# Chemical Morphing of DNA Containing Four Noncanonical Bases

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***Abstract:*** *The ability of alternative nucleic acids, in which all four nucleobases are substituted, to replicate in vitro and to serve as genetic templates in vivo was evaluated. A nucleotide triphosphate set of 5-chloro-2′-deoxyuridine, 7-deaza-2′-deoxyadenosine, 5-fluoro-2′-deoxycytidine, and 7-deaza-2′deoxy-guanosine successfully underwent polymerase chain reaction (PCR) amplification using templates of different lengths (57 or 525mer) and Taq or Vent (exo-) DNA polymerases as catalysts. Furthermore, a fully morphed gene encoding a dihydrofolate reductase was generated by PCR using these fully substituted nucleotides and was shown to transform and confer trimetho-prim resistance to E. coli. These results demonstrated that fully modified templates were accurately read by the bacterial replication machinery and provide the first example of a long fully modified DNA molecule being functional in vivo.*

Nucleic acids and their nucleoside monomers have been the subject of chemical diversification since the discovery of their structure,[1] primarily for the development of new drugs.[2] Another motivation for exploring chemical variants of nucleic acids has been the elaboration of possible scenarios for the origin of life.[3] More recently the chemical diversification of nucleic acids has been attempted in vivo to propagate additional types of nucleic acids (XNA) as templates for DNA synthesis in *E. coli*[4,5] and for transcription into RNA in mammalian cells.[6] In addition, DNA with a triazole linker instead of a phosphodiester bound have been accepted by *E. coli*[7a,b] and human[7c] cells. It has also been reported that a semisynthetic organism is able to replicate with a single unnatural base pair in vivo.[8] Although these studies show that cellular machinery is able to tolerate variants of nucleic acids, to date there have been no examples of the fully substituted DNA sequences that successfully conveyed genetic information in living organisms. Moreover, only a few examples of successful polymerase chain reaction (PCR) amplification of fully modified DNA have been demonstrated, where all four natural deoxynucleotides were replaced with its 4′-thio,[9] phosphorothioate,[10] or nucleobase analogues.[11]

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Fully morphed DNA is worth investigating as it could bring numerous scientific and technological advantages. The functional scope of aptamers and deoxyribozymes could be widened by decorating DNA monomers.[12] Restriction sites could be masked by base substitution[13] allowing efficient construction of plasmid vectors and its transformation into bacterial and mammalian cells. If such replacements could be conducted in vivo, it could enable the evolution of chemically redesigned cells and safe genetically modified organisms (GMOs) requiring unnatural nutrients.

Automated selection techniques have been previously shown to replace the full genomic content of thymine by 5-chlorouracil (5-ClU) in an *E.coli* population.[14] In the present work, we investigated whether bases other than thymine could also be substituted. Thus we studied a chemically redesigned DNA molecule with 5-substituted pyrimidines and 7-deazapurines (Figure 1), which we call "DZA", on its ability to be amplified in vitro and to serve as a template for introducing an antibiotic resistance gene in *E.coli*.
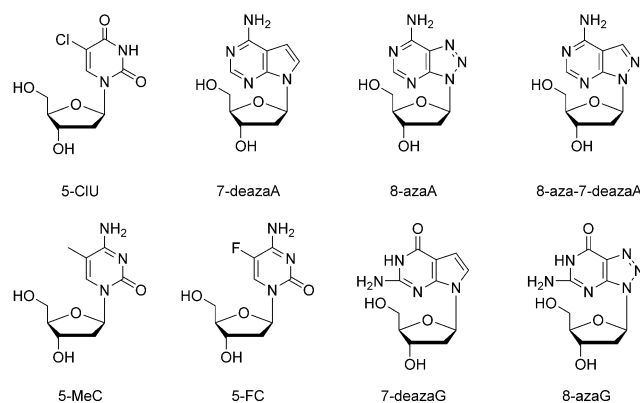


