Nucleic Acids Research, 2001, Vol. 29, No. 19 **4025–4034**

Covalent capture of a human O^6-alkylguanine alkyltransferase–DNA complex using N^1,O^6-ethanoxanthosine, a mechanism-based crosslinker

David M. Noll* and Neil D. Clarke

Department of Biophysics and Biophysical Chemistry, Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA

Received May 24, 2001; Revised and Accepted August 8, 2001

ABSTRACT

The DNA repair protein O^6-alkylguanine alkyltransferase (AGT) is responsible for removing promutagenic alkyl lesions from exocyclic oxygens located in the major groove of DNA, i.e. the O^6 and O^4 positions of guanine and thymine. The protein carries out this repair reaction by transferring the alkyl group to an active site cysteine and in doing so directly repairs the premutagenic lesion in a reaction that inactivates the protein. In order to trap a covalent AGT–DNA complex, oligodeoxyribonucleotides containing the novel nucleoside N^1,O^6-ethanoxanthosine (^{e}X) have been prepared. The ^{e}X nucleoside was prepared by deamination of 3′,5′-protected O^6-hydroxyethyl-2′-deoxyguanosine followed by cyclization to produce 3′,5′-protected N^1,O^6-ethano-2′-deoxyxanthosine, which was converted to the nucleoside phosphoramidite and used in the preparation of oligodeoxyribonucleotides. Incubation of human AGT with a DNA duplex containing ^{e}X resulted in the formation of a covalent protein–DNA complex. Formation of this complex was dependent on both active human AGT and ^{e}X and could be prevented by chemical inactivation of the AGT with O^6-benzylguanine. The crosslinking of AGT to DNA using ^{e}X occurs with high yield and the resulting complex appears to be well suited for further biochemical and biophysical characterization.

INTRODUCTION

The alkylation of exocyclic oxygens located in the major groove of DNA, i.e. the O^6 position of guanine and to a lesser extent the O^4 position of thymine, is promutagenic and can be carcinogenic. While alkylation at the O^6 position of guanine typically constitutes a small fraction of the damage produced by exogenous alkylators it is principally responsible for the GC→AT transition mutations associated with mutagenic and carcinogenic effects of many alkylating agents (1). O^6-alkylguanine-DNA alkyltransferase (AGT) is an evolutionarily conserved DNA repair protein that is the principle cellular mechanism responsible for repair of O^6-alkylguanine (see 2–6 and references therein). AGT carries out the repair reaction by transferring the alkyl group from the exocyclic oxygen to an active site cysteine in an S_N2-like transfer. This alkyl transfer results in the formation of an alkyl cysteine and inactivation of the protein. AGT therefore is a stoichiometric DNA repair molecule rather than a true enzyme. These alkyltransferases include the *Escherichia coli* proteins Ada (the C-terminal domain) and Ogt, as well as homologs identified from eukaryotic, prokaryotic and eubacterial sources (7).

First identified as a component of the adaptive response to alkylation damage in *E.coli*, alkyltransferase activity presumably evolved to protect cellular DNA from the effects of endogenous sources of alkylation. It is, however, the ability of AGT to attenuate the effect of antitumor compounds like methylating agents (e.g. dacarbazine, procarbazine and temozolomide) and chloroethylating agents [e.g. bis-chloroethylnitrosourea (BCNU), clomesone and fotemustine] that has prompted much of the recent interest in AGT (8–10). Despite detailed biochemical characterization, recent structural data and extensive screening of AGT inhibitors, the inability to prepare AGT–DNA complexes suitable for structural characterization has hampered our understanding of how these DNA repair proteins recognize and repair damaged DNA.

