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Base pairing involving artificial bases *in vitro* and *in vivo*†

Omprakash Bande,‡[b] Darren Braddick,‡[a] Stefano Agnello,‡[b] Miyeon Jang,[b] Valérie Pezo,[a] Guy Schepers,[b] Jef Rozenski,[b] Eveline Lescrinier,[b] Philippe Marlière[a] and Piet Herdewijn*[ab]

Herein we report the synthesis of N^8-glycosylated 8-aza-deoxyguanosine (N^8-8-aza-dG) and 8-aza-9-deaza-deoxyguanosine (N^8-8-aza-9-deaza-dG) nucleotides and their base pairing properties with 5-methyl-isocytosine (d-isoC^{Me}), 8-amino-deoxyinosine (8-NH$_2$-dI), 1-*N*-methyl-8-amino-deoxyinosine (1-Me-8-NH$_2$-dI), 7,8-dihydro-8-oxo-deoxyinosine (8-Oxo-dI), 7,8-dihydro-8-oxo-deoxyadenosine (8-Oxo-dA), and 7,8-dihydro-8-oxo-deoxyguanosine (8-Oxo-dG), in comparison with the d-isoC^{Me}:d-isoG artificial genetic system. As demonstrated by T_m measurements, the N^8-8-aza-dG:d-isoC^{Me} base pair formed less stable duplexes as the C:G and d-isoC^{Me}:d-isoG pairs. Incorporation of 8-NH$_2$-dI *versus* the N^8-8-aza-dG nucleoside resulted in a greater reduction in T_m stability, compared to d-isoC^{Me}:d-isoG. Insertion of the methyl group at the N^1 position of 8-NH$_2$-dI did not affect duplex stability with N^8-8-aza-dG, thus suggesting that the base paring takes place through Hoogsteen base pairing. The cellular interpretation of the nucleosides was studied, whereby a lack of recognition or mispairing of the incorporated nucleotides with the canonical DNA bases indicated the extent of orthogonality *in vivo*. The most biologically orthogonal nucleosides identified included the 8-amino-deoxyinosines (1-Me-8-NH$_2$-dI and 8-NH$_2$-dI) and N^8-8-aza-9-deaza-dG. The 8-oxo modifications mimic oxidative damage ahead of cancer development, and the impact of the MutM mediated recognition of these 8-oxo-deoxynucleosides was studied, finding no significant impact in their *in vivo* assay.

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Introduction

Non-canonical base pairing has been studied by several research groups,[1–6] based on their interest to expand the genetic alphabet. A major breakthrough in this field has been reported by Malyshev *et al.*,[7] showing replication of a synthetic hydrophobic base pair (d5SICS–dNAM) *in vivo*. This base pair system consists of *C*-nucleosides that are metabolically not easily accessible, where recognition is based on hydrophobic interactions, and no hydrogen-bond interactions (such as in canonical base pairing) are involved. Recently, we have demonstrated that a change in the backbone (HNA replacing DNA) could lead to a reduced recognition of non-canonical bases (*i.e.* isoC^{Me} and isoG) by natural bases,[8] further moving in the direction of a fully orthogonal information system. Here we have investigated new base pair systems from which one of the partners is an N^8 substituted purine base and involving hydrogen-bond formation in the recognition process.

The unnatural isoG:isoC^{Me} base pair[9,10] is not adequate to expand the genetic code and has restricted application due to facile tautomerization of isoG which enables it to form base pairs with selected natural nucleobases. Tautomerism leads to promiscuity and heterocycles (*via* tautomerism) possibly leading to pairing with natural bases, should be avoided when selecting new orthogonal information systems.[1] Therefore, we would like to replace the isoG base in the isoG:isoC^{Me} base pair by another heterocycle less prone to tautomerization. In search for new artificial genetic systems efforts have been devoted to the study of *C*-nucleosides[11–13] and nucleosides with an aberrant glycosidic linkage *i.e.* with an N^8 glycosylated nucleobase,[14–17] as they demonstrate unexpected pairing behavior and some of them may function as universal bases.[18,19] Seela *et al.*, have demonstrated that altering the glycosylation site of a nucleoside from N^9 to N^8 results in a change of the nucleobase recognition pattern, N^8-glycosylated-7-deaza-8-azaguanine behaves like isoguanine[20] and forms base pairs with isoC^{Me} and not with cytosine. Therefore, we have investigated N^8-glycosylated 8-azaguanine[21–23] **1a** together with its N^9-deaza-analogue **1b**. Removal of the N^9 nitrogen atom (giving **1b**) may have an influence on the *in vitro* and *in vivo* recognition of the

[a]*iSSB – CNRS FRE3561, University of Evry-Val-d'Essonne, 5 rue Henri Desbruères, Genopole Campus 1, Bât. 6, F-91030 Évry Cedex, France*

[b]*Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium. E-mail: piet.herdewijn@rega.kuleuven.be; Tel: +32 16 337387*

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‡ Contributed equally to this article.

N^8-glycosylated purine nucleoside. Potentially, the N^8-glycosylated purine nucleoside may be recognized by another purine nucleoside, when Hoogsteen base pairing is involved. To evaluate the potential of involving such Hoogsteen recognition[24] in an unnatural base pair, 8-amino-hypoxanthine[25,26] **2a** was selected as the pairing partner for the N^8-glycosylated bases (N^8-8-aza-dG, N^8-8-aza-9-deaza-dG) as shown in Fig. 1. For Hoogsteen pairing in a duplex structure, one of the bases should adopt the '*syn*' conformation and 8-substituted purines preferentially adopt this conformation.[27,28] The 1-Me-8-NH_2-dI nucleobase **2b** was tested in order to exclude Watson–Crick type recognition in **1a**/**1b**:**2a** base pairing. Changing the 8-amino group in **2a** in an 8-hydroxy group will, first, abolish recognition by **1a** and **1b**, which would be further demolished by changing the *aad* system of **2** in the *dda* system of **5** (the *dda* system is also present in **1a** and **1b**) (Fig. 2). Therefore, and to fully understand the base pairing pattern, we have also synthesized and evaluated the 7,8-dihydroxy-8-oxonucleosides with the 6-Oxo[29] **3**, 6-Oxo-2-amino[30–34] **4** and 6-amino[35–38] **5** base moieties. Because of the favored *syn* conformation of 8-hydroxylated purine, Hoogsteen base pairing may be preferred over Watson–Crick base pairing.[39–41] A conclusion is that Watson–Crick type base pairing as well as Hoogsteen type base pairing can be considered when selecting orthogonal base pairs *in vitro* and *in vivo*.

In order to determine the orthogonality of the nucleosides against the biological situation, they were individually analyzed *in vivo* in an XNA-dependent DNA synthesis assay. For these nucleosides the assay studied the pairing properties of each, with an extension of the study performed for the 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine bases *in vivo*, the former of which is formed by ROS-mediated damage of DNA.[42,43] Both of the 8-oxo-deoxynucleoside bases were analyzed in wild-type and knockout backgrounds of the important 8-oxo repair enzyme MutM.[44,45] The assay was to determine whether or not this enzyme in particular was influencing the base pairing results determined by tests, and confirm that the non-natural nucleosides here were not being chemically modified *in vivo*, thus affecting the results obtained.

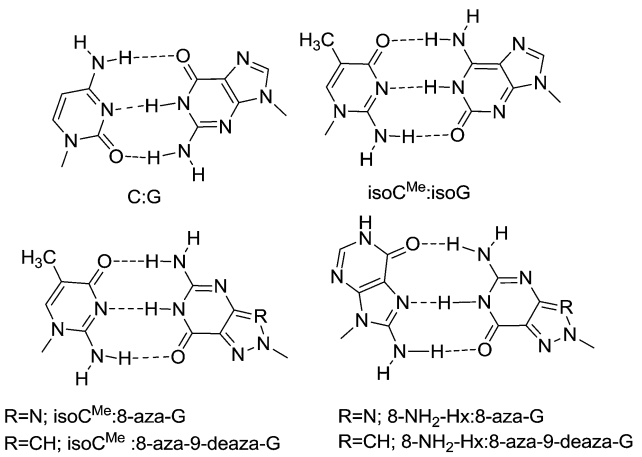


Fig. 1 Putative base pair motifs of duplexes with antiparallel strand orientation.

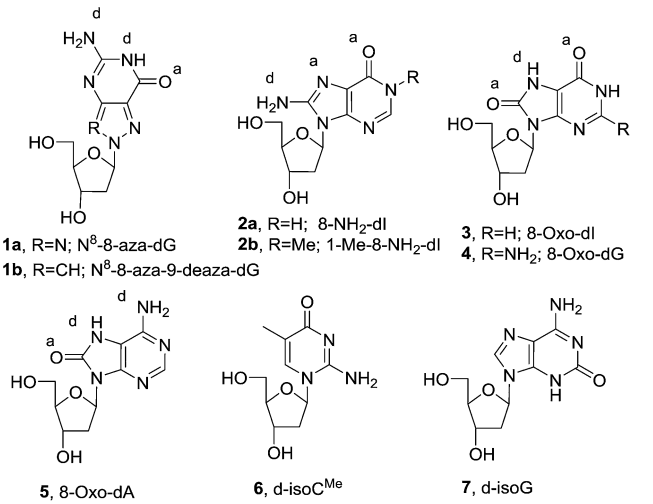
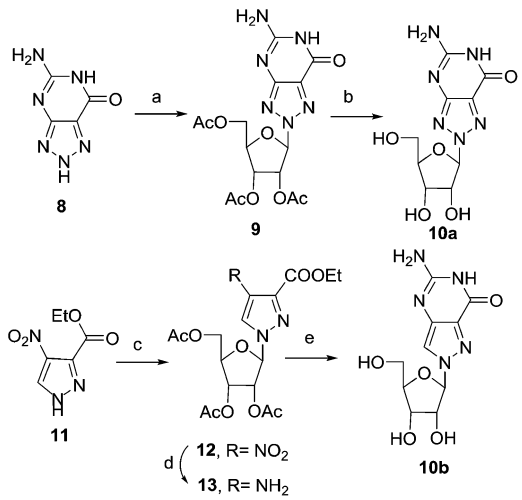


Fig. 2 2′-Deoxyribo-nucleosides, with modified purine and pyrimidine bases.

Results and discussion

Synthesis of monomers

As depicted in Scheme 1, the BF_3–Et_2O-catalysed glycosylation of commercially available 8-azaguanine (**8**) with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose afforded **9** as the major isomer. The assignment of N^8 glycosidic bond in **9** was made by comparing spectral data with the known compound **1a** in later stage. The cleavage of the acetyl-protecting group was carried out using ammonium hydroxide at room temperature for 8 hours, obtaining compound **10a** in 91% yield.[46,47] The regioselective protection of the 3′-OH and 5′-OH groups with $TIPDSiCl_2$ gave compound **14a**.



Scheme 1 Synthesis of N^8-8-aza-G and N^8-8-aza-9-deaza-G nucleoside. Reagents and conditions: (a) BF_3–Et_2O, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, CH_3CN, 75 °C, 3 h, 54%; (b) NH_4OH, rt, 8 h, 91%; (c) BSA, DCE, $SnCl_4$, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, rt, 36 h, 85%; (d) H_2, 10% Pd/C, MeOH, rt, 8 h, 100%; (e) (i) chloro-formamidine hydrochloride, DMS, 120 °C, 1 h, (ii) NH_4OH, rt, 1 h, 42%.

For the deoxygenation of the C2′-hydroxy group, the Barton–McCombie reaction was used. We first converted the nucleoside **14a** into the corresponding thiocarbamate, which, after radical reduction in the presence of tributylstannane and AIBN, gave the desired nucleoside **15a** (Scheme 2).

Subsequently, the removal of the silyl-protecting group, carried out using TBAF in THF at room temperature for 4 hours, afforded compound **1a** to 94% yield. The spectral and analytical data of **1a** were found to be in accordance with reported data.[21] Protection of the amino group of **1a** followed by the selective protection of the primary alcohol with 4,4′-dimethoxytrityl chloride (DMTrCl) in pyridine, provided the protected nucleoside **17a**,[21] which was then converted into phosphoramidite **18a** by reaction with bis(diisopropylamino)(2-cyanoethoxy)phosphine in the presence of tetrazole.

The synthesis of the N^8-8-aza-9-deaza-dG derivative started from the readily available pyrazole **11**. Vorbrüggen reaction of **11** with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, in the presence of $SnCl_4$, afforded the desired N^1-isomer **12** as the major product (91% yield).[48] The reduction of the nitro group of **12**, followed by the ring closure with chloroformamidine hydrochloride and deprotection of acetyl groups in one pot, gave the desired compound **10b** (Scheme 1). Analogously to what is described for the N^8-8-aza-guanine derivatives in Scheme 2, a series of transformations starting from **10b** allowed us to obtain the deoxy derivative **1b** and finally the corresponding phosphoramidite **18b**.

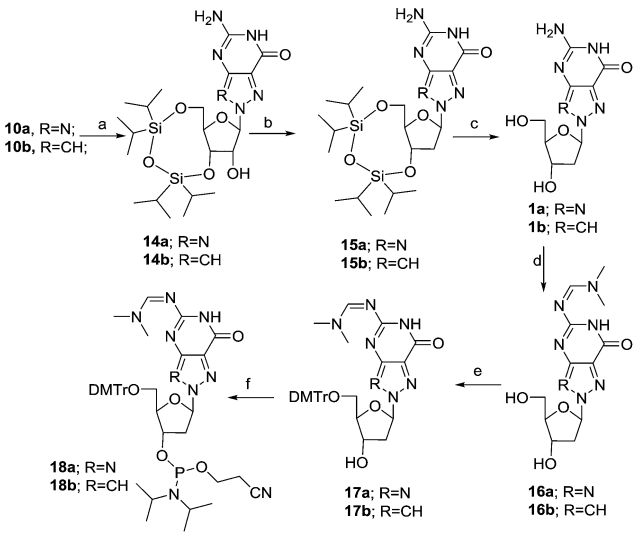
As depicted in Scheme 3, the synthesis of the phosphoramidite of 8-NH_2-dI (**2a**) started from the silylated deoxyinosine **19**.[49] The compound **19** was treated with tosyl azide and *n*-butyllithium in anhydrous THF at −78 °C for 2 hours yielding 60% of the protected 8-azido-dI nucleoside **20a**. The reduction of the azide group was carried out using sodium borohydride in methanol and further protected with an *N*,*N*-dimethylamino methylidene residue using dimethylformamide dimethyl acetal in methanol at 70 °C for 1 hour. The cleavage of the silyl protecting group was carried out using TBAF in THF at room temperature for 12 hours, to obtain compound **22a** to 60% yield. Subsequently, compound **22a** was tritylated and phosphitylated, furnishing the phosphoramidite **24a** *via* **23a**. For the synthesis of the 1-Me-8-NH_2-dI building block, the N^1 methylation of compound **20a** was carried out using methyl iodide and sodium hydride to obtain compound **20b** in 88% yield. Then similar to the synthesis of 8-NH_2-dI phosphoramidite **24a**, a series of transformations starting from **20b** yielded the corresponding phosphoramidite **24b**.