***Figure 1.*** Chemical structures of the investigated noncanonical nucleosides.

First, the 5′ triphosphodeoxyribosides of 7-deaza-, 8-aza-, and 8-aza-7-deaza-2′-deoxyadenosine (denoted 7-deazaA, 8-azaA, and 8-aza-7-deazaA, respectively) were prepared according to literature procedures (see the Supporting Information for details) and were assayed in a PCR of a short 57mer template, with or without the 5′ triphospho-deoxyribose of 5-ClU (see Figure S1 in the Supporting Information). Both 7-deazaA and 8-azaA showed sufficient yield of PCR products with the other three natural triphosphates (dT, dC, and dG; Figure S1b,c), while only 7-deazaA led to vigorous amplification pairing with 5-ClU compared to the other candidates, as judged from PCR using Vent (*exo-*) DNA polymerases (Figure S1d). This observation corresponded to previously obtained data that 8-substituted

dATPs were poor substrates for DNA polymerases.[13a,15] At this stage, we chose one of the two DNA base pairs to be morphed into the chemically distant pairing partners, 5-Cl-U:7-deazaA.

The next candidates for base-pair replacement were cytosine together with guanine. Focusing on the 5-methyl and 5-fluoro substitutions of cytosine (5-MeC and 5-FC, respectively) and 7-deaza- or 8-aza-2′-deoxyguanine (7-deazaG and 8-azaG, respectively) as guanine congeners (Figure 1), we performed PCR tests with the corresponding 5′-triphosphodeoxyribosides. All candidates were found to be incorporated together with 5-ClU and 7-deazaA in amplification assays catalyzed by Taq DNA polymerase with the 57mer DNA template (Figure S2). It should be mentioned that PCR products containing 7-deazaG stain poorly when using ethidium bromide as the dye and thus they have decreased band intensities in agarose gels compared to other samples.[13d,e] To resolve this problem, we performed amplification with both fluorescently labeled primers. The data presented in Figure S2c show slightly increased formation of both Cy3 and Cy5 labeled products during PCR cycles. This result further illustrates the ability of Taq DNA polymerase to recognize not only the initial natural DNA template, but also the Cy3-labeled newborn DZA sequence as a template for in vitro replication.

Next, we performed PCR amplification of longer DNA, up to 525 base pairs, using a pET-3a-d vector as the template and Taq or Vent (*exo-*) DNA polymerases as the catalyst (see Figure S3a). Different combinations of the canonical and noncanonical triphosphates have been examined using PCR. Only combinations of 7-deazaG with 5-MeC or 5-FC and 7-deazaA with 5-ClU-modified triphosphates yielded successful amplification with Taq polymerase, whereas PCR containing 8-azaA or 8-aza-7deaza-A and 8-azaG triphosphates did not lead to any product with 5-ClU and 5-FC or 5-MeC, neither with Taq nor Vent (*exo-*) polymerases (Figure S3a). Therefore, for subsequent studies we chose 7-deazaG as an alternative to guanosine and Taq polymerase as the catalyst. Both 5-FC and 5-MeC were found to be good candidates. However, we chose to focus on 5-FC since it is more chemically artificial than 5-MeC (5-MeC is found in natural DNA as a product of postreplicative and epigenetic modifications).[16]

We also carried out restriction enzyme cleavage assays of the resulting 525 base pair amplicons containing either only natural nucleotides, 5-ClU:7-deazaA with canonical G and C nucleotides, or all noncanonical nucleotides (Figure S3b) using six different restriction enzymes. Fully modified fragments showed complete protection even after 24 hours of incubation with all chosen restriction enzymes (Table S1). Similar results have been observed[13,17] where it was shown that the presence of 7-deazapurines or 5-substituted pyrimidines in DNA prevents the restriction enzyme cleavage. These results may be useful for creating unique restriction sites in genes of interest using DZA-containing motifs.[17b]

The successful in vitro production of DZA sequences fully modified by four noncanonical bases was thus established and shown to be applicable to different amplification templates. The results of all enzymatic studies are summarized in Table S2. We then turned to studying genetic transformation in vivo using such chemically morphed amplicons.

