP, dGTP, dTTP, dATP (lines 2, 7, 12, 17); dCTP, dGTP and dTTP (lines 3, 8, 13, 18), dCTP, dGTP, dTTP and 8-mod. dATP (lines 4, 5, 6, 9, 10, 11, 14, 15, 16, 19, 20, 21), line 1—^{32}P radiolabeled primer.

modified DNAs are summarized in Table 1.¶ The presence of the 8-Br substituent caused only very low destabilization (ΔT_m = −1.25 to 0 °C), while the methyl group resulted in more significant destabilization (ΔT_m = −5 to −3 °C). In CD spectra, the classical B structure was observed in all cases with all the sequences studied (Fig. 2).

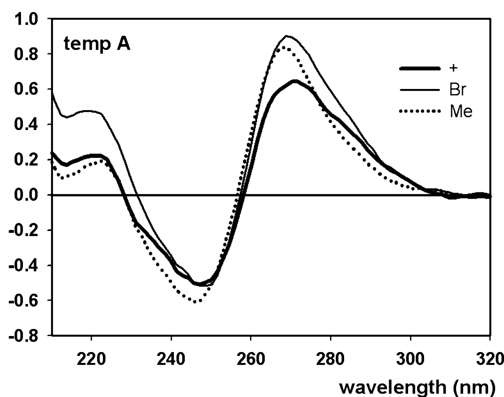


Fig. 2 CD spectra of PEX products with temp^{A} (+: natural DNA, Br: DNA bearing one bromine on A, Me: DNA bearing one methyl on A).

The successful incorporation of our 8-modified dATPs was proved by MALDI-TOF analysis (see the ESI†).‖ Single stranded DNA was prepared by PEX with biotinylated template temp^{2A} and then by using magnetoseparation.[16] Peaks at 8859.5 (calculated mass 8855.8 [M + H$^+$]), 8879.8 [M + Na$^+$] and 8897.9 [M + K$^+$] of natural ssDNA were observed. By analysis of methylated DNA (2 Me groups in one strand), the peak 8882.5 (calculated mass

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8883.8 [M + H$^+$]) was observed to confirm successful incorporation of the two modifications.

In conclusion, aqueous cross-couplings were efficiently used for attachment of a methyl or phenyl group to position 8 of dATP. The modified dATPs bearing Br, Me or Ph groups were tested as substrates for DNA polymerases to study the scope and limitations of enzymatic incorporations of 8-substituted dATPs. The results showed that 8-Br-dATP is a very good substrate, suitable for incorporation to any sequence. 8-Me-dATP is a somewhat worse substrate, but is still capable of incorporation into most sequences, while the phenyl group in 8-Ph-dATP is too bulky for the polymerase to accept this dNTP as a substrate. The resulting ONs containing 8-BrA can be further used for postsynthetic modifications (*e.g.* by nucleophilic substitutions or cross-couplings), while the methylated DNA is interesting for studying the epigenetic consequences of the unnatural methylation. Studies in both these directions will follow in the future. Despite some previous, rather scattered, examples of successful polymerase incorporations of some 8-alkylamino dATPs reported by Perrin,[19] these results confirm that 8-substituted dATP derivatives bearing bulky groups (bigger than amino, bromo or methyl) are generally poor substrates for DNA polymerases. Their replacement by 7-substituted 7-deazapurine dNTPs[12–16,21] is a much more efficient way to introduce modifications to purines in DNA by PEX or PCR.

Acknowledgements

This work is a part of the research projects Z4 055 0506. It was supported by the Ministry of Education (LC512) and by Gilead Sciences, Inc. (Foster City, CA, U. S. A.).