Previously,[29] 8-Oxo-dI **3** was prepared by enzyme-mediated deamination of 8-Oxo-dA **5**. Here, the 8-Oxo-dA[50] **5** nucleoside was deaminated by diazotization of the 6-NH_2 group using sodium nitrate in acetic acid and water at room temperature for 12 hours, affording 8-Oxo-dI **3** to 77% yield. Subsequently, compound **3** was tritylated followed by phosphitylation, furnishing the phosphoramidite **26** *via* **25** (Scheme 4). The spectral and analytical data of **26** were found to be in accordance with that reported.[29] The free amino group of 8-Oxo-dA **5** was protected with an *N*,*N*-dimethylamino methylidene residue using dimethylformamide dimethyl acetal in methanol. Subsequently, compound **27** was tritylated using DMTrCl in pyridine and DMAP as a catalyst. Furthermore, phosphitylation of the DMTr derivative **28**, furnished the phosphoramidite **29**.

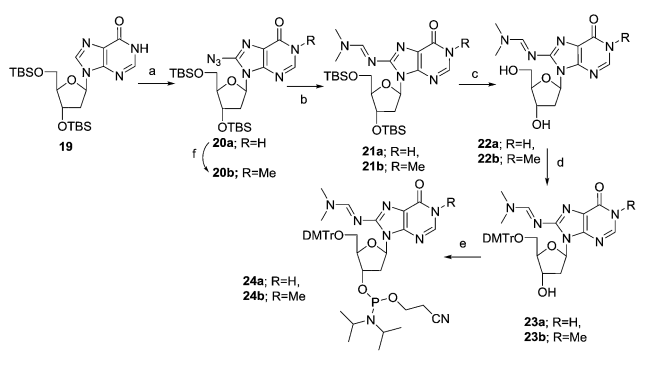
The amino group of 8-Oxo-dG[51] **4** was protected with an *N*,*N*-dimethylamino methylidene residue using dimethylformamide dimethyl acetal in methanol afforded compound **30**. Compound **30** was tritylated and phosphitylated furnishing the required phosphoramidite **32** (Scheme 5).

Oligonucleotide synthesis and hybridization properties

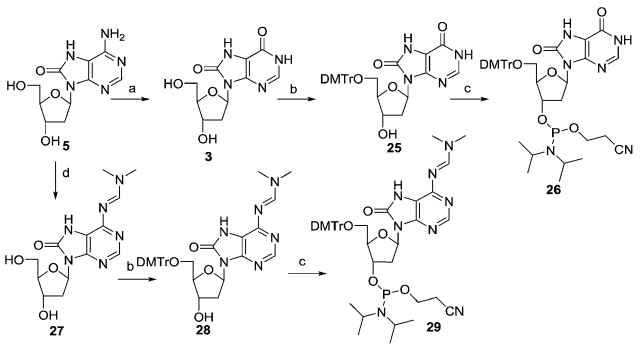
The oligonucleotides were prepared by using the common phosphoramidite method on a solid support employing a DNA



Scheme 2 Synthesis of N^8-8-aza-dG and N^8-8-aza-9-deaza-dG nucleoside. Reagents and conditions: (a) $TIPDSiCl_2$, pyridine, 0 °C to rt, 8 h, 74–77%; (b) (i) TCDI, CH_2–Cl_2, rt, 8 h; (ii) AIBN, nBu_3SnH, toluene, 75 °C, 2 h, 62–68%; (c) TBAF, THF, rt, 4 h, 94–97%; (d) DMF–DMA, MeOH, 50 °C, 1 h, 93–96%; (e) DMTrCl, pyridine, 0 °C to rt, 55 min, 2 h, 76–83%; (f) (i-$Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole, CH_2Cl_2, 0 °C to rt, 55 min, 2 h, 69–73%.



Scheme 3 Synthesis of the phosphoramidite of 8-NH_2-dI and 1-Me-8-NH_2-dI. Reagents and conditions: (a) TsN_3, BuLi, THF, −78 °C, 2 h, 60%; (b) (i) $NaBH_4$, MeOH, 0 °C to rt, 2 h; (ii) DMF–DMA, MeOH, 78 °C, 1 h, 70–73%; (c) TBAF, rt, 3 h, 60–73%; (d) $(MeO)_2TrCl$, pyridine, 0 °C to rt, 12 h, 89–96%; (e) (i-$Pr_2N)_2POC_2H_4CN$, 1*H*-tetrazole, CH_2Cl_2, 0 °C to rt, 1 h, 75–77%; (f) MeI, NaH, 0 °C to rt, 12 h, 88%.

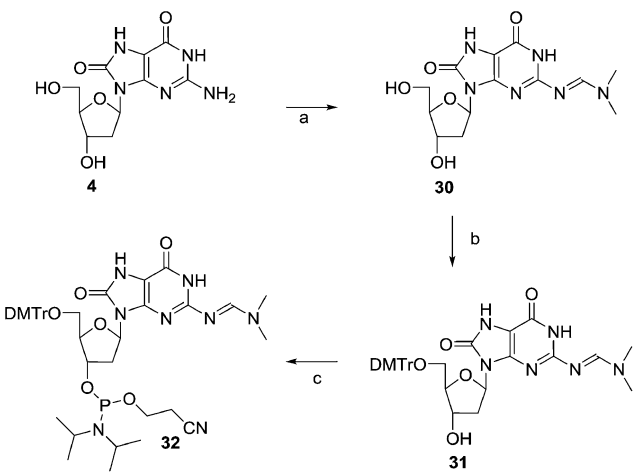


Scheme 4 Synthesis of the phosphoramidite of 8-Oxo-dI and 8-Oxo-dA. Reagents and conditions: (a) $NaNO_2$, acetic acid: H_2O, 12 h, 77%; (b) $(MeO)_2TrCl$, pyridine, 0 °C to rt, 12 h, 69–73%; (c) $(i\text{-}Pr_2N)_2POC_2H_4CN$, 1*H*-tetrazole, CH_2Cl_2, 0 °C to rt, 1 h, 77–80%; (d) DMF–DMA, MeOH, 3 h, 65 °C, 91%.

synthesizer. The base pairing properties of N^8-8-aza-9-deaza-dG and N^8-8-aza-dG nucleosides with d-isoC^{Me},[8] 8-NH_2-dI, 1-Me-8-NH_2-dI, 8-Oxo-dI, 8-Oxo-dA, and 8-Oxo-dG were examined by hybridizing oligomers with their complementary strands and determining the T_m of the hybrids by temperature-dependent UV spectroscopy. In order to investigate the base pairing properties, the N^8-8-aza-dG **1a**, N^8-8-aza-9-deaza-dG **1b** and d-isoG **7** nucleosides were incorporated in the antiparallel duplex 5′-d(GGT AGC A**G***C GGT G)-3′ replacing the G residue. The 8-NH_2-dI **2a**, 1-Me-8-NH_2-dI **2b**, 8-Oxo-dI **3**, 8-Oxo-dA **5**, 8-Oxo-dG **4**, and d-isoC^{Me} **6** nucleosides were incorporated in the sequence 3′-d(CCA TCG T**C***G CCA C)-5′ replacing the C residue. The strength of hybridization was studied by thermal denaturation experiments, which were determined at 260 nm in NaCl (0.1 M) buffer with KH_2PO_4 (20 mM, pH 7.5) and EDTA (0.1 mM) at a concentration of 4 μm for each strand. The stability of the duplexes was compared to the stability of the natural DNA duplex containing dC:dG (Table 1, entry 1) and unnatural d-isoC^{Me}:d-isoG (Table 1, entry 2). The N^8-8-aza-dG:d-isoC^{Me} base pair gave a less stable duplex than the dC:dG (−3.1 °C) pair and d-isoC^{Me}:d-isoG (−5.4 °C). Incorporation of an 8-NH_2-dI **2a** *versus* an N^8-8-aza-dG nucleoside gave a greater decrease in T_m (−9.5 °C) than incorporation of d-isoC^{Me} *versus* N^8-8-aza-dG nucleoside (−5.4 °C), both decreasing on comparison to the d-isoC^{Me}:d-isoG base pair. Insertion of the methyl group at N^1 position of 8-NH_2-dI did not affect the duplex stability, which suggests that the base pairing of 8-NH_2-dI with N^8-8-aza-dG takes place through Hoogsteen base pairing (Fig. 1). Surprisingly, 8-Oxo-dI and 8-Oxo-dG also show similar duplex stability, compared to 8-NH_2-dI. As shown in Fig. 3, this could be explained by Wobble type base pairing in which two hydrogen bonds are involved. The sudden decrease in T_m of the duplexes when N^8-8-aza-dG is placed opposite to 8-Oxo-dA (−11.2 °C), demonstrated that the C6 acceptor (C=O) of 8-Oxo-dI is necessary for base pairing. The experiment proved that the base pair stability for N^8-8-aza-dG decreases in the order d-isoC^{Me} > 8-Oxo-dG ≥ 8-NH_2-dI ≈ 1-Me-8-NH_2-dI ≥ 8-Oxo-dI ≫ 8-Oxo-dA. Approximately similar results were observed for N^8-8-aza-9-deaza-dG **1b**, which indicates that the N^9 nitrogen atom does not play a significant role in duplex stabilization (Table 2).

In order to investigate the selectivity of base pairing, the stability of the duplexes with the modified base N^8-8-aza-9-deaza-dG, N^8-8-aza-dG, d-isoC^{Me}, 8-NH_2-dI, 1-Me-8-NH_2-dI, 8-Oxo-dI, 8-Oxo-dA, and 8-Oxo-dG and the four canonical bases (A, T, C and G) was investigated by T_m determination (Table 3). This study is done in function of the potential use of the artificial base pairs *in vivo*. Among the four inosine derivatives (**2a**, **2b**, **3** and **4**) the mismatch discrimination (ΔT_m) is the lowest for 8-NH_2-dI and the highest for 1-Me-8-NH_2-dI. For the N^8 nucleosides; the N^8-8-aza-9-deaza-dG showed better mismatch discrimination than N^8-8-aza-dG.

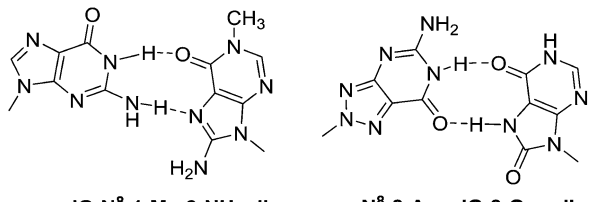
The selectivity of the **1a**:**2a** base pair is low, as the T_m of the duplexes containing **1a**:**2a** are only 1.0–3.4 °C higher than the



Scheme 5 Synthesis of the phosphoramidite of 8-Oxo-dG. Reagents and conditions: (a) DMF–DMA, MeOH, 50%; (b) $(MeO)_2TrCl$, pyridine, 0 °C to rt, 12 h, 71%; (c) $(i\text{-}Pr_2N)_2POC_2H_4CN$, 1*H*-tetrazole, CH_2Cl_2, 0 °C to rt, 1 h, 81%.

Table 1 T_m values (in °C) of non-self-complementary antiparallel-stranded oligonucleotide duplexes containing dA, dT, dC, dG, **6** (d-isoC^{Me}), and **7** (d-isoG)

Entry	Duplex	T_m (°C)
1	5′-d(GGT AGC A**G**C GGT G)-3′ 3′-d(CCA TCG T**C**G CCA C)-5′	61.3
2	5′-d(GGT AGC A**7**C GGT G)-3′ 3′-d(CCA TCG T**6**G CCA C)-5′	63.6



dG:N^8-1-Me-8-NH_2-dI N^8-8-Aza-dG:8-Oxo-dI

Fig. 3 Putative base pairs between dG:N^8-1-Me-8-NH_2-dI and N^8-8-aza-dG:8-Oxo-dI.

Table 2 T_m values (in °C) of non-self-complementary antiparallel-stranded oligonucleotide duplexes containing **1a**, **1b** {5′-d[GGT AGC A(**1a** or **1b**)C GGT G]-3′} and **6**, **2a**, **2b**, **3**, **4**, **5** {3′-d[CCA TCG T(**6** or **2a** or **2b** or **3** or **4** or **5**)G CCA C]-5′} and ΔT_m with respect to entry 1 (Table 1)

	N^8-8-aza-dG (**1a**)	ΔT_m	N^8-8-aza-9-deaza-dG (**1b**)	ΔT_m
d-isoC^{Me} (**6**)	58.2	−3.1	58.3	−3.0
8-NH_2-dI (**2a**)	54.1	−7.2	54.4	−6.9
1-Me-8-NH_2-dI (**2b**)	54.0	−7.3	54.0	−7.3
8-Oxo-dI (**3**)	53.4	−7.9	54.0	−7.3
8-Oxo-dG (**4**)	54.9	−6.4	55.4	−5.9
8-Oxo-dA (**5**)	47.0	−14.3	48.6	−12.7

Table 3 T_m values (in °C) of antiparallel-stranded oligonucleotide duplexes containing **1a**, **1b** {5′-d[GGT AGC A(**1a** or **1b**)C GGT G]-3′} and **2a**, **2b**, **3**, **4**, **5** {3′-d[CCA TCG T(**2a**, or **2b** or **3** or **4**, or **5**)G CCA C]-5′} hybridized against complementary oligonucleotides with natural bases

	A	T	C	G	ΔT_m[a]
N^8-8-aza-dG (**1a**)	47.1	53.8	46.9	54.6	7.7
N^8-8-aza-9-deaza-dG (**1b**)	46.3	53.0	47.0	55.8	9.5
8-NH_2-dI (**2a**)	50.7	51.6	53.1	52.9	2.4
1-Me-8-NH_2-dI (**2b**)	46.2	43.7	43.1	52.1	9.0
8-Oxo-dI (**3**)	55.8	50.3	53.5	47.9	7.9
8-Oxo-dG (**4**)	54.7	50.0	57.0	54.9	7.0
8-Oxo-dA (**5**)	45.8	54.7	44.5	51.2	10.2

[a] In this case ΔT_m corresponds to the difference between lowest and highest T_m (mismatch discrimination).

