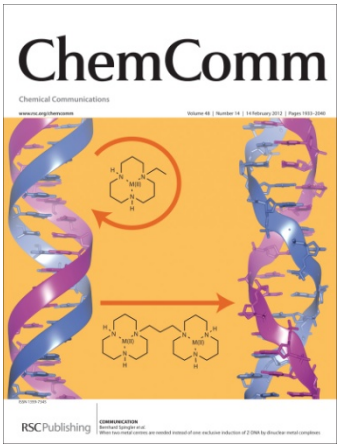

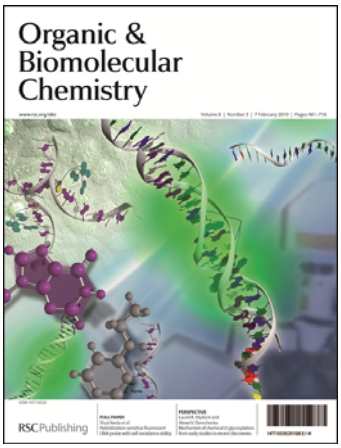

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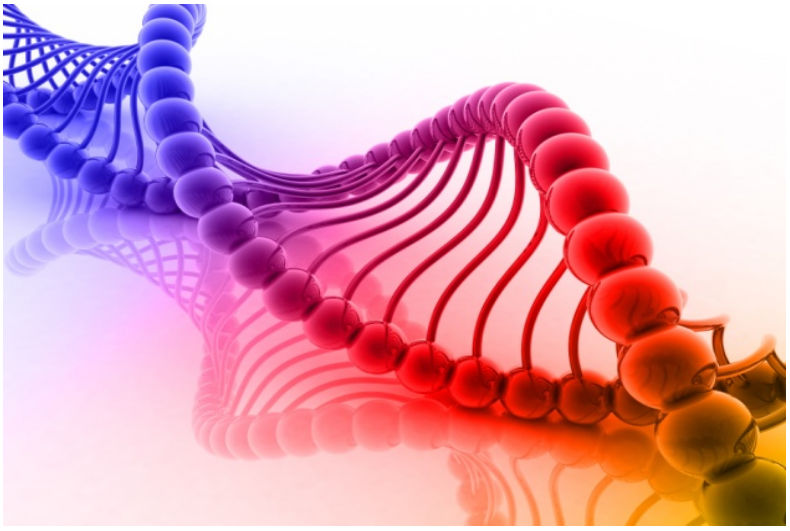

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COMMUNICATION

Synthesis of oligodeoxyribonucleotides containing a conformationally-locked *anti* analogue of O^6-methyl-2′-deoxyguanosine and their recognition by MGMT and Atl1†‡§

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We show that DNA containing a conformationally-locked *anti* analogue of O^6-alkylguanine is a poor substrate for human O^6-methylguanine–DNA methyltransferase (MGMT) and the alkyltransferase-like protein, Atl1. This highlights the requirement for the *syn* conformation and rationalises why certain O^6-alkylguanines are poor MGMT substrates.

Exposure of DNA to alkylating agents can lead to the formation of O^6-alkylguanines (Fig. 1) which are both mutagenic and toxic due to their ability to mispair with thymine during replication.[1] In humans such DNA damage is repaired by an O^6-alkylguanine–DNA alkyltransferase (hAGT or MGMT) that irreversibly transfers the alkyl group to an active site Cys regenerating guanine in the DNA.[2,3] MGMT repairs a wide variety of different O^6-alkylguanines and displays similar binding affinity for most substrates.[4] The repair reaction involves rapid flipping the damaged base from the duplex into the active site (approx. 350 s^{-1})[5] followed by a rate-limiting alkyl transfer reaction. For O^6-alkylguanines with smaller alkyl groups the relative rates of alkyl transfer are consistent with S_N2 reaction kinetics. However some O^6-alkylguanines are much poorer substrates for MGMT than would be expected. Examples include oligodeoxyribonucleotides (ODNs) containing the epoxide-derived lesion O^6-(hydroxyethyl)-guanine (HOEtG, **1**)[6] and O^6-[4-oxo-4-(3-pyridyl)but-1-yl]-guanine (pobG, **2**)[7] formed following exposure to the nitrosamine (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) found in tobacco smoke. For pobG the repair reaction is almost 3500-fold slower than for O^6-(methyl)guanine (MeG).[4]