We exploited the bacterium *E. coli* and its sensitivity toward the antibiotic trimethoprim (Tmp). This compound inhibits the enzyme dihydrofolate reductase (DHFR), encoded by the chromosomal gene *folA*. The toxicity of Tmp cannot be alleviated by spontaneous mutation at a concentration of 150 μM.[18] The DHFR gene from the R67 resistance plasmid (R67 DHFR) encodes an alternative version of dihydrofolate reductase (type II) that is structurally and phylogenetically unrelated to the *folA* gene product and is totally resistant to trimethoprim inhibition.[18] The R67 DHFR gene is quite small, containing only 78 codons, making it a good candidate for in vivo tests (Figure S5).[19]

First, we performed PCR amplification and agarose gel purification of the R67 DHFR with R67 primers leading to 237 bp-long duplexes (Figures 2a; bp = base pair), part of which were nuclease-digested, dephosphorylated, and analyzed by HPLC–MS to confirm the nucleotide composition of the obtained synthetic R67 DHFR genes (Figure 2b–d; Figures S6, S7). A HPLC–MS assay confirmed the presence of all four modified nucleosides in a ratio in good agreement with calculations, although it was not possible to distinguish peak areas corresponding to 5-ClU and thymidine as they overlap (Table S3).

The DZA genes were then ligated to the pJET1.2 ampicillin-resistant vector by T4 DNA ligase (Figure S8) and transformed in *E. coli*, following by growth on Luria–Bertani (LB) agar plates either with ampicillin only (AmpR) or ampicillin together with 50 μg mL⁻¹ of the trimethoprim antibiotic (AmpR + TmpR; Scheme 1). The viable colonies
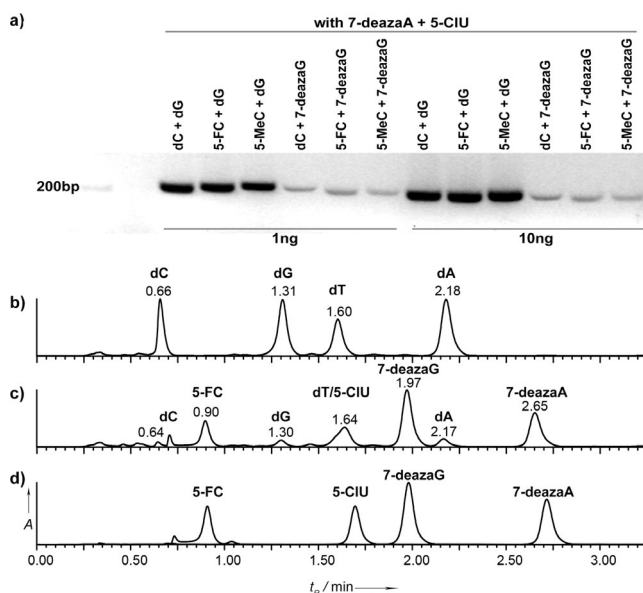


*Figure 2.* In vitro experiments with the synthetic R67 DHFR gene. a) PCR amplification of the R67 DHFR gene in the presence of 200 μM natural or modified triphosphates, 25U ml⁻¹ Taq DNA polymerase, and 1 or 10 ng of the pXEN156 plasmid template. b–c) HPLC chromatograms of deoxynucleosides from digested DNA (b) or fully substituted DZA R67 DHFR genes (c). d) Modified nucleoside standards. All HPLC chromatograms can be found in Figure S6.
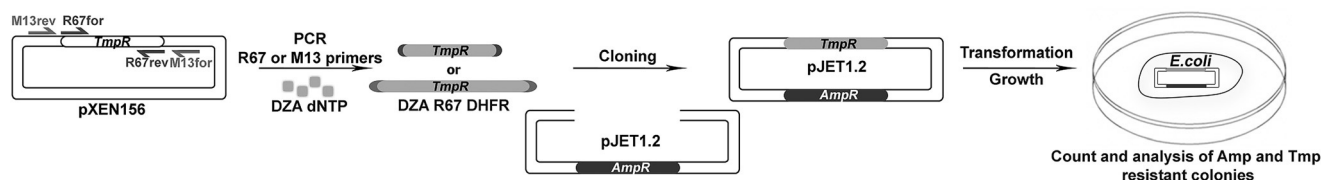
**Scheme 1.** The general method employed for the in vivo studies. We first synthesized the morphed R67 DHFR genes conferring trimethoprim resistance (*TmpR*) by PCR with R67 or M13 primers. The genes were cloned into a pJET1.2 plasmid with ampicillin resistance (*AmpR*), followed by transformation into TG1 *E.coli*. The samples were grown in LB or MH media with ampicillin only and ampicillin with trimethoprim antibiotics. For further details see Figure S10.