T_m of the duplexes of **2a** and the natural bases (A, T, C and G). The selectivity of the **1a**:**2b** base pair is much better, due to a large drop in the T_m of duplexes containing the **2b**:**A**, **2b**:**T** and **2b**:**C** base pairs. However, discrimination with G (**2b**:**G** base pair) stays low. In case of 8-Oxo-dI **3**, the stability of the **3**:**A** is higher than of **3**:**1a** (which is similar than **3**:**C**). Hence the mismatch discrimination is highest with the G base (**3**:**G**). 8-Oxo-dG (**4**) shows better base pairing with C (**4**:**C**) and with G (**4**:**C**), than 8-Oxo-dI (**3**:**C** and **3**:**G**). Base pairing of 8-Oxo-dG with canonical bases has been described previously.[32] Base pair stability of **4**:**A** and **4**:**G** is the same as of **4**:**1a**. The base pair stability of 8-Oxo-dA *versus* canonical bases decreases in the order of T > G ≫ A > C. The results with **1b** are similar to the results with **1a**, except that **1b**:**5** is somewhat more stable than **1a**:**5** and **1b**:**G** is somewhat more stable than **1a**:**G**. The result, however, could be sequence dependent. Based on the T_m data, the 1-Me-8-NH_2-dI:N^8-8-aza-dG (or N^8-8-aza-9-deaza-dG) base pair is the most discriminative *versus* A, T, and C, except *versus* G. However this does not mean that it would be the best orthogonal base pair *in vivo*.

Recognition of modified bases *in vivo*

A range of modified oligonucleosides were chemically synthesized with flanking DNA backbones and subsequently analysed for their biological characteristics *in vivo*. These oligonucleotides included separately each of 1-Me-8-NH_2-dI, 8-NH_2-dI, N^8-8-aza-dG, N^8-8-aza-9-deaza-dG, 8-Oxo-dI, 8-Oxo-dA and 8-Oxo-dG. The DNA backbone represented the 18-base long coding strand encoding the active site of the essential ThyA enzyme, whereby the nucleosides indicated replaced DNA within various codons (Fig. 4).

XNA-dependent DNA synthesis *in vivo*

We investigated the relationship between the nature of the chemical modifications of the 8-derivatives and the response of the *E. coli* genetic system to base pair with sufficient recognition and selectivity to enable survival *in vivo*. The aim in this study was to identify which of these modified nucleosides lacked such recognition, and possessed poor base-pairing ability with the canonical bases so as to approach orthogonality away from the biological system.

The xenobiotic nucleosides were tested using the published and established gapped-vector assay.[52,53] In summary, DNA base/modified nucleoside mosaic oligomers were ligated enzymatically into a gapped form of the inactive thyA gene, which encodes an essential section of the ThyA active site. The inactive thyA gene was located on a plasmid vector (the pAK1/pAK2 heteroduplex) together with the bla ampicillin resistance gene permitting selection on two criteria. Once ligated and circularized, the plasmid was transformed into a strain of *E. coli* lacking thyA, a lethal growth phenotype in thymidine (dT) deficient media (Fig. 5).

The completion of the thyA gene finalizes the plasmid, which permits bacteria survival in the dT deficient media. The ratio between bacterial colony numbers in ±dT media indicates the success of the mosaic xenobiotic oligonucleotide to serve as a template for DNA polymerization. The mosaic oligonucleotides all took the form of the six middle codons that encode the active site of ThyA around the catalytically essential cysteine (codon TGC or TGT). The forms of each of the oligomers tested follow, including the nucleosides and their positions within the oligomer (Table 4).

The thyA plasmid genes from sixteen clones selected on dT deficient media were sequenced for each mosaic oligonucleotide tested (Fig. 6).

1-Me-8-NH_2-dI and 8-NH_2-dI

The assay and interpretation of the 8-NH_2-dI and 1-Me-8-NH_2-dI and nucleotides *in vivo* follows (Fig. 7).

Analysis of the 1-Me-8-NH_2-dI nucleotide *in vivo* reveals faint recognition as G and even less as T, with nothing apparent as C or A. Comparison to the related inosine-derivative 8-NH_2-dI, which lacks the N^1 methyl group, shows that recognition is

wild-type *E. coli* ThyA: 143 144 145 146 147 148 — *Amino acid number*
sites of modification in mosaic oligos: 5'-P-CTA-**G**C**G**-C**C**G-T**G**C-C**A**T-GCA-3' — -DNA base-
Leu – Ala – Pro – Cys – His – Ala — - Amino acid -

Fig. 4 The general structure of the mosaic test oligos. The 18 bases derive from the wild-type active site of *E. coli* thyA. Their positions and encoded amino acids are shown, including the catalytically essential Cys residue at 146. The various positions within this sequence where the modified nucleosides were tested within are bolded and underlined.

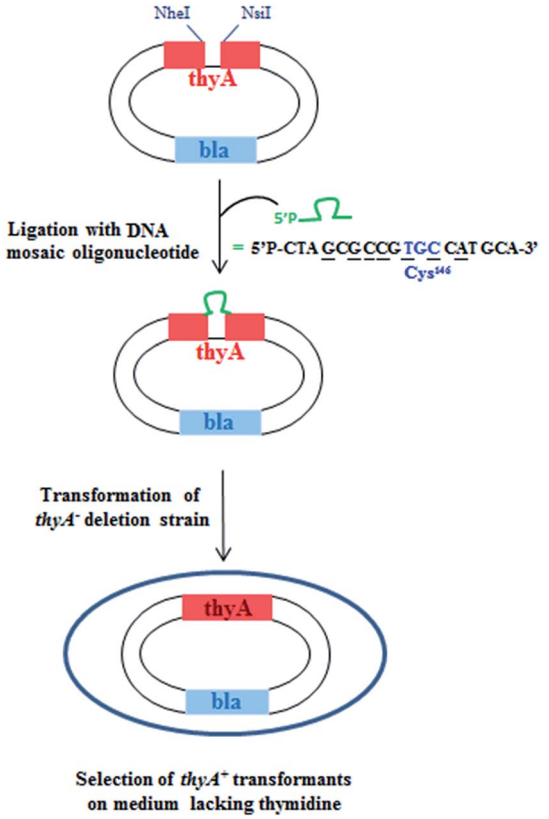


Fig. 5 Selection screen to test DNA oligonucleotides bearing modified base(s) in templates for DNA synthesis *in vivo*.

entirely as G (and none with the other canonical bases), demonstrating that the presence of this 1-Me group is sufficient to reduce recognition as G but increase slightly recognition as T.

The 8-NH_2-dI nucleotide base pairs the canonical bases *in vivo* preferentially of the order C $\ggg$ T = G = A, whereas the methylated form 1-Me-8-NH_2-dI nucleotide appears to base pair preferentially in the order C $\gg$ A > G = T. These findings agree with the *in vitro* T_m data interpretations (Table 3), indicating perseverance of the recognition patterns between terminal stabilization in the artificial base pairs and enzymatic polymerization *in vivo*. Sequencing analysis was performed on purified plasmids from positive transformants and in both experiments revealed only wild-type sequences, indicating no forced changes of the genetic code by the bases.

N^8-8-aza-dG and N^8-8-aza-9-deaza-dG

The assay and interpretation of the two N^8 nucleosides, N^8-8-aza-dG and N^8-8-aza-9-deaza-dG nucleotides *in vivo* follow (Fig. 8 and 9).

Analysis shows that N^8-8-aza-dG appears to behave as an A or C nucleotide, and lacks any recognition as a T or G nucleotide. Sequencing of positive colonies revealed the presence of incorporated A's into the XCG codon (changing the amino acid to Thr from Ala, tolerated to approximately 30% of comparable wild-type survival through DNA control tests). In the GCX codon changes were also observed, with mispairing resulting in the inclusion of silent C and A residues (tolerated to 100% as wild-type) for an Ala residue in the enzyme primary structure. The N^8-8-aza-dG nucleoside base pairs in the order of T $\gg$ G > C = A. The N^8-8-aza-9-deaza-dG nucleotide appears to behave similarly to the derivative N^8-8-aza-dG, with an interpreted base pairing preferentiality of T $\gg$ G = C = A, and similarly a proportion of changes to A nucleotides as observed through sequencing, also in the XCG codon (tolerated to Thr).

We hypothesized that the N^9 nitrogen atom may assist Hoogsteen base pairing, although the similar findings *in vivo* between the two N^8 nucleosides suggests the N^9 nitrogen atom does not offer a significant role in recognition, agreeing with the previously indicated findings *in vitro* for this pair with regards to duplex stabilization. Modifications in recognition primarily occur on the XCG, CXT and GCX codons, but the influence remains marginal, as other codons are not affected, indicating little difference *in vivo* of the recognition of these two N^8-glycosylated nucleosides. These findings do not discredit the *in vitro* T_m data, where N^8-8-aza-dG showed worse mismatch discrimination than its N^9-deaza-analogue. The general appearance of the *in vivo* results indicate an agreement to the *in vitro* findings, where general mismatch is comparably lower in N^8-8-aza-9-deaza-dG except towards T, which appears easier to pair. Between the two nucleosides, the N^8-8-aza-9-deaza-dG is the most orthogonal of this pair, and one of the most orthogonal of all of those tested in this study.

8-Oxo-dI, 8-Oxo-dG and 8-Oxo-dA

The assay and interpretation of the 8-Oxo-dI, 8-Oxo-dG and 8-Oxo-dA nucleotides *in vivo* follow (Fig. 10 and 11).

The response of the 8-Oxo-dI nucleotides reveals the worst *in vivo* mismatch discrimination of all of the nucleotides tested

Table 4 Sequence of the oligomers used in the XNA-dependent DNA synthesis experiments

Sequence (5′–3′)	X
d(P-CTA **X**CG CCG TGC CAT GCA)	1-Me-8-NH_2-dI, 8-NH_2-dI, 8-Oxo-dI, N^8-8-aza-dG, or N^8-8-aza-9-deaza-dG
d(P-CTA GCG CCG **X**GC CAT GCA)	1-Me-8-NH_2-dI, 8-NH_2-dI, 8-Oxo-dI, N^8-8-aza-dG, or N^8-8-aza-9-deaza-dG
d(P-CTA GCG CCG TG**X** CAT GCA)	1-Me-8-NH_2-dI, 8-NH_2-dI, 8-Oxo-dI, N^8-8-aza-dG, or N^8-8-aza-9-deaza-dG
d(P-CTA GCG CCG TGC C**X**T GCA)	1-Me-8-NH_2-dI, 8-NH_2-dI, 8-Oxo-dI, N^8-8-aza-dG, or N^8-8-aza-9-deaza-dG
d(P-CTA GC**X** CCT TGT CAT GCA)	8-Oxo-dI, N^8-8-aza-dG, N^8-8-aza-9-deaza-dG, 8-Oxo-dA, or 8-Oxo-dG
d(P-CTA GC**X** C**X**T TGT CAT GCA)	8-Oxo-dI, N^8-8-aza-dG, N^8-8-aza-9-deaza-dG8-Oxo-dA, or 8-Oxo-dG
d(P-CTA GC**X** **X**CT TGT CAT GCA)	8-Oxo-dI, N^8-8-aza-dG, N^8-8-aza-9-deaza-dG, 8-Oxo-dA, or 8-Oxo-dG
d(P-CTA GC**X** **XX**T TGT CAT GCA)	8-Oxo-dI, N^8-8-aza-dG, 8-Oxo-dA, or 8-Oxo-dG

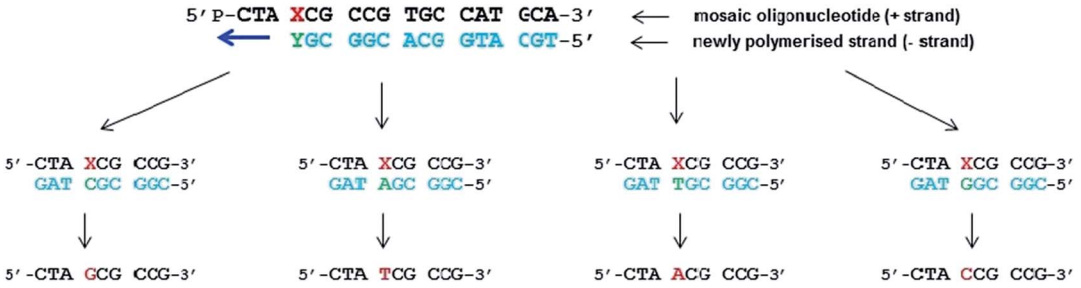


Fig. 6 Fate of mosaic oligomers *in vivo*. The first step *in vivo* is the polymerisation of the complementary DNA strand followed by canonical replication. Different possible incorporations of canonical nucleotides facing the base modified DNA nucleoside during the synthesis of the first DNA strand and the resulting coding strand are presented.