We here describe a strategy for covalently trapping a post-repair AGT–DNA complex. This strategy is based on the observation that covalent protein–DNA complexes form when chloroethylnitrosourea (CENU)-treated DNA is incubated with AGT (11). The CENUs are a class of bifunctional crosslinking agents that produce interstrand crosslinks via N^1,O^6-ethanoguanine (^{e}**G**), an activated compound that results from intramolecular cyclization of the initial chloroethylation at the O^6 position of guanine (12). As shown in Figure 1, intramolecular cyclization of the initial CENU alkylation product O^6-chloroethylguanine (pathway a) gives rise to ^{e}**G**. The positively charged ^{e}**G** is highly reactive and can undergo nucleophilic attack by the cytosine located on the opposite strand of DNA (pathway b) to produce a G-C interstrand crosslink (12). This G-C crosslink is believed to be the principal

*To whom correspondence should be addressed. Tel: +1 410 614 1760; Fax: +1 410 502 6910; Email: dmndawit@jhmi.edu

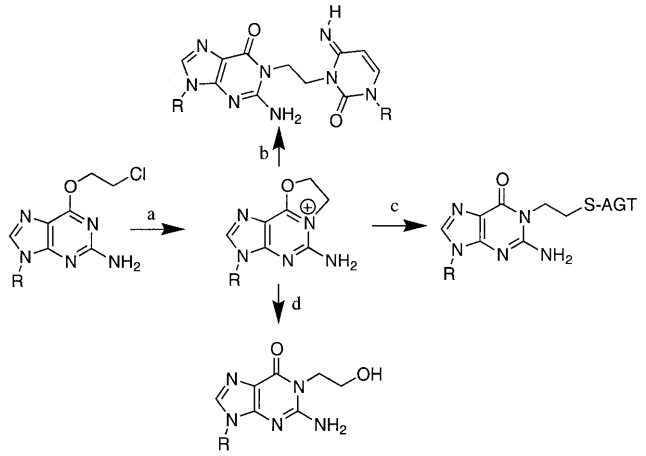

Figure 1. Formation and reactions of N^1,O^6-ethanoguanine. Intramolecular cyclization of the initial chloroethylnitrosourea adduct (a) generates the reactive ethano intermediate which can crosslink DNA (b), be 'repaired' by AGT (c) or react with water (d).

Figure 2. Structures of N^1,O^6-ethanoguanine (**^{e}G**) and N^1,O^6-ethanoxanthine (**^{e}X**).

cytotoxic lesion produced by CENU. The observation that DNA treated with BCNU in the presence of AGT produced a small amount of covalently trapped AGT–DNA suggested that **^{e}G** might be a substrate for AGT (13–15). The 'repair' of **^{e}G** (pathway c) results in a covalently trapped protein–DNA complex. Characterization of the crosslinked species confirmed the structure of the covalent protein–DNA crosslink and suggested that crosslinking occurred as a result of nucleophilic attack by the active site cysteine on the O^6-CH_2- portion of the ethano bridge (11). The hyper-reactivity of **^{e}G** also results in its hydrolysis (pathway d), producing N^1-hydroxyethylguanine (16).

The ability of the N^1,O^6-ethano bridge to be both a substrate for AGT and a covalent tether is the basis of our crosslinking strategy. Unfortunately, the hydrolytic sensitivity of **^{e}G** as well as its incompatibility with the methods associated with automated synthesis of oligodeoxyribonucleotides greatly reduce its potential as a mechanism-based crosslinker. Therefore, we set out to design a nucleoside analog that would retain the major groove structure of **^{e}G** but be stable enough to withstand both chemical and enzymatic manipulation. Replacement of the exocyclic amino group of guanine with oxygen to give N^1,O^6-ethanoxanthine (**^{e}X**) (Fig. 2) satisfied these conditions. This xanthine derivative maintains the major groove structure while taking advantage of the uncharged tautomeric form accessible to xanthine.

Below we report the synthesis of the nucleoside phosphoramidite of **^{e}X**, the preparation of oligodeoxyribonucleotides containing **^{e}X** and the formation of covalent protein–DNA complexes between human AGT and an oligodeoxyribonucleotide containing a single **^{e}X**.