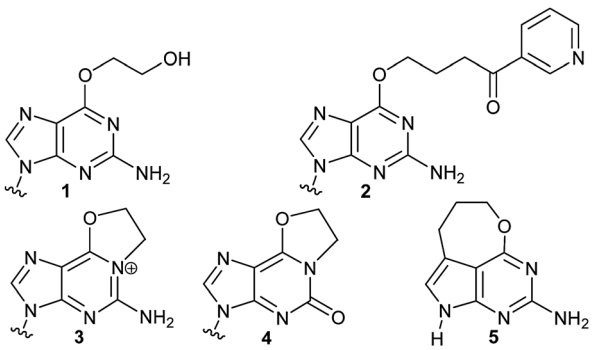

Fig. 1 Selected O^6-alkylguanines and analogues: O^6-hydroxyethyl-guanine (**1**), O^6-[4-oxo-4-(3-pyridyl)but-1-yl]guanine (**2**) and N^1,O^6-ethanoguanine (**3**), N^1,O^6-ethanoxanthine (**4**) and **5**.

In the crystal structure of the catalytically inactive C145S MGMT mutant bound to O^6-methylguanine-containing DNA the alkyl group adopts the *syn* conformation (Fig. 2), aligning it with Ser145 and is positioned for in-line displacement by Cys145 in wild-type MGMT (Fig. 3).[8] In this orientation the alkyl group fits into a hydrophobic binding pocket defined by a Met134 side chain and an active site loop (Val155–Gly160). Although relatively large alkyl groups can be accommodated, Ser159 might experience a steric clash with an alkyl group in the *anti* conformation (Fig. 2 and 3). Notably, N^1,O^6-ethano-guanine (**3**) and N^1,O^6-ethanoxanthine (**4**) which are locked in the *syn* conformation are recognised by MGMT and result in protein–DNA crosslinking following alkyl transfer. Recently it has been suggested that HOEtG and pobG are repaired less effectively by displaying higher proportions of *anti*

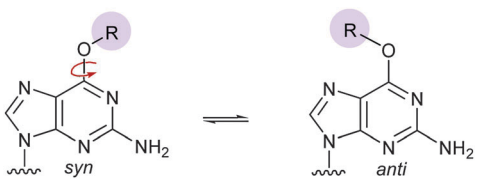

Fig. 2 *Syn* and *anti* conformations of O^6-alkylguanines.

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† In memory of Professor Har Gobind Khorana (1922–2011), acknowledging his legacy to the scientific community.

‡ This article is part of the 'Nucleic acids: new life, new materials' web themed issue.

§ Electronic supplementary information (ESI) available: Full Methods and NMR spectra of compounds synthesised, ODN mass spectra and MGMT/ATL assays. See DOI: 10.1039/c2cc36252j

¶ The authors wish it to be known that in their opinion the first two authors should be regarded as joint first authors.

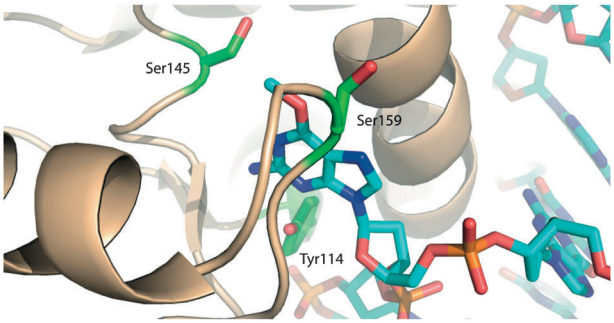

Fig. 3 Complex of C145S MGMT with O^6-methylguanine-containing DNA duplex ref. (PDB accession number IT38).

conformation or that this conformation may be stabilised by MGMT perhaps *via* hydrogen bonding interactions of the side chains to the Ser159 OH group.[4]

Here we describe the chemical synthesis of an ODN containing the analogue **5** in which the alkyl group is locked in the *anti* conformation and test the hypothesis that the orientation of the O^6-alkoxy group is critical to MGMT-mediated repair.

Typically 7-deazapurine-containing 2′-deoxyribonucleosides are prepared by *N*-alkylation of the deazapurine anion with 1-chloro-2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranose (chlorosugar) in acetonitrile.[9,10] Unfortunately the heterocycle **5**[11] displayed poor solubility in acetonitrile and was only soluble in DMF, a solvent in which anomerisation of the chlorosugar competes with S_N2 displacement.[12] Consequently we envisaged that the known pyrrolopyrimidine **6**[11] could be converted into **7** that after glycosylation and removal of the benzyl group, could be cyclised. Following reaction of compound **6** with $POCl_3$, compound **7** was isolated by silica chromatography and although a single component by silica tlc, ESI-MS revealed two mass ions corresponding to **7** and the bis-chloro compound **8**. ^{1}H NMR revealed a 3 : 1 ratio of **7** : **8** based on the integration of the CH_2 protons attached to the benzyloxy and chloro substituents respectively. Glycosylation of this mixture by reaction with chlorosugar in acetonitrile in the presence of NaH afforded two nucleosides **9** and **10** which could be separated by silica chromatography. The benzylated nucleoside **9** was then deprotected by treatment with a mixture of aq. ammonia solution and methanol followed by BCl_3 to afford nucleoside **11**. However, when nucleoside **11** was treated with NaH in DMF it showed little evidence of reaction, despite heating for 48 h.