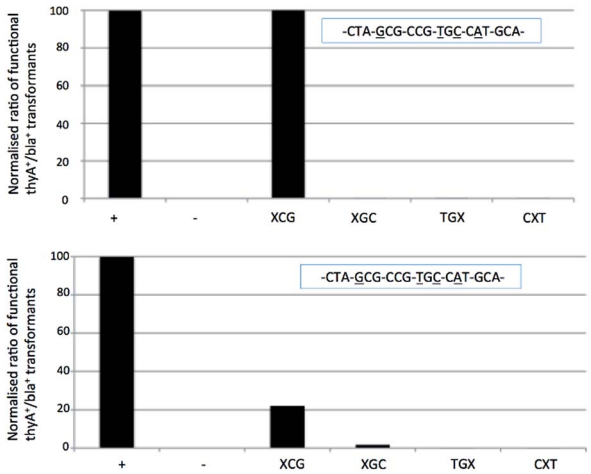


Fig. 7 The *in vivo* interpretation of the 8-NH_2-dI nucleotide (top) and 1-Me-8-NH_2-dI nucleotide (bottom) within the essential thyA gene. The normalized ratio is the experimentally derived average number of thymidine-prototrophic colonies (bla$^+$ thyA$^+$) from the average total number of colonies (bla$^+$ thyA$^-$ and bla$^+$ thyA$^+$). The modified section of the oligomer sequence indicates the position/s of the 8-NH_2-dI or 1-Me-8-NH_2-dI nucleotide as appropriate.

here. Wide base-pairing capabilities with the canonical bases is shown by the apparent survival rates recovered through the tests. As with the previous tests, sequencing was performed of the now thymidine-prototrophic colonies (*bla$^+$ thyA$^+$*), showing

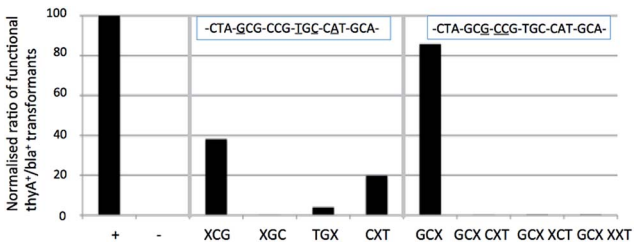


Fig. 8 The *in vivo* interpretation of the N^8-8-aza-dG nucleotide within the essential thyA gene. The normalized ratio is the experimentally derived average number of thymidine-prototrophic colonies (bla$^+$ thyA$^+$) from the average total number of colonies (bla$^+$ thyA$^-$ and bla$^+$ thyA$^+$). The modified section of the oligomer sequence indicates the position of the N^8-8-aza-dG nucleotide/s.

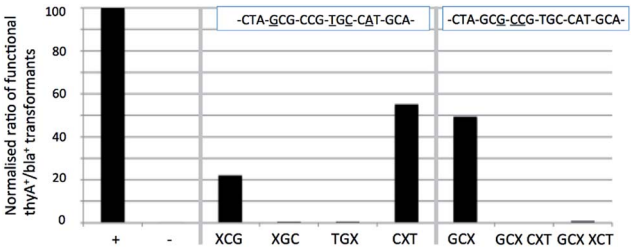


Fig. 9 The *in vivo* interpretation of the N^8-8-aza-9-deaza-dG nucleotide within the essential thyA gene. The normalized ratio is the experimentally derived average number of thymidine-prototrophic colonies (bla$^+$ thyA$^+$) from the average total number of colonies (bla$^+$ thyA$^-$ and bla$^+$ thyA$^+$). The modified section of the oligomer sequence indicates the position of the N^8-8-aza-9-deaza-dG nucleotide/s.

widespread changes to the gene. In the XCG codon, over 80% of the 8-Oxo-dI nucleotides had become T, changing the amino acid to Ser (tolerated lightly compared to wild-type Ala). Similarly, the codons GCX and (GCX) XCT returned around 15% each changes to T (silent change). This nucleotide has a base-pairing preferential order of A = C > G ≫ T, which support Hoogsteen recognition *in vivo*.

The *in vitro* findings and hypothesis of 8-Oxo-dI base pairing stabilities of the order A > C > G agree with the *in vivo* data. As with all of the nucleotides tested, the contribution of the oligo sequence is important as it imposes restrictions on the result obtained that is not observable in the *in vitro* work, underlining the importance of these vital tests in the study of modified nucleotides.

The 8-Oxo-dG and 8-Oxo-dA nucleotides show different patterns of base-pairing discrimination. For 8-Oxo-dG, recognition as an G appears strongest, with weak evidence it behaves as a T, C or A. Base-pairing preferentiality *in vivo* appears in the order of C > T > A ≫ G, with an aversion to base-pairing G also shown in the sequencing data across the double (GCX) XCT codons, which showed changes towards G, T and A in descending order. The finding of mismatches are largely further agreed upon by the *in vitro* dataset, although differences imposed by the primary sequence structure are observed in these tests.

The 8-Oxo-dA nucleotide in contrast shows a much cleaner and less promiscuous base-pairing profile than 8-Oxo-dG. The

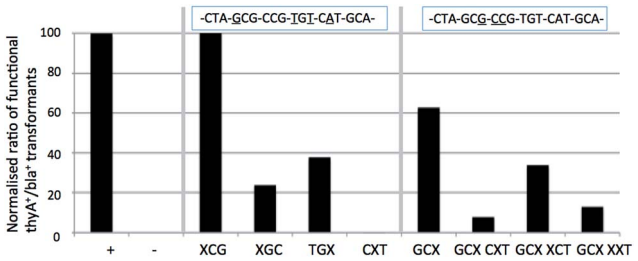


Fig. 10 The *in vivo* interpretation of the 8-Oxo-dG nucleotide (left) and 8-Oxo-dA nucleotide (right) within the essential thyA gene. The normalized ratio is the experimentally derived average number of thymidine-prototrophic colonies (bla$^+$ thyA$^+$) from the average total number of colonies (bla$^+$ thyA$^-$ and bla$^+$ thyA$^+$). The modified section of the oligomer sequence indicates the position of the 8-Oxo-dG and 8-Oxo-dA nucleotide/s, for single (left) and double (right) codons for each.

in vivo data indicates that this nucleotide is strongly recognized as an A, affording a base-pairing order of T $\gg$ A $\gg$ G = C. Sequencing of the positive clones showed widespread changes to A, especially within the GCX codon (tolerating the silent change) and in (GCX) XCT which promoted a change to Thr (with poor *in vivo* survival compared to wild-type). As with the other nucleotides, the base pairing stability determined *in vitro* of T > G $\gg$ A > C agrees well with the findings *in vivo*, possibly with sequence dependent effects.

It was suspected that the DNA repair pathways of the host test strain of *E. coli* might have been interacting with and perhaps modifying the 8-oxo modifications of these compounds. It is widely reported that the gene MutM (encoding the MutM DNA glycosylase) can recognize 8-Oxo-dG and remove it from DNA towards protection against mutagenic lesions.[44,45]

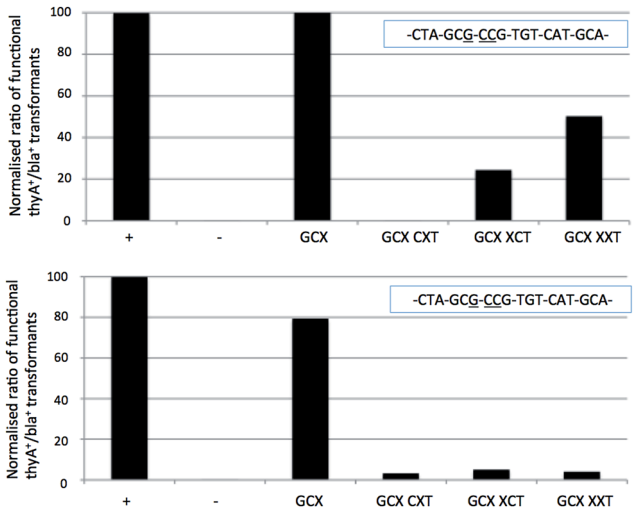


Fig. 11 The *in vivo* interpretation of the 8-Oxo-dI nucleotide within the essential thyA gene. The normalized ratio is the experimentally derived average number of thymidine-prototrophic colonies (bla$^+$ thyA$^+$) from the average total number of colonies (bla$^+$ thyA$^-$ and bla$^+$ thyA$^+$). The modified section of the oligomer sequence indicates the position of the 8-Oxo-dI nucleotide/s, for single (top) and double (bottom) codons.

Accordingly, both 8-Oxo-dG and 8-Oxo-dA nucleotides were tested in the same assay in the equivalent strain of *E. coli* that had been modified in the MutM locus, deleting the gene. On following the same experimental procedure it was revealed that there was in fact no significant difference between the assay response in the strain with and the strain without MutM, indicating that the impact of MutM in this assay format was negligible. This demonstrated that the known mutagenic base (8-Oxo-dG) was not being modified *in vivo* during our assay by MutM, and therefore not modifying our findings.

Conclusions

We have developed an efficient route for the synthesis of oligonucleotides containing N^8-8-aza-dG, N^8-8-aza-9-deaza-dG, 8-NH_2-dI, 1-Me-8-NH_2-dI, 8-Oxo-dI, 8-Oxo-dA, and 8-Oxo-dG nucleotides on solid phase support by using phosphoramidite building blocks **18a**, **18b**, **24a**, **24b**, **26**, **29**, and **32** respectively. The base pairing and mismatch discrimination of N^8-8-aza-9-deaza-dG, N^8-8-aza-dG with d-isoC^{Me}, 8-NH_2-dI, 1-Me-8-NH_2-dI, 8-Oxo-dI, 8-Oxo-dA, and 8-Oxo-dG in duplex DNA with antiparallel chain orientation were determined by T_m measurements. The new base pairs form less stable duplexes compared to the dC:dG and d-isoC^{Me}:d-isoG pairs. Incorporation of 8-NH_2-dI *versus* N^8-8-aza-dG nucleoside gives a greater decrease in T_m than of d-isoC^{Me} *versus* N^8-8-aza-dG. Insertion of the methyl group at N^1 position of 8-NH_2-dI does not affect duplex stability, which suggests that H-bonding with N^8-8-aza-dG takes place *via* Hoogsteen base pairing. As could be expected there is no straight correlation between duplex stability (T_m measurement) and base pair recognition *in vivo*. For example, N^8-8-aza-dG is best recognized by G *in vitro* (T_m) and by T *in vivo* (*E. coli*). This, of course, reflects the contribution of the polymerase in base-pairing recognition.

From all of the tests *in vivo*, the compounds belonging to the family of 8-amino-deoxyinosines (1-Me-8-NH_2-dI and 8-NH_2-dI) and N^8-8-aza-9-deaza-dG have shown to be the most orthogonal from the *E. coli* genetic system and the base-pairing rules of the canonical bases (A, T, G and C). The 8-aza-deoxyguanosines (N^8-8-aza-dG and N^8-8-aza-9-deaza-dG) were recognized largely as A (base pairing T), although the 9-deaza modifications affected general pairing with canonical bases and afforded more orthogonality against the unsubstituted N^8-8-aza-dG. The 9-deaza modifications did not change base pairing preferentiality, maintaining the findings observed *in vitro*.

In testing the 8-Oxo-dG and 8-Oxo-dA nucleosides, a side test was performed after deleting the repair enzyme MutM. This revealed that at least this enzyme was not modifying the 8-oxo-deoxynucleosides and therefore not giving an incorrect view into the orthogonality of the nucleoside (as if it had been corrected to a G through the repair mechanism).

Between the *in vitro* and *in vivo* tests it appears that a base pairing of either of the N^8-glycosylated nucleosides against 8-NH_2-dI or 1-Me-8-NH_2-dI may identify a near orthogonal pair. Poor responses *in vivo* for these two pairs of nucleosides suggest that they are not strongly pairing with the canonical bases. The biggest problem foreseeable in this pair is the tendency for

these bases to lack strong discrimination against the canonical base G, although these pairs would be discriminative against A, T and C.

Experimental section

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on 300 MHz (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz; ^{31}P NMR, 121 MHz) or 500 MHz (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) or 600 MHz (^{1}H NMR, 600 MHz; ^{13}C NMR, 150 MHz) spectrometers. 2D NMRs (H-COSY, HSQC and HMBC) were used for the assignment of all the intermediates and final compounds. Mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer. Column chromatographic separations were carried out by gradient elution with suitable combination of two/three solvents and silica gel (100–200 mesh or 230–400 mesh). Solvents for reactions were distilled prior to use: THF and toluene from Na/benzophenone; CH_2Cl_2 and CH_3CN from CaH_2; Et_3N and pyridine from KOH.

5-Amino-2-(2,3,5-*O*-triacetyl-β-D-ribofuranosyl)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (9)

β-D-Ribofuranose-1,2,3,5-tetraacetate (8.06 g, 23.01 mmol) was added to a solution of compound **8** (3.50 g, 25.31 mmol) in acetonitrile (115 mL). The reaction mixture was heated to 75 °C and boron trifluoride diethyl etherate (3.03 mL, 24.16 mmol) was added. After 3 h the volatiles were removed *in vacuo* and the residue was purified by flash chromatography (hexane/EtOAc 7 : 3) to give the product **9** (5.10 g, 54%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.01 (br s, 1H, NH), 6.67 (br s, 2H, NH_2), 6.32 (d, J = 2.8 Hz, 1H, H-1′), 5.84–5.75 (m, 1H, H-2′), 5.68–5.49 (m, 1H, H-3′), 4.54–4.33 (m, 2H, H-4′, H-5a′), 4.07 (dd, J = 12.1, 4.8 Hz, 1H, H-5b′), 2.09 (s, 6H, CH_3), 1.98 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.9 (C=O), 169.5 (C=O), 169.2 (C=O), 159.6 (C7), 156.5 (C3a), 154.6 (C5), 128.0 (C7a), 93.6 (C1′), 80.0 (C4′), 73.2 (C3′), 70.3 (C2′), 62.3 (C5′), 20.4, 20.3 (CH_3); HRMS (ESI+) calcd for $C_{15}H_{18}N_6NaO_8$ $[M + Na]^+$ 433.1078, found 433.1076.