MATERIALS AND METHODS

Materials

2′-Deoxyguanosine was purchased from US Biochemicals (Cleveland, OH). All other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous solvents were used as supplied. TLC was conducted on EM plastic-backed sheets (silica gel 60 F254, 0.2 mm). The 5′-*O*-dimethoxytrityl-2′-deoxyribonuceoside-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites of *N*-acetyl-2′-deoxycytidine, *N*-phenoxyacetyl-2′-deoxyadenosine and *N*-(4-isopropyl-phenoxyacetyl)-2′-deoxyguanosine and thymidine as well as the appropriately protected deoxyribonucleoside controlled pore glass supports were purchased from Glen Research Inc. (Sterling, VA). Anhydrous acetonitrile for use in the oligodeoxyribonucleotide synthesis was stored over calcium hydride. The enzymes shrimp alkaline phosphatase (SAP) and T4 polynucleotide kinase (PNK) were purchased from New England Biolabs (Beverly, MA); snake venom phosphodiesterase (SVP) from *Crotalus durissus* was purchased from Boehringer Mannheim (Indianapolis, IN). Human AGT was a gift from Dr Phil Potter (St Jude).

General procedures

Flash column chromatography was performed on Merck silica gel 60, 230–400 mesh. Absorption spectra were recorded using a Varian DMS100S UV/Vis spectrophotometer. HPLC separations and analyses were conducted on a Varian 9010/50 system. ^{1}H, ^{13}C and ^{31}P NMR were performed on a Bruker AMX300 spectrometer; chemical shifts are in p.p.m. and proton chemical shifts are reported relative to internal tetramethylsilane ($CDCl_3$) or to residual solvent peaks (DMSO); ^{31}P spectra were collected with broadband proton noise decoupling and referenced to an external 85% H_3PO_4 standard. High resolution mass spectrometry (MALDI-FTMS) was performed by the Scripps Research Institute Mass Spectrometry Facility.

Synthesis of 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-2′-deoxyguanosine (3)

The 3′- and 5′-hydroxyls of commercially available 2′-deoxyguanosine were protected using t-butyldimethylsilylchloride (17). 3′,5′-TBDMSi-dG (**2**) (3 g, 6 mmol) was dried by rotary evaporation (*in vacuo*) with anhydrous pyridine, dissolved in 60 ml of anhydrous pyridine and cooled in an ice bath. To this stirred mixture, trifluoroacetic anhydride (3.0 ml, 21 mmol) was added drop-wise. After stirring for 30 min, 150 ml of a freshly prepared solution of sodium in ethylene glycol (12.5 g sodium in 250 ml of anhydrous ethylene glycol dissolved in 3 g batches over a 24 h period) was added. The reaction was stirred overnight at room temperature. The resulting reddish-brown mixture was poured into 500 ml of water and extracted with four 200 ml portions of chloroform. The combined organic layers were dried over sodium sulfate and, following filtration, the solvent was removed by rotary evaporation (*in vacuo*). Traces of pyridine were removed by co-evaporation with two 50 ml portions of toluene. The resulting oil was

dissolved in 10 ml of chloroform/methanol (95:5) and purified by flash column chromatography on a silica gel column using chloroform/methanol (95:5). Fractions containing product were pooled and the solvent removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-2′-deoxyguanosine (**3**) as a yellow foam (2.75 g, 5.4 mmol; yield 90%). UV_{max} 279, 247 in CH_3OH; R_f 0.15 in $CHCl_3$/CH_3OH (95:5); ^1H NMR (d_6-DMSO) δ (p.p.m.) 8.05 [s, 1.0H, *H8*], 6.42 [s, 1.9H, N^2H_2], 6.21 [t, 1.0H, *H1′*], 4.89 [t, 0.5H, $O^6CH_2CH_2OH$], 4.52 [m, 1.0H, *H3′*], 4.41 [t, 1.9H, $O^6CH_2CH_2OH$], 3.82 [m, 1.0H, H4′], 3.62–3.77 [m, 3.9H, *H5′H5″*/$O^6CH_2CH_2OH$], 2.72 [m, 1.0, *H2′*], 2.27 [m, 1.0H, *H2′*], 0.89–0.87 [s&s, 17.7H, $(CH_3)_3C(CH_3)_2Si$], 0.10 [s, 5.8H, $(CH_3)_3C(CH_3)_2Si$], 0.04 [s, 5.8H, $(CH_3)_3C(CH_3)_2Si$].