In contrast, when nucleoside **10** was heated in a mixture of 1 M aq. NaOH and dioxane for 3 days a fluorescent component was observed by silica tlc along with three other nucleosides. The fluorescent nucleoside **12** was isolated in 29% yield following silica chromatography together with other nucleosides identified by ^{1}H NMR and ESI-MS as **11** and **13**. Monitoring the reaction by silica tlc revealed that the disappearance of starting nucleoside **10** coincided with the formation of tricyclic nucleoside **12** and nucleoside **13**. A fourth faster-running nucleoside was also observed which was identified following ESI-MS analysis of the reaction mixture as deprotected bis-chloro nucleoside **10** *i.e.* having lost the *p*-toluoyl protecting groups. We concluded that the cyclisation reaction proceeded by nucleophilic displacement of chloride by the pyrrolopyrimidine O4 lactam oxygen rather than *via* nucleoside **11**. Consequently we prepared the bis-chloro

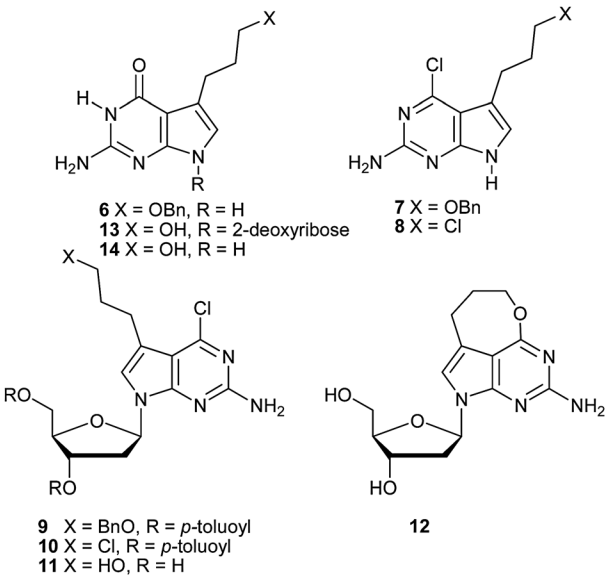

Fig. 4 Structures of compounds **9–14**.

compound **8** from the alcohol **14**[11] by heating with $POCl_3$ under reflux at 80 °C for two hours, to give **8** in 44% yield following silica chromatography. Glycosylation of **8** afforded nucleoside **10** in 60% yield after chromatography, which was then converted to the tricyclic nucleoside **12** as described above (Fig. 4).

When nucleoside **12** was reacted with *N*,*N*-dimethylformamide dimethylacetal in dry DMF the amidine **15** was formed, but this partially hydrolysed to the *N*-formyl compound **16** during silica chromatography. Equally, attempts to protect **15** as its 5′-dimethoxytrityl derivative also led to partial decomposition of the amidine function. The instability of the formamidine derivative of the O^6-methyl-7-deaza-2′-deoxyguanosine has also been noted previously.[13] Consequently it was decided to convert amidine **15** into the *N*-formyl compound **16** prior to 5′-protection. This was achieved by stirring **15** overnight in 20% aq. acetic acid. The formyl group could be completely removed from **16** with 33% aq. ammonia solution at 50 °C for 12 hours producing **12** as the sole product, showing its suitability as a protecting group for DNA synthesis. The 5′-OH group of nucleoside **16** was then protected with dimethoxytrityl chloride (DMTrCl) and DMAP in pyridine to give **17** which was subsequently reacted with 2-cyanoethyl-*N*,*N*-diisopropylamine chlorophosphoramidite to give phosphoramidite **18** as a mixture of two diastereoisomers that were characterised by ^{31}P NMR and ESI-MS (Fig. 5).

The ODNs 5′-d(GCCATG**5**CTAGTA) bearing either a 5-DMTr protecting group (ODN-1) or a fluorescent 5′-SIMA(HEX) (dichlorodiphenylfluorescein) label (ODN-2) were prepared using standard protocols on a DNA synthesiser using a 0.15 M (rather than standard 0.1 M) concentration of amidite **18**. "Base-labile" protecting groups were used for the other bases (phenoxyacetyl for G and A and acetyl for C). The ODNs were deprotected with 33% aq. ammonia at 50 °C overnight and purified by reverse-phase HPLC. The 5′-DMTr protecting group was then removed from ODN-1 using 20% aq. acetic acid and the ODNs then desalted and characterised by ESI-MS (ESI§).