5-Amino-2-(β-D-ribofuranosyl)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (10a)[46]

Concentrated ammonium hydroxide (48 mL) solution was added to compound **9** (5 g, 12.19 mmol) and the mixture was stirred for 8 h at room temperature. The aqueous solution was dried by lyophilization. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 8.5 : 1.5) to yield the title compound **10a** as a white solid (3.17 g, 91%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.96 (br s, 1H, NH), 6.57 (br s, 2H, NH_2), 5.87 (d, J = 3.8 Hz, 1H, H-1′), 5.60 (br s, 1H, OH), 5.24 (br s, 1H, OH), 4.79–4.77 (m, 1H, OH), 4.49–4.48 (m, 1H, H-2′), 4.25–4.21 (m, 1H, H-3′), 4.00–3.97 (m, 1H, H-4′), 3.60–3.56 (m, 1H, H-5a′), 3.50–3.44 (m, 1H, H-5b′); ^{13}C (75 MHz, DMSO-d_6): δ 159.6 (C7), 156.8 (C3a), 154.5 (C5), 127.5 (C7a), 96.8 (C1′), 86.2 (C4′), 74.6 (C3′), 70.8 (C2′), 62.2 (C5′); HRMS (ESI+) calcd for $C_9H_{12}N_6NaO_5$ $[M + Na]^+$ 307.0761, found 307.0772.

5-Amino-2-[3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]-2*H*-[1,2,3]triazolo[4,5-*d*] pyrimidin-7-one (14a)

To a suspension of the intermediate **10a** (3 g, 10.55 mmol), in anhydrous pyridine (52 mL) under argon atmosphere, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.63 mL, 11.61 mmol) at 0 °C was added and then the mixture was stirred at room temperature for 8 h. The reaction was quenched by the addition of MeOH. The solvents were evaporated and the product **14a** (4.3 g, 77%) was isolated by flash column chromatography (hexane/EtOAc 1 : 1). ^{1}H NMR (600 MHz, DMSO-d_6): δ 11.09 (br s, 1H, NH), 6.62 (br s, 2H, NH_2), 5.89 (s, 1H, H-1′), 5.75 (d, J = 4.6 Hz, 1H, OH), 4.89 (dd, J = 8.5, 4.6 Hz, 1H, H-3′), 4.44 (t, J = 3.7 Hz, 1H, H-2′), 4.05–3.99 (m, 1H, H-5a′), 3.95–3.79 (m, 2H, H-4′, H-5b′), 1.12–0.87 [m, 28H, 4 × $CH(CH_3)_2$]; ^{13}C NMR (151 MHz, DMSO-d_6): δ 159.7 (C7), 156.8 (C3a), 154.7 (C5), 127.5 (C7a), 96.2 (C1′), 81.4 (C4′), 74.4 (C3′), 71.5 (C2′), 61.6 (C5′), 17.4, 17.3, 17.2, 17.1, 17.0, 16.96, 16.91, 12.8, 12.5, 12.3, 12.2, 12.1 [$CH(CH_3)_2$]; HRMS (ESI+) calcd for $C_{21}H_{38}N_6NaO_6Si_2$ $[M + Na]^+$ 527.2463, found 527.2469.

5-Amino-2-[3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-D-ribofuranosyl]-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (15a)

Compound **14a** (2 g; 3.80 mmol) was treated with 1,1′-thiocarbonyldiimidazole (0.676 g; 3.80 mmol) in dry CH_2Cl_2 (38 mL) for 8 h at room temperature under argon atmosphere. The solvent was evaporated and the residue was dissolved in CH_2Cl_2 (20 mL) and washed with H_2O (10 mL). The aqueous layer was extracted twice with CH_2Cl_2 (10 mL); the combined organic layers were dried over Na_2SO_4 and evaporated. The crude product obtained as slightly yellow foam, was used without further purification in the next step. The crude product was dissolved in dry toluene (76 mL), AIBN (0.125 g; 0.76 mmol) and Bu_3SnH (2.56 mL; 9.50 mmol) were added under argon atmosphere and the mixture was degassed with argon for 15 min. Afterwards, the reaction was heated at 75 °C for 2 h. The solution was evaporated *in vacuo*, and the product was purified by flash chromatography (hexane/EtOAc 1 : 1). The compound **15a** was obtained as white solid (1.20 g, 62% over two steps). ^{1}H NMR (600 MHz, DMSO-d_6): δ 11.46 (s, 1H, NH), 6.87 (s, 2H, NH_2), 6.30 (d, J = 7.5 Hz, 1H, H-1′), 5.12 (m, 1H, H-3′), 3.91–3.79 (m, 2H, H-4′, H-5a′), 3.72 (dd, J = 12.3, 8.8 Hz, 1H, H-5b′), 2.79 (m, 1H, H-2a′), 2.64–2.50 (m, 1H, H-2b′), [m, 28H, 4 × $CH(CH_3)_2$]. ^{13}C NMR (151 MHz, DMSO-d_6): δ 159.6 (C7), 157.0 (C3a), 155.1 (C5), 127.3 (C7a), 91.0 (C1′), 84.8 (C4′), 72.6 (C3′), 63.6 (C5′), 40.1 (C2′), 17.3, 17.3, 17.2, 17.1, 16.9, 16.83, 16.81, 12.7, 12.5, 12.2, 12.1, 12.0 [$CH(CH_3)_2$]. HRMS (ESI+) calcd for $C_{21}H_{39}N_6O_5Si_2$ $[M + H]^+$ 511.2514, found 511.2517.

5-Amino-2-(2-deoxy-β-D-ribofuranosyl)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (1a)[21]

Compound **15a** (1.1 g; 2.15 mmol) was dissolved in dry THF (14 mL) and tetrabutylammonium fluoride solution 1.0 M in THF (6.46 mL, 6.46 mmol) was added. The reaction was stirred

for 3 h at room temperature. The solvent was evaporated *in vacuo*, and the crude residue was purified by flash chromatography (CH_2Cl_2/MeOH 9 : 1) to yield the product **1a** as white solid (0.560 g, 97%). Spectral and analytical data were in agreement with previous report.[21]

2-(2-Deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (16a)[21]

To a solution of compound **1a** (0.50 g, 1.86 mmol) in MeOH (12 mL) was added dimethylformamide dimethyl acetal (499 μL, 3.73 mmol), and the mixture was heated at 50 °C for 1 h. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (CH_2Cl_2/MeOH 95 : 5) to yield the compound **16a** as a white solid (0.56 g, 93%). Spectral and analytical data were in agreement with previous report.[21]

2-(5-*O*-Dimethoxytrityl-2-deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one (17a)[21]

The compound **16a** (0.50 g, 1.55 mmol) was co-evaporated with dry pyridine twice under argon atmosphere and then dissolved in dry pyridine (15 mL). 4,4′-Dimethoxytrityl chloride (0.524 g, 1.55 mmol) in CH_2Cl_2 (1.5 mL) was slowly added drop wise under argon atmosphere at 0 °C, then the mixture was stirred at room temperature for 2 h. The reaction was quenched by the addition of MeOH and the solvents were evaporated. The residue was dissolved in CH_2Cl_2 and washed with H_2O, the organic layers were dried on Na_2SO_4 and evaporated under argon atmosphere. The compound **17a** was isolated by column chromatography (CH_2Cl_2/MeOH/TEA 97 : 2 : 1) as a white solid (0.81 g, 83%). Spectral and analytical data were in agreement with previous report.[21]

2-(5-*O*-Dimethoxytrityl-2-deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-one-4-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite (18a)

To a stirred solution of **17a** (0.320 g, 0.511 mmol) and 1 M solution of bis(diisopropylamino)(2-cyanoethoxy)phosphine (767 μL, 0.767 mmol) in anhydrous CH_2Cl_2 (4 mL), under argon atmosphere and at 0 °C, was added 0.45 M solution of 1*H*-terazole (1.13 mL, 0.562 mmol) dropwise. After 10 min, the ice bath was removed and reaction mixture was allowed to stir at room temperature for 45 min. The reaction mixture was diluted with CH_2Cl_2 and was washed with 1 M TEAB solution. The extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude mixture was purified by flash column chromatography (hexane/acetone/TEA, 65 : 34 : 1) to afford amidite **18a** (0.310 g, 73% yield). ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.3, 149.2. HRMS (ESI+) calcd for $C_{42}H_{53}N_9O_7P$ $[M + H]^+$ 826.3799, found 826.3818.[3]

Ethyl 1-(2,3,5-*O*-triacetyl-β-D-ribofuranosyl)-4-nitro-1*H*-pyrazole-3-carboxylate (12)

To the compound **11** (1.85 g, 10 mmol) in 66 mL of 1,2-dichloroethane, *N*,*O*-bis(trimethylsilyl) acetamide (4.9 mL, 20 mmol) was added and the reaction was stirred for 30 minutes at room temperature. Afterwards, β-D-ribofuranose-1,2,3,5-tetraacetate (3.18 g, 10 mmol) was added into the reaction mixture, followed by 1 M solution of stannic chloride in CH_2Cl_2 (8 mL, 8 mmol) and the resulting mixture was stirred at room temperature for 36 h. The reaction solution was quenched with cold saturated aq. $NaHCO_3$ (20 mL) and extracted with CH_2Cl_2. The organics were washed with sat. aq. $NaHCO_3$ (2 × 15 mL) and brine (2 × 15 mL), dried over $MgSO_4$, filtered, concentrated, and purified by flash column chromatography (hexane/EtOAc 7 : 3) to give compound **12** (3.8 g, 85%) as light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (s, 1H, H-5), 5.91 (d, J = 2.9, 1H, H-1′), 5.70 (dd, J = 5.2, 2.9 Hz, 1H, H-2′), 5.45 (dd, J = 6.2, 5.2 Hz, 1H, H-3′), 4.47–4.22 (m, 4H, H-4′, H-5a′, OCH_2), 4.25 (dd, J = 12.5, 3.6 Hz, 1H, H-5b′), 2.15 (s, 6H, CH_3), 2.07 (s, 3H, CH_3), 1.38 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C (75 MHz, $CDCl_3$): δ 170.2, 169.2, 169.0 (C═O), 159.8 (C═O), 140.1 (C4), 134.2 (C3), 129.3 (C5), 92.5 (C1′), 80.6 (C4′), 74.2 (C3′), 69.5 (C2′), 62.5 (C5′), 62.0 (CH_2O), 20.5, 20.2, 13.8 (CH_3); HRMS (ESI+) calcd for $C_{17}H_{21}N_3O_{11}$ $[M + Na]^+$ 466.1068, found 466.1063.

Ethyl 4-amino-1-(2,3,5-*O*-triacetyl-β-D-ribofuranosyl)-1*H*-pyrazole-3 (13)

To a solution of compound **12** (3.7 g, 8.35 mmol) in MeOH (41 mL) was added 10% Pd/C (88 mg), and the mixture was stirred at room temperature for 8 h under a hydrogen atmosphere (1 atm). The catalyst was removed by filtration over celite. The filtrate was concentrated and compound **13** (3.45 g, 100%) was used as such in the next step. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.38 (s, 1H, H-5), 5.99 (d, J = 4.2 Hz, 1H, H-1′), 5.70 (dd, J = 5.2, 2.9 Hz, 1H, H-2′), 5.45 (dd, J = 6.2, 5.2 Hz, 1H, H-3′), 4.47–4.22 (m, 4H, H-4′, H-5a′, OCH_2), 4.25 (dd, J = 3.6, 12.5 Hz, 1H, H-5b′), 2.15 (s, 6H, CH_3), 2.07 (s, 3H, CH_3), 1.28 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C (75 MHz, DMSO-d_6): δ 170.1, 169.5, 169.3, 162.8 (C═O), 135.7 (C4), 130.6 (C3), 116.2 (C5), 90.7 (C1′), 79.6 (C4′), 72.9 (C3′), 70.4 (C2′), 62.8 (C5′), 59.8 (CH_2O), 20.5, 20.3, 14.3 (CH_3); HRMS (ESI+) calcd for $C_{17}H_{23}N_3O_9$ $[M + Na]^+$ 436.1326, found 436.1331.

5-Amino-2-(β-D-ribofuranosyl)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (10b)

A mixture of **13** (1.2 g, 2.9 mmol), chloroformamidine hydrochloride (0.84 g, 7.26 mmol) and dimethylsulfone (1.37 g, 14.5 mmol), was heated for 1 hour at 120 °C in an open flask with magnetic stirring. After the mixture was cooled to room temperature, H_2O (5 mL) was added. The solution was then neutralized with NH_4OH, stirred for 1 h and then the aqueous solution was dried by lyophilization. The crude residue was purified by flash column chromatography (CH_2Cl_2/MeOH 8 : 2) to yield the compound **10b** as a white solid (0.35 g, 42%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.95 (br s, 1H, NH), 8.00 (s, 1H, H-9), 6.07 (br s, 2H, NH_2), 5.72 (d, J = 4.1 Hz, 1H, H-1′), 5.54 (d, J = 5.0 Hz, 1H, OH), 5.19 (m, 1H, OH), 4.98 (t, J = 5.8 Hz, 1H, OH), 4.31–4.29 (m, 1H, H-2′), 4.14–4.09 (m, 1H, H-3′), 3.95–3.91 (m, 1H, H-4′), 3.65–3.60 (m, 1H, H-5a′), 3.54–3.33 (m, 1H, H-5b′); ^{13}C (75 MHz, DMSO-d_6): δ 157.3 (C7), 151.2 (C5), 139.3 (C3a), 133.4

(C7a), 119.5 (C3), 94.9 (C1′), 85.4 (C4′), 75.1 (C3′), 70.3 (C2′), 61.4 (C5′); HRMS (ESI+) calcd for $C_{10}H_{13}N_5O_5$ $[M + Na]^+$ 306.0809, found 306.0810.