Synthesis of 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-2′-deoxyxanthosine (4)

Deamination was accomplished by dissolving **3** (2.75 g, 5.4 mmol) in 10 ml of acetone, which was transferred to a reaction vessel containing a solution of $NaNO_3$ (10 g) in 30 ml of water. To this mixture 15 ml of glacial acetic acid was added and the solution was vigorously agitated until the evolution of nitrogen ended, ~2 h. The reaction was neutralized by adding sodium bicarbonate in batches, poured into 500 ml of water and extracted with two 200 ml portions of chloroform. The combined organic layers were dried over sodium sulfate and, after filtration, the solvent removed by rotary evaporation (*in vacuo*). The resulting foam was dissolved in 5 ml of chloroform/methanol (95:5) and purified by flash column chromatography on a silica gel column using chloroform/methanol (95:5). Fractions containing product were pooled and solvent removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-2′-deoxyxanthosine (**4**) as a tan foam (1.76 g, 3.5 mmol; yield 65%). UV_{max} 266, 237 in CH_3OH; R_f 0.38 in $CHCl_3$/CH_3OH (90:10); ^1H NMR (d_6-DMSO) δ (p.p.m.) 11.57 [bs, 0.4H, N^3H], 8.22 [s, 1.0H, *H8*], 6.25 [t, 1.0H, *H1′*], 4.91 [bs, 0.3H, $O^6CH_2CH_2OH$], 4.60 [m, 1.0H, *H3′*], 4.47 [t, 2.0H, $O^6CH_2CH_2OH$], 3.82 [m, 1.0H, H4′], 3.78–3.62 [m, 3.9H, *H5′H5″*/$O^6CH_2CH_2OH$], 2.77 [m, 1.0, *H2′*], 2.30 [m, 1.0H, *H2′*], 0.89–0.86 [s&s, 17.7H, $(CH_3)_3C(CH_3)_2Si$], 0.11 [s, 5.8H, $(CH_3)_3C(CH_3)_2Si$], 0.02 [d, 5.8H, $(CH_3)_3C(CH_3)_2Si$].

Synthesis of 3′,5′-t-butyldimethylsilyl-O^6-(dimethoxytrityl-*O*-ethyl)-2′-deoxyxanthosine (5)