from both types of plates were counted and analyzed (Table 1 A; Figure S9). All sample cultures produced colonies, but with different efficiencies. The incorporation of purine-modified nucleotides (7-deazaA and 7-deazaG) led to a clearer decrease in the total number of colonies than the incorporation of pyrimidine-modified nucleotides (5-ClU or 5-FC). Apparently, the difference in colony numbers is associated with variations in the cloning efficiency and also the recognition ability of DZA containing fragments by natural enzymes.

It should be noticed that after transformation in *E.coli*, the DZA-containing plasmid produce natural DNA clones which were used for subsequent analysis. Sequencing of plasmids found in resistant transformants showed that mainly transition mutations (A:T→G:C, 91.2% of all mutations

within all samples; see Table S4 and Table 2) had been accumulated through PCR by Taq DNA polymerase and were less likely through plasmid replication by host DNA polymerases. Most mutations led to amino acid substitution with

**Table 2:** Nucleobase and amino acid substitution in the synthetic R67 DHFR genes. Cells were grown in A) LB media or B) MH media.[a]

|   | Sample | | No. of colonies | No. of mutants | Mutation[b] | Gene position |
|---|--------|---|---|---|---|---|
| A) | DNA | | 36 | 2 | T→C | 48 |
|   |   |   |   |   | T→C | 212 |
|   | DZA | | 42 | 9 | A→G | 33 |
|   |   |   |   |   | T→C | 39 |
|   |   |   |   |   | T→C | 45 |
|   |   |   |   |   | T→C | 50 |
|   |   |   |   |   | A→G | 67 |
|   |   |   |   |   | A→G | 81 |
|   |   |   |   |   | A→G | 122 |
|   |   |   |   |   | T→C | 177 |
|   |   |   |   |   | T→C*2 | 213 |
| B) | R67 | DNA | 22 | 1 | C→T | 24 |
|   |   | DZA | 9 | 1 | G→T | 123 |
|   | M13 | DNA | 10 | 1 | A→T | 232 |
|   |   | DZA | 4 | 4 | G→A | 90 |

[a] The entire tables of sequencing data can be found in Table S4 and S5. [b] The type of mutation is given with respect to the plus DNA strand.