5-Amino-2-[3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (14b)

Reaction of compound **10b** (0.8 g, 2.82 mmol), in anhydrous pyridine (14 mL) under argon atmosphere with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (970 μL, 3.11 mmol), was carried out as described for compound **14a** and purified by flash column chromatography (CH_2Cl_2/MeOH 95 : 5) affording compound **14b** as white solid (1.1 g, 74%) by ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.60 (br s, 1H, NH), 7.83 (s, 1H, H-9), 5.96 (br s, 2H, NH_2), 5.79 (s, 1H, H-1′), 5.69 (d, J = 4.4 Hz, 1H, OH), 4.59 (dd, J = 8.2, 4.6 Hz, 1H, H-3′), 4.29 (t, J = 4.4 Hz, 1H, H-2′), 4.03–3.98 (m, 2H, H-4′, H-5a′), 3.90–3.87 (m, 1H, H-5b′), 1.05–0.94 [m, 28H, 4 × $CH(CH_3)_2$]; ^{13}C (75 MHz, DMSO-d_6): δ 156.9 (C7), 150.9 (C5), 139.1 (C3a), 133.9 (C7a), 119.8 (C3), 94.0 (C1′), 80.9 (C4′), 75.8 (C2′), 70.1 (C3′), 60.3 (C5′), 17.3, 17.2, 17.1, 17.1, 16.9, 16.8, 13.0, 12.8, 12.7, 12.4, 12.3, 12.2 [$CH(CH_3)_2$]; HRMS (ESI+) calcd for $C_{22}H_{40}N_5O_6Si_2$ $[M + H]^+$ 526.2511, found 526.2507.

5-Amino-2-[3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-β-D-ribofuranosyl]-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (15b)

Compound **14b** (1.2 g; 2.28 mmol) was treated with 1,1′-thiocarbonyldiimidazole (0.406 g; 2.28 mmol) in dry CH_2Cl_2 (22 mL) for 8 h at 35 °C then with AIBN (75 mg; 0.456 mmol) and Bu_3SnH (1.54 mL; 5.70 mmol) in dry toluene (45 mL), was carried out as described for compound **15a** and purified by flash column chromatography (1% to 3% MeOH in CH_2Cl_2). The compound **15b** was obtained as white powder (0.790 g, 68% over two steps). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.83 (br s, 1H, NH), 7.82 (s, 1H, H-9), 6.16 (d, J = 7.3 Hz, 1H, H-1′), 6.13 (s, 2H, NH_2), 4.97 (m, 1H, H-3′), 3.85–3.79 (m, 2H, H-4′, H-5a′), 3.74 (dd, J = 12.4, 8.3 Hz, 1H, H-5b′), 2.74–2.70 (m, 1H, H-2a′), 2.50–2.45 (m, 1H, H-2b′), 1.05–0.94 [m, 28H, 4 × $CH(CH_3)_2$]; ^{13}C NMR (125 MHz, DMSO-d_6): δ 157.0 (C7), 151.0 (C5), 139.2 (C3a), 133.6 (C7a), 119.7 (C3), 88.6 (C1′), 84.8 (C4′), 72.1 (C3′), 63.3 (C5′), 40.1 (C2′), 17.4, 17.3, 17.2, 17.1, 17.0, 16.9, 16.8, 13.1, 12.8, 12.5, 12.3, 12.0 [$CH(CH_3)_2$]; HRMS (ESI+) calcd for $C_{22}H_{40}N_5O_5Si_2$ $[M + H]^+$ 510.2562, found 510.2561.

5-Amino-2-(2-deoxy-β-D-ribofuranosyl)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (1b)

Reaction of compound **15b** (0.8 g; 1.57 mmol) with tetrabutylammonium fluoride in THF (4.71 mL, 4.71 mmol) in dry THF (9.4 mL) and was carried out as described for compound **1a** and purified by flash column chromatography (CH_2Cl_2/MeOH 8 : 2) to yield the product **1b** as white solid (0.395 g, 94%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.38 (br s, 1H, NH), 7.95 (s, 1H, H-9), 6.14 (t, J = 4.1 Hz, 1H, H-1′), 5.94 (br s, 2H, NH_2), 5.28 (d, J = 4.3 Hz, 1H, OH), 4.87 (t, 1H, J = 5.5 Hz, OH), 4.37–4.34 (m, 1H, H-3′), 3.86–3.82 (m, 1H, H-4′), 3.57–3.50 (m, 1H, H-5a′), 3.46–3.40 (m, 1H, H-5b′), 2.62–2.54 (m, 1H, H-2a′), 2.32–2.28 (m, 1H, H-2b′); ^{13}C (75 MHz, DMSO-d_6): δ 157.3 (C7), 150.9 (C5), 139.2 (C3a), 133.1 (C7a), 119.3 (C3), 90.6 (C1′), 88.1 (C4′), 70.5 (C3′), 61.8 (C5′), 40.0 (C2′); HRMS (ESI+) calcd for $C_{10}H_{14}N_5O_4$ $[M + H]^+$ 268.1040, found 268.1055.

2-(2-Deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (16b)

Reaction of compound **1b** (0.430 g, 1.61 mmol) with dimethylformamide dimethyl acetal (430 μL, 3.22 mmol) in MeOH (10 mL) was carried out as described for compound **16a** and purified by flash column chromatography (CH_2Cl_2/MeOH 95 : 5) to yield the compound **16b** as a white solid (0.50 g, 96%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.11 (br s, 1H, NH), 8.54 (s, 1H, N=CH), 8.16 (s, 1H, H-9), 6.19 (t, J = 6.1 Hz, 1H, H-1′), 5.32 (d, J = 4.3 Hz, OH), 4.92 (t, J = 5.4 Hz, 1H, OH), 4.38 (m, 1H, H-3′), 3.87–3.85 (m, 1H, H-4′), 3.62–3.51 (m, 1H, H-5a′), 3.49–3.40 (m, 1H, H-5b′), 3.12 (s, 3H, NCH_3), 3.00 (s, 3H, NCH_3), 2.62–2.57 (m, 1H, H-2a′), 2.34–2.29 (m, 1H, H-2b′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 158.0 (N=CH), 157.4 (C7), 155.1 (C5), 138.3 (C3a), 134.6 (C7a), 121.0 (C3), 90.7 (C1′), 88.2 (C4′), 70.4 (C3′), 61.7 (C5′), 40.4 (NCH_3), 39.8 (C2′), 34.5 (NCH_3); HRMS (ESI+) calcd for $C_{13}H_{19}N_6O_4$ $[M + H]^+$ 323.1462, found 323.1455.

2-(5-*O*-Dimethoxytrityl-2-deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (17b)

The reaction of compound **16b** (0.450 g, 1.4 mmol) in dry pyridine (14 mL) and 4,4′-dimethoxytrityl chloride (0.473 g, 1.4 mmol) was carried out as described for compound **17a** and purified by flash column chromatography (CH_2Cl_2/MeOH/TEA 97 : 2 : 1), affording compound **17b** as a white solid (0.665 g, 76%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.20 (br s, 1H, NH), 8.53 (s, 1H, N=CH), 8.12 (s, 1H, H-9), 7.35–7.08 (m, 9H, ArH), 6.80–6.73 (m, 4H, ArH), 6.26 (dd, J = 6.7, 3.8 Hz, 1H, H-1′), 5.35 (s, 1H, OH), 4.46–4.43 (m, 1H, H-3′), 4.00–3.84 (m, 1H, H-4′), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.13 (s, 3H, NCH_3), 3.10–3.04 (m, 2H, H-5a′,b′), 3.01 (s, 3H, NCH_3), 2.85–2.67 (m, 1H, H-2a′), 2.34–2.30 (m, 1H, H-2b′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 158.0 (N=CH), 157.9, 157.5, 157.4 (Ar, C7), 155.2, 155.1 (Ar, C5), 144.9, 135.7, 135.6, 135.5, 134.7, 134.6 (C7a, C3a, Ar), 129.8, 129.5, 127.7, 126.5, 121.4, 113.1, 91.1 (Ar), 89.9 (C1′), 85.9 (C4′), 85.3 (CPh_3), 70.3 (C3′), 63.9 (C5′), 55.0 (2 × OCH_3), 40.5 (NCH_3), 40.0 (C2′), 39.8 (NCH_3); HRMS (ESI+) calcd for $C_{34}H_{37}N_6O_6$ $[M + H]^+$ 625.2768, found 625.2748.

2-(5-*O*-Dimethoxytrityl-2-deoxy-β-D-ribofuranosyl)-5-(dimethylaminomethylidene)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-one-3-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (18b)

The reaction of compound **17b** (0.250 g, 0.40 mmol) and 1 M bis(diisopropylamino)(2-cyanoethoxy)phosphine (600 μL, 0.60 mmol) and 0.45 M 1*H*-terazole (880 μL, 0.440 mmol) in anhydrous CH_2Cl_2 (4 mL), was carried out as described for compound **18a** and purified by flash column chromatography (hexane/acetone/TEA, 55 : 44 : 1) afforded amidite **18b** (0.230 g, 69% yield). ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.1, 148.8. HRMS (ESI+) calcd for $C_{43}H_{53}N_8O_7P$ $[M + H]^+$ 825.3847, found 825.3849.

8-Azido-3′-5′-*O*-di(*tert*butyldimethylsilyl)-2′-deoxyinosine (20a)

To a solution of compound **19** (1.0 g, 2.08 mmol) in dry THF (40 mL) under argon atmosphere at −78 °C was added *n*-BuLi (2.5 M solution in hexane, 4.2 mL, 10.4 mmol) and the solution was stirred 1 h at −78 °C. *p*-Toluenesulfonyl azide (0.1 M in toluene, 13.7 mL, 6.24 mmol) was added drop wise and the reaction mixture was stirred for 2 h at −78 °C and quenched with NH_4Cl. After removing volatiles, the residue was extracted with CH_2Cl_2. The combined organic layers were washed with water, brine and dried over Na_2SO_4 and the solvent was removed under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/MeOH 50/1) to yield compound **20a** as light yellow solid (0.65 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 13.5 (s, 1H, NH), 8.02 (s, 1H, H-2), 6.20 (t, J = 6.9 Hz, 1H, H-1′), 4.69–4.74 (m, 1H, H-3′), 3.89–3.92 (m, 1H, H-4′), 3.82 (dd, J = 10.8, 6.5 Hz, 1H, H-5a′), 3.69 (dd, J = 10.8, 4.7 Hz, 1H, H-5b′), 3.25 (quint, J = 6.8 Hz, 1H, H-2a′), 2.14–2.21 (m, 1H, H-2b′), 0.92 (s, 9H, CCH_3), 0.86 (s, 9H, CCH_3), 0.12 (s, 6H, $SiCH_3$), 0.019 (s, 3H, $SiCH_3$), −0.006 (s, 3H, $SiCH_3$); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 158.4 (C6), 149.3, 145.7, 143.7 (C4, C8, C2), 123.1 (C5), 87.9, 83.7 (C4′, C1′), 72.3 (C3′), 62.8 (C5′), 37.4 (C2′), 26.0, 25.9 (CH_3), 18.5, 18.1 (CCH_3), −4.5, −4.6, −5.2, −5.3 ($SiCH_3$). HRMS: calcd for $C_{22}H_{40}N_7O_4Si_2$ $[M + H]^+$ 522.2675, found 522.2682.

8-Amino-N^8-(dimethylaminomethylidene)-3′-5′-*O*-di(*tert*butyldimethylsilyl)-2′-deoxyinosine (21a)

To a pre-cooled solution of compound **20a** (600 mg, 1.15 mmol) in MeOH (6 mL) was added $NaBH_4$ (0.218 g, 5.75 mmol) portion wise at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 2 h. After the completion of the reaction, excess $NaBH_4$ was carefully quenched with saturated NH_4Cl (2 mL) and the solvents were removed under reduced pressure. The crude product was used without further purification for the next step. The solution of crude product in *N*,*N*-dimethylforamide-dimethylacetal (5 mL) was heated at 70 °C for 1 h. After removing solvent, the crude residue was purified by flash column chromatography (CH_2Cl_2/MeOH 30/1) to yield compound **21a** (448 mg, 70%) as light yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 13.4 (s, 1H, NH), 8.71 (s, 1H, N=CH), 7.75 (s, 1H, H-2), 6.64 (t, J = 7.3 Hz, 1H, H-1′), 4.71–4.72 (m, 1H, H-3′), 3.83–3.92 (m, 2H, H-4′, H-5a′), 3.68 (dd, J = 4.2, 9.7 Hz, 1H, H-5b′), 3.39 (quint, J = 5.9 Hz, 1H, H-2a′), 2.08–2.15 (m, 1H, H-2b′), 0.91 (s, 9H, CCH_3), 0.86 (s, 9H, CCH_3), 0.10 (s, 6H, $SiCH_3$), −0.01 (s, 3H, $SiCH_3$), −0.02 (s, 3H, $SiCH_3$); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 158.7, 157.6, 155.8, 148.7, 141.6 (C6, C=N, C2, C8, C4), 123.1 (C5), 87.4, 83.1 (C1′, C4′), 73.2 (C3′), 63.4 (C5′), 40.9 (NCH_3), 36.9 (C2′), 34.8 (NCH_3), 26.0, 25.9 (CH_3), 18.5, 18.1 (CCH_3), −4.5, −5.1, −5.2 ($SiCH_3$). HRMS: calcd for $C_{25}H_{47}N_6O_4Si_2$ $[M + H]^+$ 551.3191, found 551.3198.

8-Amino-N^8-(dimethylaminomethylidene)-2′-deoxyinosine (22a)

To a solution of compound **21a** (0.55 g, 1.0 mmol) in THF (10 mL) was added TBAF (1 M in THF, 2.2 mL, 2.2 mmol) and the solution was stirred overnight at room temperature. After removing volatiles, the crude residue was purified by flash column chromatography (CH_2Cl_2/MeOH 20/1) to yield compound **22a** (0.19 g, 60%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.56 (s, 1H, N=CH), 7.82 (s, 1H, H-2), 6.50 (t, J = 6.4 Hz, 1H, H-1′), 4.41–4.42 (m, 1H, H-3′), 3.82 (br s, 1H, H-4′), 3.64 (dd, J = 11.8, 4.2 Hz, 1H, H-5a′), 3.48 (dd, J = 11.7, 4.3 Hz, 1H, H-5b′), 3.14 (s, 3H, NCH_3), 3.02 (s, 4H, NCH_3, H-2a′), 1.98–2.05 (m, 1H, H-2b′); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 159.0, 156.8, 153.0, 147.3, 145.1 (C6, C=N, C2, C8, C4), 122.3 (C5), 87.7, 82.8 (C1′, C4′), 71.6 (C3′), 62.5 (C5′), 40.2 (NCH_3), 37.4 (C2′), 34.2 (NCH_3). HRMS: calcd for $C_{13}H_{19}N_6O_4$ $[M + H]^+$ 323.1462, found 323.1474.