Notes and references

‡ **Suzuki cross-couplings of 8-Br-dATP:** a water–acetonitrile mixture (2 : 1, 0.4 ml) was added through a septum to an argon-purged vial containing 8-Br-dATP sodium salt (22 mg, 0.034 mmol), methylboronic acid (4.1 mg, 0.068 mmol) or phenylboronic acid (8.3 mg, 0.068 mmol), and Cs_2CO_3 (55.4 mg, 0.17 mmol). After the solids had dissolved, a solution of $Pd(OAc)_2$ (0.8 mg, 0.0034 mmol) and TPPTS (9.7 mg, 0.017 mmol) in water–acetonitrile (2 : 1, 0.3 ml) was added and the mixture was stirred and heated up to 120 °C for 30 minutes. Products were isolated from the crude reaction mixture by HPLC on a C18 column with the use of a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O–MeOH (1 : 1) as eluent. Several co-distillations with water and conversion to sodium salt form (Dowex 50WX8 in Na+ cycle) followed by freeze-drying from water gave white solid products. 8-Me-dATP: yield 40%. MS(ESI–): 504.1 (100, M – 1), 526.1 (M + Na), HRMS: for $C_{11}H_{17}N_5O_{12}P_3$ calculated 504.0087, found 504.0098. ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.40 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 7.0, $J_{2'b,3'}$ = 3.7, H-2′b); 2.69 (s, 3H, CH_3); 3.10 (dt, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 7.6, H-2′a); 4.19–4.32 (m, 3H, H-4′ and H-5′); 4.84 (bm, 1H, H-3′); 6.48 (dd, 1H, $J_{1'2'}$ = 7.6, 7.0, H-1′); 8.19 (s, 1H, H-2). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref$_{H3PO4}$ = 0 ppm, pH = 7.1): –21.28 (dd, J = 19.4, 19.2, P_β); –10.32 (d, J = 19.2, P_α); –6.73 (d, J = 19.4, P_γ). 8-Ph-dATP: yield 65%. MS(ESI–): 566.4 (25, M – 1), 486.4 (100, M – PO_3H_2 – 1), 588.4 (25, M + Na), HRMS: for $C_{16}H_{19}N_5O_{12}P_3$ calculated 566.0243, found 566.0245. ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH = 7.1): 2.23 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 7.2, $J_{2'b,3'}$ = 4.1, H-2′b); 3.26 (dt, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 7.5, H-2′a); 4.12–4.22 (m, 2H, H-4′ and H-5′b); 4.29 (dt, 1H, J_{gem} = 10.5, $J_{5'a,4'}$ = $J_{H,P}$ = 4.0, H-5′a); 4.64 (ddd, 1H, $J_{3',2'}$ = 7.5, 4.1, $J_{3',4'}$ = 3.8, H-3′); 6.33 (dd, 1H, $J_{1'2'}$ = 7.5, 7.2, H-1′); 7.57–7.66 (m, 3H, H-*m*,*p*-Ph); 7.69 (m, 2H, H-*o*-Ph); 8.26 (s, 1H, H-2). ^{31}P (^{1}H dec.) NMR (202.3 MHz, D_2O, ref$_{H3PO4}$ = 0 ppm, pH = 7.1): –21.41 (dd, J = 19.1, 17.9, P_β); –10.22 (d, J = 19.1, P_α); –7.46 (d, J = 17.9, P_γ).

§ **PEX:** the reaction mixture (20 µl) contained a polymerase (0.1 unit), 8-modified dATP (1 mM), dTTP, dCTP and dGTP (0.1 mM), primer (0.15 µM), template (0.225 µM) in polymerase reaction buffer supplied by the manufacturer. Primer was labeled by use of [γ^{32}P]-ATP. The mixtures were incubated for 30 min at 37 °C (Klenow(exo-)) or 60 °C (other polymerases) in a thermal cycler and were stopped by addition of stop solution (40 µL, 80% [v/v] formamide, 20 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol). Reaction mixtures were separated by use of a 12.5% denaturing PAGE. Visualization was performed by phosphoimaging.

¶ T_m**:** for preparative purposes, a total volume of 500 µl PEX using higher concentrations of primer (2 µM) and template (2 µM) was run and purification was carried out with a QIAquick Nucleotide Removal Kit (Qiagen). Samples were eluted with 100 µl H_2O (pH 7.5) and then freeze-dried. DNA duplexes were first dissolved in 160 µl of phosphate buffer (10 mM) and 1 M NaCl (pH 7) and further diluted with the buffer to optimum concentration – OD_{260} between 0.08 and 0.1. Thermal denaturation studies were performed on a Cary 100 Bio (UV–Visible spectrometer with temperature controller, Varian). Data were obtained from six individual cooling–heating cycles. Melting temperatures (T_m values in °C) were obtained by plotting temperature *versus* absorbance and by applying a sigmoidal curve fit.

‖ **Preparation of samples for MALDI-TOF analysis:** PEX on a large scale followed by magnetoseparation.[16] For desalting of samples, we used the ZipTip technique.[22]

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