**Table 1:** Number of colonies formed after transformation and cell growth in A) LB media or B) MH media.[a]

|   | Sample | | No. of colonies | | Ratio (AmpR+TmpR): (AmpR×10⁻²) |
|---|--------|---|---|---|---|
|   |   |   | AmpR | AmpR+ TmpR | |
| A) | DNA[b] | | 2704 | 171 | 6.3 |
|   | 5-ClU | | 3077 | 177 | 5.8 |
|   | 7-deazaA | | 1087 | 35 | 3.2 |
|   | (7-deazaA) + (5-ClU) | | 493 | 24 | 4.8 |
|   | 5-FC | | 3227 | 171 | 5.3 |
|   | 7-deazaG | | 613 | 27 | 4.4 |
|   | (7-deazaG) + (5-FC) | | 650 | 26 | 3.9 |
|   | (7-deazaA) + (5-ClU) + (5-FC) | | 1690 | 56 | 3.3 |
|   | Fully modified (DZA) | | 118 | 4 | 3.0 |
|   | (7-deazaA) + (5-ClU) + (7-deazaG) | | 123 | 2 | 1.3 |
|   | C1 (pUC19) | | 81 | 0 | – |
|   | C2 (without plasmid) | | 0 | 0 | – |
| B) | R67 | DNA | 490 | 19.8 | 4.0 |
|   |   | DZA | 45 | 0.5 | 1.1 |
|   |   | NC | 144 | 0 | 0 |
|   | M13 | DNA | 1070 | 8.1 | 0.8 |
|   |   | DZA | 54 | 0.1 | 0.2 |
|   |   | NC | 226 | 0 | 0 |
|   | C1 (pUC19) | | 53 | 0 | – |
|   | C2 (without plasmid) | | 0 | 0 | – |

[a] Normalized number of colonies shown as a mean. C1-pUC19 transformant carrying AmpR only and C2-nontransformed cells. The R67 primer was used in the PCR in (A) and in B) DNA or DZA fragments were PCR synthesized with R67 or M13 primers containing signatures. NC is the control PCR sample performed without Taq DNA polymerase. [b] Unmodified system (i.e. DNA) employed.

the same entity, but about 25% resulted in a different type of amino acid. It is interesting that among the sequenced samples, the incorporation of 5-FC or 7-deazaG into the gene sequence did not cause any nucleotide substitution (Table S4), while the incorporation of 7-deazaA caused the highest mutation frequency. Despite the accumulation of mutations during amplification, the DZA fragments could still be read by the bacterial machinery to yield the correct DNA and mRNA followed by the synthesis of functional R67 DHFR proteins, thus the DZA transfer scheme is sufficiently accurate.

To eliminate the influence of thymidine, the cause of trimethoprim inhibition,[20] we next performed the same experiments as described above in Mueller–Hinton (MH) agar plates instead of LB agar. MH media has minimal thymine and thymidine content, thus markedly reducing the inactivation of trimethoprim. We also examined the effect of elongated DZA inserts on the bacterial DNA polymerase recognition ability using M13 primers lying outside the gene sequence (Scheme 1; Figure S10). The 241 bp or 360 bp DNA

or DZA fragments were synthesized using PCR with Taq DNA polymerase and R67 or M13 primer sets containing additional two-base mutations at the 5′-end different from the original pXEN156 sequence. These mutations have been used as signatures to identify and confirm the synthetic nature of DNA or DZA inserts. The resultant PCR products were digested by the Dpn I restriction enzyme to remove the initial pXEN156 from the samples.

All samples produced some colonies with different efficiencies (see Table 1B), while negative controls (denoted NC, involving PCR with all components but without Taq DNA polymerase) had no colonies in MH agar plates with trimethoprim. Moreover, in all experiments, control plates containing Tmp either with nontransformed cells (C2) or with only ampicillin-resistant transformant (C1; cells with the pUC19 plasmid), did not give any colonies even after 48 hours of incubation (Table 1).

Colonies from MH agar plates were also analyzed and the trimethoprim marker gene together with appropriate 5′-end signatures were found in each sequenced sample (Table S5). These results confirm that the colonies obtained expressed functional protein conferring resistance to Tmp, and that these colonies were not the result of trimethoprim suppression, media contamination, or the presence of the parent pXEN156 template.

To summarize, our results demonstrate the potential of fully morphed sequences to replicate in vitro with sufficient yield. Such DZA libraries can produce functional aptamers and DNAzymes with improving target affinities[12] and can be used for the delivery of prodrugs into cells.[21] Simple ligation by T4 DNA ligase allows direct cloning of DZA inserts without the reverse transcription step, which further simplifies in vitro selection of functional sequences. We also showed that DZA fragments can efficiently block restriction sites from cleavage that can be useful for programmable vector construction. Moreover, we demonstrated that it is possible to introduce DZA motifs into living organisms as genetic messengers bearing new properties by incorporation into *E. coli*. The next attempts to explore DZA sequences in vivo as a safe genetic material should include diversification of the 5-position of pyrimidines and the 7-position of purines as well as the engineering of cells able to selectively accept or intercellularly biosynthesize the artificial triphosphates.