8-Amino-N^8-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-2′-deoxyinosine (23a)

Reaction of compound **22a** (0.16 g, 0.50 mmol) with 4,4′-dimethoxytrityl chloride (0.168 g, 0.50 mmol) in dry pyridine/DMF (16 mL, 7/1 v/v) was carried out as described for compound **17a** and purified by flash column chromatography (CH_2Cl_2/MeOH/TEA 80/2/1) to yield compound **23a** (0.3 g, 96%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.53 (s, 1H, N=CH), 7.93 (s, 1H, H-2), 7.27 (d, J = 7.3 Hz, 2H, ArH), 7.30–7.27 (m, 8H, ArH), 6.85–6.74 (m, 4H, ArH), 6.52 (t, J = 7.2 Hz, 1H, H-1′), 5.25 (d, J = 5.25 Hz, OH), 4.47–4.43 (m, 1H, H-3′), 3.97–3.70 (m, 1H, H-4′), 3.72 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.451–3.26 (m, 2H, H-H-5′), 3.14–3.09 (m, 4H, CH_3, H-2a′), 2.94 (s, 3H, NCH_3), 2.14–2.06 (m, 1H, H-2b′). ^{13}C NMR (125 MHz, DMSO-d_6) δ 158.3, 157.4, 156.0, 154.9 (Ar, C6, C=N, C2, C8, C4), 147.0, 145.5, 136.2, 136.1, 130.1, 130.0, 128.0, 127.9, 126.8 (Ar), 122.0 (C5), 113.3, 113.3 (Ar), 86.0, 85.6 [C1′, $C(CH_3)_3$], 82.5 (C4′), 71.8 (C3′), 64.9 (C5′), 55.4 (OCH_3), 36.9, 34.7, 33.7 ($2NCH_3$, C2′). HRMS: calcd for $C_{34}H_{37}N_6O_6$ $[M + H]^+$ 625.2769, found 625.2770.

8-Amino-N^8-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-2′-deoxyinosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite (24a)

Reaction of compound 23a (0.2 g, 0.32 mmol) with 1 M bis (diisopropylamino)(2-cyanoethoxy)phosphine (1 M in CH_3CN, 0.96 mL, 0.96 mmol) and 1*H*-tetrazole (0.45 M in CH_3CN, 1.56 mL, 0.70 mmol) in anhydrous CH_2Cl_2 (10 mL), was carried out as described for compound **18a** and purified by flash column chromatography (CH_2Cl_2/MeOH/TEA 80/2/1) to yield compound **24a** (0.20 g, 75%) as white solid. ^{31}P NMR (121 MHz, $CDCl_3$) δ 148.5, 148.0. HRMS: calcd for $C_{13}H_{19}N_6O_4$ $[M + H]^+$ 323.1462, found 323.1474.

8-Azido-3′-5′-*O*-di(*tert*butyldimethylsilyl)-N^1-methyl-2′-deoxyinosine (20b)

To a solution of sodium hydride (344 mg, 8.62 mmol) in dry THF (30 mL), compound **20a** (3.0 g, 5.75 mmol) in THF (50 mL) was added drop wise at 0 °C and stirred for 15 min. Methyl iodide (0.72 mL, 11.50 mmol) was added slowly to the reaction mixture at 0 °C. The reaction mixture was stirred at room temperature for 12 h and then NH_4Cl (5 mL) was added to the reaction mixture to quench the excess sodium hydride and the reaction mixture was

evaporated to dryness. The residue obtained was extracted with CH_2Cl_2 (2 × 100 mL), washed with water (2 × 15 mL), brine (10 mL) and dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure and the residue was purified by flash column chromatography (*n*-hexane/ethyl acetate 7/3) to yield compound **20b** as a yellow solid (2.71 g, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (s, 1H, H-2), 6.16 (t, J = 6.9 Hz, 1H, H-1′), 4.74–4.69 (m, 1H, H-3′), 3.90–3.86 (m, 1H, H-4′), 3.79 (dd, J = 10.9, 6.4 Hz, 1H, H-5b′), 3.68 (dd, J = 10.9, 4.6 Hz, 1H, H-5a′), 3.63 (s, 3H, NCH_3), 3.18 (pent, J = 6.9 Hz, 1H, H-2b′), 2.15 (ddd, J = 10.8, 6.9, 3.9 Hz, 1H, H-2a′), 0.92 (s, 9H, CCH_3), 0.85 (s, 9H, CCH_3), 0.11 (s, 6H, $SiCH_3$), 0.02 (s, 3H, $SiCH_3$), −0.01 (s, 3H, $SiCH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.1 (C6), 147.7 (C4), 146.0 (C2), 145.1 (C8), 122.9 (C5), 87.6 (C4′), 83.4 (C1′), 72.1 (C3′), 62.7 (C5′), 37.3 (C2′), 34.4 (NCH_3), 26.0, 25.9 (CCH_3), 18.4, 18.1 (CH_3C), −4.5, −4.6, −5.2, −5.3 (CH_3Si); HRMS (ESI+): calcd for $C_{23}H_{41}N_7O_4Si_2$ $[M + H]^+$ 536.2831; found 536.2832.

8-Amino-N^8-(dimethylaminomethylidene)-3′-5′-*O*-di(*tert*butyldimethylsilyl)-N^1-methyl-2′-deoxy-inosine (21b)

The reaction of compound **20b** (2.5 g, 4.67 mmol) with $NaBH_4$ (882 mg, 23.33 mmol) in MeOH (26 mL) and then with *N*,*N*-dimethylformamide-dimethylacetal (15 mL) was carried out as described for compound **21a**, and purified by flash column chromatography (CH_2Cl_2/MeOH 15/1) to yield yellow solid (1.92 g, 73%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (s, 1H, CH═N), 8.18 (s, 1H, H-2), 6.47 (t, J = 7.0 Hz, 1H, H-1′), 4.71 (dt, J = 6.7, 3.6 Hz, 1H, H-3′), 3.80 (dd, J = 10.4, 6.7 Hz, 1H, H-5b′), 3.77–3.72 (m, 1H, H-4′), 3.59 (dd, J = 10.4, 4.9, 1H, H-5a′), 3.47 (s, 3H, NCH_3), 3.28–3.21 (m, 1H, H-2b′), 3.15 (s, 3H, NCH_3), 3.02 (s, 3H, NCH_3), 2.09 (ddd, J = 10.9, 7.0, 3.6 Hz, 1H, H-2a′), 0.90 (s, 9H, CCH_3) 0.83 (s, 9H, CCH_3), 0.11 (s, 6H, $SiCH_3$), −0.01 (s, 3H, $SiCH_3$), −0.03 (s, 3H, $SiCH_3$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 157.1 (C═N), 155.7 (C6), 154.6 (C4), 146.8 (C8), 145.5 (C2), 121.7 (C5), 86.3 (C4′), 81.9 (C1′), 72.5 (C3′), 62.8 (C5′), 40.2 (NCH_3), 36.2 (C2′), 34.3 (NCH_3), 33.4 (NCH_3), 25.7 (CH_3), 18.0 (CCH_3), 17.7 (CCH_3), −4.6 ($SiCH_3$), −4.7 ($SiCH_3$). HRMS (ESI+): calcd for $C_{23}H_{43}N_5O_4Si_2$ $[M + H]^+$ 565.3348; found 565.3358.

8-Amino-N^8-(dimethylaminomethylidene)-N^1-methyl-2′-deoxyinosine (22b)

The reaction of compound **21b** (565 mg, 1.0 mmol) with TBAF (1 M in THF, 2.2 mL, 2.2 mmol) in THF (10 mL) was carried out as described for compound **22a** and purified by flash column chromatography (CH_2CI_2/MeOH, 9 : 1) to give **22b** (246 mg, 73%) as a pale yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (s, 1H, CH═N), 8.21 (s, 1H, H-2), 6.49 (t, J = 6.4 Hz, 1H, H-1′), 5.25 (s, 1H, OH), 4.96 (s, 1H, OH), 4.43 (br s, 1H, H-3′), 3.81 (br s, 1H, H-4′), 3.64 (dd, J = 11.4, 4.6 Hz, 1H, H-5b′), 3.47 (br s, 4H, NCH_3, H-5a′), 3.14 (s, 3H, NCH_3), 3.09–2.90 (m, 4H, NCH_3, H-2b′), 2.05 (ddd, J = 8.8, 6.4, 2.0 Hz, 1H, H-2a′); ^{13}C NMR (125 MHz, DMSO-d_6) δ 157.1 (C═N), 155.5 (C6), 154.4 (C8), 146.5 (C4), 145.6 (C2), 121.7 (C5), 87.5 (C4′), 82.5 (C1′), 71.3 (C3′), 62.3 (C5′), 40.3 (NCH_3), 37.1 (NCH_3), 34.4 (C2′), 33.4 (NCH_3); HRMS (ESI+): calcd for $C_{14}H_{20}N_6O_4$ $[M + Na]^+$ 359.1438; found 359.1444.

8-Amino-N^8-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-N^1-methyl-2′-deoxyinosine (23b)

Reaction of **22b** (420 mg, 1.25 mmol) with 4,4′-dimethoxytrityl chloride (465 mg, 1.37 mmol) in dry pyridine (45 mL), in dry CH_2Cl_2 (5 mL) was carried out as described for compound **17a** and purified by flash column chromatography (CH_2Cl_2/MeOH/TEA 80/2/1) to yield compound **23b** (710 mg, 89%) as white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.68 (s, 1H, N═CH), 7.45 (s, 1H, H-2), 7.41 (d, J = 7.2 Hz, 2H, Ar), 7.31–7.15 (m, 7H, Ar), 6.78–6.73 (m, 4H, Ar), 6.66 (t, J = 6.9 Hz, 1H, H-1′), 4.85–4.80 (m, 1H, H-3′), 4.11–4.06 (m, 1H, H-4′), 3.77 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 3.54 (s, 3H, NCH_3), 3.52–3.45 (m, 1H, H-5b′), 3.32–3.25 (m, 2H, H-2b′, H-5b′), 3.07 (m, 3H, NCH_3), 3.02 (m, 3H, NCH_3), 2.28 (ddd, J = 12.3, 6.9, 4.5 Hz, 1H, H-2a′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.5 (Ar), 157.5 (N═C), 156.7 (C6), 155.4 (C8), 146.9 (C4), 145.0 (Ar), 143.7 (C2), 136.3, 136.1, 130.2, 128.2, 127.8, 126.7 (Ar), 122.8 (C5), 113.1 (Ar), 86.3 (CPh_3), 85.5 (C4′), 82.6 (C1′), 73.6 (C3′), 64.6 (C5′), 55.3 (OCH_3), 40.9 (NCH_3), 37.2 (C2′), 34.8, 34.1 (NCH_3); HRMS (ESI+): calcd for $C_{35}H_{38}N_6O_6$ $[M + H]^+$ 639.2925; found 639.2926.

8-Amino-N^8-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-N^1-methyl-2′-deoxyinosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (24b)

The reaction of compound **23b** with 1 M bis(diisopropylamino)(2-cyanoethoxy)phosphine (1.18 mL, 1.40 mmol) and 0.45 M 1*H*-tetrazole (1.45 mL, 0.77 mmol) in CH_2Cl_2 (25 mL) was carried out as described for compound **18a** and purified by flash column chromatography (CH_2Cl_2/MeOH/TEA 40/1/1) to yield compound **24b** (0.380 g, 77%) as white solid. ^{31}P NMR (202 MHz, $CDCl_3$) δ 148.6, 148.2. HRMS (ESI+): calcd for $C_{44}H_{55}N_8O_7P$ $[M + H]^+$ 839.4003; found 839.4014.

7,8-Dihydro-8-oxo-2′-deoxyinosine (3)[29]

A solution of $NaNO_2$ (516 mg, 7.48 mmol) in (2 mL) of water was added to a stirred solution of 1 g (3.74 mmol) of compound **5** in 50 mL of 95% aqueous acetic acid. The reaction mixture was stirred overnight. The solvent was removed under reduced pressure and the oily residue was purified by flash column chromatography (CH_2CI_2/MeOH, 4 : 1) to give **3** (780 mg, 77%) as a white solid. Spectral and analytical data were in agreement with previous report.[29]

7,8-Dihydro-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxyinosine (25)[29]

Reaction of compound **3** (350 mg, 1.35 mmol) with 4,4′-dimethoxytrityl chloride (486 mg, 1.44 mmol) in dry pyridine (70 mL) was carried out as described for compound **17a** and purified by flash column chromatography (CH_2CI_2/MeOH/TEA, 95 : 4 : 1) to give **25** (510 mg, 69%) as a white foam. Spectral and analytical data were in agreement with previous report.[29]

7,8-Dihydro-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxyinosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (26)[29]

The reaction of compound **25** (400 mg, 0.70 mmol) with 1 M bis(diisopropylamino)(2-cyanoethoxy)phosphine (1.40 mL, 1.40

mmol) and 0.45 M 1*H*-tetrazole (1.7 mL, 0.77 mmol) in CH_2Cl_2 (25 mL) was carried out as described for compound **18a** and purified by flash column chromatography (*n*-hexane/acetone/TEA, 39 : 60 : 1) to give **26** (430 mg, 80%) as a white foam. Spectral and analytical data were in agreement with previous report.[29]

7,8-Dihydro-N^6-(dimethylaminomethylidene)-8-oxo-2′-deoxyadenosine (27)

To a suspension of compound **5** (1 g, 3.74 mmol) in MeOH (35 mL), *N*,*N*-dimethylformamide dimethyl acetal (2.5 mL, 18.7 mmol) was added. The reaction mixture was stirred at 65 °C for 3 h. The reaction mixture was evaporated to dryness and purified by flash column chromatography (CH_2Cl_2/MeOH : TEA, 90 : 9 : 1) to give **27** (1.1 g, 91%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.72 (s, 1H, HC═N), 8.19 (s, 1H, H-2), 6.17 (dd, J = 8.2, 6.5 Hz, 1H, H-1′), 5.20 (s, 1H, OH), 5.08 (s, 1H, OH), 4.42–4.38 (m, 1H, H-3′), 3.83–3.79 (m, 1H, H-4′), 3.62 (dd, J = 11.7, 4.5 Hz, 1H, H-5b′), 3.47 (br s, 1H, H-5a′), 3.14 (s, 1H, NCH_3), 3.10 (s, 1H, NCH_3), 3.10–2.95 (m, 1H, H-2b′), 2.01 (ddd, J = 9.2, 6.5, 2.6 Hz, H-2a′); ^{13}C NMR (125 MHz, DMSO-d_6) δ 156.0 (C═N), 152.2 (C8), 149.6 (C2), 148.9 (C6), 148.3 (C4), 111.7 (C5), 87.5 (C4′), 81.4 (C1′), 71.4 (C3′), 62.4 (C5′), 40.4 (NCH_3), 36.0 (C2′), 34.3 (NCH_3); HRMS (ESI+): calcd for $C_{13}H_{18}N_6O_4$ $[M + H]^+$ 323.1462; found 323.1455.