To assess the repair of ODN-1 by MGMT we used the standard assay that involves pre-incubation of the protein with

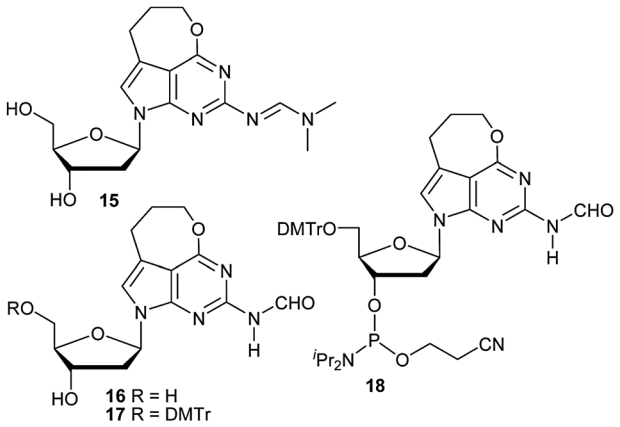

Fig. 5 Structures of compounds **15–18**.

the ODN, followed by the addition of excess DNA containing tritium-labelled O^6-methylguanine.[6,14] Since the MGMT reaction is irreversible the residual protein activity can be quantified by measuring radioactivity transferred to the protein, allowing IC_{50} values to be determined. The MeG-containing ODN exhibited an IC_{50} value of 45 nM. While there was insufficient MGMT inactivation to determine an IC_{50} for both the MeG–ODN and ODN1, an extrapolation of the IC_{20} (inactivation of 20% of the MGMT used in the assay) gave values of 0.27 nM for the MeG ODN and 4000 nM for ODN-1 (ESI§). Thus the former was around 15 000 times more potent than the latter. Furthermore we saw no evidence for complex formation between MGMT and DNA containing **5** following analysis by non-denaturing polyacrylamide gel electrophoresis (data not shown), suggesting that MGMT shows very poor recognition of the base **5**.

Recently a family of AGT-related proteins known as alkyltransferase-like (ATL) proteins have been discovered in which the active site Cys is replaced typically by Trp or Ala.[15,16] ATL proteins are consequently catalytically inactive and in organisms such as *S.pombe* that lack an AGT protein, the ATL protein (Atl1) recruits nucleotide excision repair proteins to repair O^6-alkylguanines.[17] Structural data of Atl1–DNA complexes[15,17] reveal many of the features of MGMT–DNA complexes: The target base is flipped into the active site and the alkyl group adopts the *syn* conformation. However, the hydrophobic binding pocket for the alkyl group is much larger than that found in MGMT. We were also interested to assess the recognition of the conformationally-locked analogue **5** by Atl1. Titration of native wild-type Atl1 protein into a solution containing SIMA-labelled ODNs resulted in a concentration-dependent decrease in fluorescence from which we derived an equilibrium dissociation constant (Table 1 and ESI§).[18] Whilst Atl1 binds to natural DNA (unmodified G-containing ODN) typically ODNs containing O^6-alkylguanines such as MeG and pobG are bound almost three orders of magnitude more tightly (Table 1). In contrast, Atl1 has approximately a 2-fold higher affinity for ODN-2 (containing **5**) compared to the natural sequence and an affinity that is dramatically decreased relative to ODNs containing MeG or pobG. This highlights the requirement for O^6-alkylguanine-containing substrates to be able to adopt the *syn* conformation of the damaged base for effective binding by Atl1.

In summary we have described the synthesis of DNA containing the first example of a conformationally-locked *anti* analogue of O^6-methyl-2′-deoxyguanosine. Our data shows that the *anti* orientation of the alkyl group appears to block repair by MGMT and adversely affects binding by Atl1. This lends further support to the suggestion that MGMT repairs the *syn* conformation of O^6-alkylguanines and that analogues which have higher amounts of the *anti* conformation are repaired less effectively. In common with MGMT, Atl1 also shows preferential recognition of the *syn* conformation.

Table 1 Equilibrium dissociation constants for Atl1–ODN complexes

ODN sequence	K_D (nM)
5′-SIMA-d(GCCATG(*MeG*)CTAGTA)[a]	2.4 ± 1.2
5′-SIMA-d(GCCATG(*pobG*)CTAGTA)[a]	1.1 ± 0.02
5′-SIMA-d(GCCATG(**5**)CTAGTA)	430 ± 41
5′-SIMA-d(GCCATGGCTAGTA)[a]	740 ± 91

[a] Data taken from ref. 18.

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