[1] J. D. Watson, F. H. C. Crick, *Nature* **1953**, *171*, 964–967.

[2] a) J. B. Opalinska, A. M. Gewirtz, *Nat. Rev. Drug Discovery* **2002**, *1*, 503–514; b) L. P. Jordheim, D. Durantel, F. Zoulim, C. Dumontet, *Nat. Rev. Drug Discovery* **2013**, *12*, 447–464.

[3] a) L. E. Orgel, *The Origins of Life: Molecules and Natural Selection*, Wiley, New York, NY, **1973**; b) M. W. Powner, B. Gerland, J. D. Sutherland, *Nature* **2009**, *459*, 239–242.

[4] a) V. Pezo, F. W. Liu, M. Abramov, M. Froeyen, P. Herdewijn, P. Marlière, *Angew. Chem. Int. Ed.* **2013**, *52*, 8139–8143; *Angew. Chem.* **2013**, *125*, 8297–8301; b) V. Pezo, G. Schepers, C. Lambertucci, P. Marlière, P. Herdewijn, *ChemBioChem* **2014**, *15*, 2255–2258.

[5] a) T. W. Kim, J. C. Delaney, J. M. Essigmann, E. T. Kool, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 15803–15808; b) J. C. Delaney, J. Gao, H. Liu, N. Shrivastav, J. M. Essigmann, E. T. Kool, *Angew. Chem. Int. Ed.* **2009**, *48*, 4524–4527; *Angew. Chem.* **2009**, *121*, 4594–4597; c) J. Chelliserrykattil, H. Lu, A. H. F. Lee, E. T. Kool, *ChemBioChem* **2008**, *9*, 2976–2980; d) A. T. Krueger, L. W. Peterson, J. Chelliserry, D. J. Kleinbaum, E. T. Kool, *J. Am. Chem. Soc.* **2011**, *133*, 18447–18451.

[6] H. Maruyama, K. Furukawa, H. Kamiya, N. Minakawa, A. Matsuda, *Chem. Commun.* **2015**, *51*, 7887–7890.

[7] a) A. H. El-Sagheer, A. P. Sanzone, R. Gao, A. Tavassoli, T. Brown, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 11338–11343; b) A. P. Sanzone, A. H. El-Sagheer, T. Brown, A. Tavassoli, *Nucleic Acids Res.* **2012**, *40*, 10567–10575; c) C. N. Birts, A. P. Sanzone, A. H. El-Sagheer, J. P. Blaydes, T. Brown, A. Tavassoli, *Angew. Chem. Int. Ed.* **2014**, *53*, 2362–2365; *Angew. Chem.* **2014**, *126*, 2394–2397.

[8] D. A. Malyshev, K. Dhami, T. Lavergne, T. Chen, N. Dai, J. M. Foster, I. R. Corrêa, F. E. Romesberg, *Nature* **2014**, *509*, 385–388.

[9] T. Kojima, K. Furukawa, H. Maruyama, N. Inoue, N. Tarashima, A. Matsuda, N. Minakawa, *ACS Synth. Biol.* **2013**, *2*, 529–536.

[10] a) M. Andreola, C. Calmels, J. Michel, J.-J. Toulmé, S. Litvak, *Eur. J. Biochem.* **2000**, *267*, 5032–5040; b) F. J. Ghadessy, N. Ramsay, F. Boudsocq, D. Loakes, A. Brown, S. Iwai, A. Vaisman, R. Woodgate, P. Holliger, *Nat. Biotechnol.* **2004**, *22*, 755–759.

[11] a) S. Jäger, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, *J. Am. Chem. Soc.* **2005**, *127*, 15071–15082; b) S. Jäger, M. Famulok, *Angew. Chem. Int. Ed.* **2004**, *43*, 3337–3340; *Angew. Chem.* **2004**, *116*, 3399–3403.