7,8-Dihydro-N^6-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxy adenosine (28)

The reaction of compound **27** (600 mg, 1.86 mmol) with 4,4′-dimethoxytrityl chloride (693 mg, 2.05 mmol) in dry pyridine (60 mL), was carried out as described for compound **17a** and purified by flash column chromatography (CH_2CI_2/MeOH/TEA, 95 : 4 : 1) to give **28** (850 mg, 73%) as a white foam. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70 (s, 1H, HC═N), 8.04 (s, 1H, H-2), 7.36–7.34 (m, 2H, Ar), 7.24–7.14 (m, 7H, Ar), 6.71–6.81 (m, 4H, Ar), 6.17 (dd, J = 7.2, 6.2 Hz, 1H, H-1′), 4.52 (br s, 1H, H-3′), 3.94–3.88 (m, 1H, H-4′), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.20–3.05 (m, 9H, 2 × NCH_3, H-5a′,b′, H-2b′), 2.09 (ddd, J = 12.7, 7.2, 4.8 Hz, 1H, H-2a′). ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.9, 157.8, 155.8, 152.3, 149.7, 148.5, 145.1 (C2, C4, C8, C6, C═N, Ar), 135.8, 135.6, 129.7, 129.5, 128.9, 127.6, 127.6, 127.41, 126.4, 113.0, 112.9, 112.7 (Ar), 111.7 (C5), 85.2 (CPh_3), 85.1 (C4′), 80.7 (C1′), 71.0 (C3′), 64.2 (C5′), 55.0, 54.9 (OCH_3), 40.4 (NCH_3), 35.6 (C2′), 34.3 (NCH_3); HRMS (ESI+): calcd for $C_{34}H_{36}N_6O_6$ $[M + H]^+$ 625.2768; found 625.2776.

7,8-Dihydro-N^6-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxyadenosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (29)

The reaction of compound **28** (900 mg, 1.44 mmol), 1 M bis(diisopropylamino)(2-cyanoethoxy)phosphine (2.88 mL, 2.88 mmol) and 0.45 M solution of 1*H*-tetrazole (3.5 mL, 1.58 mmol) in CH_2Cl_2 (30 mL) was carried out as described for compound **18a** and purified by flash column chromatography (*n*-hexane/acetone/TEA, 49 : 50 : 1) to give **29** (920 mg, 77%) as a white foam. ^{31}P NMR (202 MHz, DMSO-d_6) δ 147.4, 146.8. HRMS (ESI+): calcd for $C_{43}H_{53}N_8O_7P$ $[M + H]^+$ 825.3847; found 825.3842.

7,8-Dihydro-N^2-(dimethylaminomethylidene)-8-oxo-2′-deoxyguanosine (30)

To a suspension of compound **4** (1 g, 3.53 mmol) in MeOH (25 mL), *N*,*N*-dimethylformamide dimethyl acetal (4.7 mL, 35.3 mmol) was added. The reaction mixture was stirred at 25 °C for 12 h. The resulting precipitate was isolated, and was washed with CH_2Cl_2 to give **30** (600 mg, 50%) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.46 (s, 1H, N═CH), 6.10 (t, J = 7.3 Hz, 1H, H-1′), 5.16 (s, 1H, OH), 4.75 (s, 1H, OH), 4.39–4.33 (m, 1H, H-3′), 3.76–3.71 (m, 1H, H-4′), 3.56 (dd, J = 11.5, 4.6 Hz, 1H, H-5b′), 3.44 (dd, J = 11.5, 4.2 Hz, 1H, H-5a′), 3.12 (s, 3H, NCH_3), 3.00 (s, 3H, NCH_3), 2.99–2.91 (m, 1H, H-2b′), 1.94 (ddd, J = 9.6, 6.7, 2.7 Hz, 1H, H-2a′); ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.8 (C═N), 156.4 (C6), 152.2 (C8), 151.7 (C2), 145.6 (C4), 102.2 (C5), 87.3 (C4′), 81.1 (C1′), 71.2 (C2′), 62.3 (C5′), 40.7 (NCH_3), 36.0 (C2′), 34.6 (NCH_3); HRMS (ESI+): calcd for $C_{13}H_{18}N_6O_5$ $[M + Na]^+$ 361.1231; found 361.1227.

7,8-Dihydro-N^2-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxy guanosine (31)

The reaction of compound **30** (480 mg, 1.42 mmol) with 4,4′-dimethoxytrityl chloride (528 mg, 1.56 mmol) in dry pyridine (60 mL) was carried out as described for compound **17a** and purified by flash column chromatography (CH_2CI_2/MeOH/TEA, 97 : 2 : 1) to give **31** (650 mg, 71%) as a white foam. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.53 (s, 1H, NH), 10.82 (s, 1H, NH), 8.33 (s, 1H, C═N), 7.36–7.31 (m, 2H, Ar), 7.25–7.15 (m, 7H, Ar), 6.72–6.82 (m, 4H, Ar), 6.13 (dd, J = 7.9, 5.0 Hz, 1H, H-1′), 5.24 (d, J = 4.9 Hz, 1H, OH), 4.46 (quin, J = 4.9 Hz, 1H, H-3′), 3.81–3.85 (m, 1H, H-4′), 3.72, 3.71 (OCH_3), 3.20 (dd, J = 9.9, 7.5 Hz, 1H, H-5b′), 3.07 (dd, J = 9.9, 3.3 Hz, 1H, H-5a′), 3.02, 2.99 (NCH_3), 2.90–2.96 (m, 1H, H-2b′), 2.09 (ddd, J = 13.5, 7.9, 5.0 Hz, 1H, H-2a′); ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.9, 157.8, 157.4, 156.0, 152.1, 151.6, 145.5, 145.1 (C6, C2, C═N, C8, C4, Ar), 135.8, 135.7, 129.6, 129.5, 127.7, 127.6, 126.4, 113.0, 112.9 (Ar), 112.7 (C5), 102.2 (CPh_3), 85.1 (C4′), 80.3 (C1′), 70.9 (C3′), 64.5 (C5′), 54.9, 54.9 (OCH_3), 40.6 (NCH_3), 36.4 (C2′), 34.6 (NCH_3); HRMS (ESI+): calcd for $C_{34}H_{36}N_6O_7$ $[M + Na]^+$ 663.2537; found 663.2537.

7,8-Dihydro-N^2-(dimethylaminomethylidene)-5′-*O*-dimethoxytrityl-8-oxo-2′-deoxyguanosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (32)

The reaction of compound **31** (440 mg, 0.68 mmol), 1 M bis(diisopropylamino)(2-cyanoethoxy)phosphine (1.37 mL, 1.37 mmol) and 0.45 M solution of 1*H*-tetrazole (1.7 mL, 0.75 mmol) in CH_2Cl_2 (20 mL) was carried out as described for compound **18a** and purified by flash column chromatography (*n*-hexane/acetone/TEA, 49 : 50 : 1) to give **32** (470 mg, 81%) as a white foam. ^{31}P NMR (202 MHz, DMSO-d_6) δ 147.8, 147.6. HRMS (ESI+): calcd for $C_{43}H_{53}N_8O_8P$ $[M + Na]^+$ 863.3616; found 863.3620.

Oligonucleotide synthesis

Oligonucleotide assembly was performed with an Expedite DNA synthesizer (Applied Biosystems) by using the phosphoramidite approach. The oligomers were deprotected and cleaved from the solid support by treatment with aqueous ammonia (30%) for 2 h. After gel filtration on a NAP-25 column (Sephadex G25-DNA grade; Pharmacia) with water as eluent, the crude mixture was analysed by using a Mono-Q HR 5/5 anion exchange column, after which purification was achieved by using a Mono-Q HR 10/100 GL column (Pharmacia) with the following gradient system: $A = 10$ mM $NaClO_4$ in 15% CH_3CN, pH 7.4, $B =$ 600 mM $NaClO_4$ in 15% CH_3CN, pH 7.4. The low-pressure liquid chromatography system consisted of a Merck-Hitachi L-6200A intelligent pump, a Mono-Q HR 10/100 GL column (Pharmacia), an Uvicord SII 2138 UV detector (Pharmacia-LKB) and a recorder. The product-containing fraction was desalted on a NAP-25 column and lyophilised. Analysis by mass spectrometry followed.

UV melting experiments

Oligomers were dissolved in a buffer solution containing NaCl (0.1 M), potassium phosphate (0.02 M, pH 7.5) and EDTA (0.1 mM). The concentration was determined by measuring the absorbance in Milli-Q water at 260 nm at 80 °C. The following extinction coefficients were used: dA, $\varepsilon = 15\,000$; dT, $\varepsilon = 8500$; C, $\varepsilon = 7100$; dG, $\varepsilon = 12\,100$; N^8-8-aza-dG, $\varepsilon = 2200$; N^8-8-aza-9-deaza-dG, $\varepsilon = 2500$; 8-NH_2-dI, $\varepsilon = 6800$; 1-Me-8-NH_2-dI, $\varepsilon = 7600$; d-isoC^{Me}, $\varepsilon = 6300$; d-isoG, $\varepsilon = 4600$; 8-Oxo-dI, $\varepsilon = 7500$; 8-Oxo-dA, $\varepsilon = 11\,100$; 8-Oxo-dG, $\varepsilon = 5900$. The concentration for each strand was 4 μM in all experiments. Melting curves were determined with a Varian Cary 100 BIO spectrophotometer. Cuvettes were maintained at constant temperature by water circulation through the cuvette holder. The temperature of the solution was measured with a thermistor that was directly immersed in the cuvette. Temperature control and data acquisition were carried out automatically with an IBM-compatible computer by using Cary WinUV thermal application software. A quick heating and cooling cycle was carried out to allow proper annealing of both strands. The samples were then heated from 10 to 80 °C at a rate of 0.2 °C min^{-1}, and were cooled again at the same speed. Melting temperatures were determined by plotting the first derivative of the absorbance as a function of temperature; data plotted were the average of two runs. Up and down curves showed identical T_m values.

In vivo assay of XNA-oligonucleotides

The oligomers were dissolved in MilliQ water to 100 mM. Before assay, they were diluted ten-fold in the same medium. Testing was performed with a gapped heteroduplex vector produced through the enzymatic digestion and PCR assisted denaturation and hybridization of the pAK1 and pAK2 plasmids.[45] A mix of equimolar (25 ng each) purified pAK1 (NheI and NsiI cut) and purified pAK2 (two-fold EcoRI cut and dephosphorylated) was diluted into 10 mM Tris–HCl (pH 7.5) with 100 mM NaCl. The mixture was denatured at 95 °C for 5 min before cooling to ambient temperature over 2 h, before water dialysis on 0.05 μM nitrocellulose filters (Millipore) for 30 min. The oligomers (0.02 pmol), as well as a positive d(CTAGCGCCGTGCCATGCA) and negative d(CTAGCGCCG···CATGCA) oligomer controls, were added separately to the dialyzed heteroduplex mixture diluted into 1× DNA ligase T4 reaction buffer (NEB) for 20 μL per sample. The mixture was denatured at 85 °C as before. Ligation was performed by adding 5 U T4 DNA ligase (NEB) supplemented with 1 mM ATP to the samples before overnight incubation (16 °C). Ligated mixtures were dialyzed as before, and transformed by electroporation into electro-competent *E. coli* K12 (Δ*thyA*:*aadA*). Incubation of the electroporated bacteria was made at 37 °C for 1 hour, before plating 100 μL of a serial dilution of the suspension onto Muller-Hinton (MH) media containing 100 μg mL^{-1} ampicillin (spreading the 10^0 and 10^{-1} dilutions) and onto the same media supplemented additionally with 0.3 mM thymidine (10^{-3} and 10^{-4} dilutions).

In vivo assay of control DNA-oligonucleotides

Control DNA oligomers were designed by modifying the 18-long standard oligomer to incorporate single changes at the codons tested. All DNA oligomers were ordered at Eurofins MWG and dissolved in MilliQ water to a concentration of 100 mM before dilution ten-fold for testing. Assay of these oligos followed that of the XNA-oligonucleotides, following the same protocol as above.

Data analysis and sequencing

As before,[8] the total numbers of thymidine-prototrophic colonies and ampicillin resistant colonies were taken from the plate counts, and the ratio of the former over the latter was calculated. Positive thymidine-prototrophic colonies were restreaked twice to MH ampicillin, before growth in 2 mL liquid cultures of the same medium. Plasmids were recovered by Miniprep (Qiagen) and the plasmids sequenced using an oligo for pAK1 d(AACAGTGGCGCGCCTGG).

Generation of the ΔMutM strain

The MutM gene was deleted from the *E. coli* K12 strain (Δ*thyA*:*aadA*) through pKD46 meditation recombination, using a deletion construct containing flanking regions of the gene around an apramycin resistance cassette (*aac(3)IV*). Recombination was made by the published procedure.[54] Deletion was confirmed by external primers through colony-PCR.

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