[12] a) M. Kimoto, R. Yamashige, K. Matsunaga, S. Yokoyama, I. Hirao, *Nat. Biotechnol.* **2013**, *31*, 453–457; b) J. D. Vaught, C. Bock, J. Carter, T. Fitzwater, M. Otis, D. Schneider, J. Rolando, S. Waugh, S. K. Wilcox, B. E. Eaton, *J. Am. Chem. Soc.* **2010**, *132*, 4141–4151; c) L. Gold, D. Ayers, J. Bertino, C. Bock, A. Bock, E. N. Brody, J. Carter, A. B. Dalby, B. E. Eaton, T. Fitzwater, et al., *PLoS One* **2010**, *5*, e15004; d) L. Zhang, Z. Yang, K. Sefah, K. M. Bradley, S. Hoshika, M.-J. Kim, H.-J. Kim, G. Zhu, E. Jimenez, S. Cansiz, et al., *J. Am. Chem. Soc.* **2015**, *137*, 6734–6737; e) M. Hollenstein, C. J. Hipolito, C. H. Lam, D. M. Perrin, *ChemBioChem* **2009**, *10*, 1988–1992.

[13] a) H. Macíčková-Cahovthoma, M. Hocek, *Nucleic Acids Res.* **2009**, *37*, 7612–7622; b) H. Macíčková-Cahová, R. Pohl, M. Hocek, *ChemBioChem* **2011**, *12*, 431–438; c) P. Kielkowski, N. L. Brock, J. S. Dickschat, M. Hocek, *ChemBioChem* **2013**, *14*, 801–804; d) M. Mačková, S. Boháčová, P. Perlíková, L. Poštová Slavětínská, M. Hocek, *ChemBioChem* **2015**, *16*, 2225–2236; e) F. Seela, A. Röling, *Nucleic Acids Res.* **1992**, *20*, 55–61.

[14] P. Marlière, J. Patrouix, V. Döring, P. Herdewijn, S. Tricot, S. Cruveiller, M. Bouzon, R. Mutzel, *Angew. Chem. Int. Ed.* **2011**, *50*, 7109–7114; *Angew. Chem.* **2011**, *123*, 7247–7252.

[15] H. Cahová, R. Pohl, L. Bednárová, K. Nováková, J. Cvacka, M. Hocek, *Org. Biomol. Chem.* **2008**, *6*, 3657–3660.

[16] J. A. Law, S. E. Jacobsen, *Nat. Rev. Genet.* **2010**, *11*, 204–220.

[17] a) V. Valinluck, W. Wu, P. Liu, J. W. Neidigh, L. C. Sowers, *Chem. Res. Toxicol.* **2006**, *19*, 556–562; b) W. H. Ang, S. J. Lippard, *Chem. Commun.* **2009**, 5820–5822; c) S. K. Grime, R. L. Martin, B. L. Holaway, *Nucleic Acids Res.* **1991**, *19*, 2791; d) T. Gourlain, A. Sidorov, N. Mignet, S. J. Thorpe, S. E. Lee, J. A. Grasby, D. M. Williams, *Nucleic Acids Res.* **2001**, *29*, 1898–1905.

[18] E. E. Howell, *ChemBioChem* **2005**, *6*, 590–600.

[19] N. Brisson, T. Hohn, *Gene* **1984**, *28*, 271–275.

[20] A. E. Koch, J. J. Burchall, *Appl. Microbiol.* **1971**, *22*, 812–817.

[21] a) S. Kruspe, U. Hahn, *Angew. Chem. Int. Ed.* **2014**, *53*, 10541–10544; *Angew. Chem.* **2014**, *126*, 10711–10715; b) M. E. Drew, J. C. Morris, Z. Wang, L. Wells, M. Sanchez, S. M. Landfear, P. T. Englund, *J. Biol. Chem.* **2003**, *278*, 46596–46600.