4 (1.7 g, 3.4 mmol) was dried by rotary evaporation (*in vacuo*) with anhydrous pyridine and dissolved in 20 ml of anhydrous pyridine. Dimethoxytrityl chloride (1.5 equiv., 1.7 g) was added and the reaction stirred overnight at room temperature. The reaction was quenched by addition of 3 ml of 95% ethanol and allowed to stir for 15 min. The reaction mixture was concentrated by rotary evaporation (*in vacuo*), dissolved in 200 ml of ethyl acetate and extracted with two 200 ml portions of 5% sodium bicarbonate and one 200 ml portion of a saturated NaCl solution. The resulting organic layer was dried over sodium sulfate and, after filtration, the solvent removed by rotary evaporation (*in vacuo*). The resulting foam was dissolved in 5 ml chloroform/methanol (99:1) with a trace of triethylamine and purified by flash column chromatography using chloroform/methanol (99:1) in the presence of trace triethylamine. Fractions containing product were pooled and the solvent was removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-O^6-(dimethoxytrityl-*O*-ethyl)-2′-deoxyxanthosine (**5**) (1.9 g, 2.35 mmol; yield 70%). ^1H NMR (d_6-DMSO) δ (p.p.m.) 11.61 [bs, 0.6H, N^3H], 8.29 [s, 0.8H, *H8*], 7.39–6.81 [m, 12.9, Ph-*H*], 6.27 [t, 1.0H, *H1′*], 4.68 [bs, 1.9H, $O^6CH_2CH_2OH$], 4.57 [m, 1.0H, *H3′*], 3.77 [m, 1.0H, H4′], 3.76–3.63 [m, 7.9H, *H5′H5″*/Ph-OCH_3], 3.3 [bs, 1.5H, $O^6CH_2CH_2OH$], 2.80 [m, 1.0, *H2′*], 2.32 [m, 1.0H, *H2′*], 0.96–0.80 [s&s, 18.1H, $(CH_3)_3C(CH_3)_2Si$], 0.12 [s, 5.1H, $(CH_3)_3C(CH_3)_2Si$], 0.02 [d, 5.0H, $(CH_3)_3C(CH_3)_2Si$].

Synthesis of 3′,5′-t-butyldimethylsilyl-O^6-(dimethoxytrityl-*O*-ethyl)-O^2-nitrophenethyl-2′-deoxyxanthosine (6)

5 (1.9 g, 2.35 mmol) was dissolved in 25 ml of anhydrous 1,4-dioxane and stirred at room temperature. To the reaction vessel 3 equiv. of triphenylphosphine (1.85 g), 3 equiv. of diethyl azodicarboxylate (1.11 ml) was added, followed by 3 equiv. of nitrophenylethanol (1.17 g). Following a rapid evolution of heat, the reaction vessel was stirred overnight at room temperature. The reaction was quenched by the addition of 5 ml of 95% ethanol and stirred for 5 min. The solvents were removed by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 5 ml of chloroform and purified using flash column chromatography on silica gel using chloroform. Pure fractions were pooled; fractions containing product and contaminating reaction components were pooled, dried and re-chromatographed. Fractions containing product were pooled and the solvent was removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-O^6-(dimethoxytrityl-*O*-ethyl)-O^2-nitrophenethyl-2′-deoxyxanthosine (**6**) as a white foam (1.4 g, 1.46 mmol; yield 62%). ^1H NMR (d_6-DMSO) δ (p.p.m.) 8.36 [s, 1.0H, *H8*], 8.14 [d, 1.7H, Ph-*H*], 7.74–6.78 [m, 19.4, Ph-*H*], 6.30 [t, 1.0H, *H1′*], 4.71 [bs, 2.1H, $O^6CH_2CH_2OH$], 4.56 [m, 3.0H, *H3′*, O^2-CH_2CH_2-Ar], 3.83 [m, 1.1H, H4′], 3.73–3.63 [m, 8.8H, *H5′H5″*/Ph-OCH_3/O^2-CH_2CH_2-Ar], 3.3 [t, 2.5H, $O^6CH_2CH_2OH$], 2.91 [m, 1.3, *H2′*], 2.31 [m, 1.1H, *H2′*], 0.95–0.80 [s&s, 20.6H, $(CH_3)_3C(CH_3)_2Si$], 0.12 [s, 5.8H, $(CH_3)_3C(CH_3)_2Si$], –0.02 [d, 5.6H, $(CH_3)_3C(CH_3)_2Si$].

Synthesis of 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-O^2-nitrophenethyl-2′-deoxyxanthosine (7)

6 (1.4 g, 1.46 mmol) was dissolved in 3 ml of dichloromethane and transferred to a separation funnel. To the separation funnel was added 50 ml of 2.5% dichloroacetic acid in dichloromethane. The solution turned bright orange and was allowed to stand for 5 min. Water (50 ml) was added to the separation funnel followed by small batches of solid sodium bicarbonate. Following agitation the pH of the aqueous layer was checked and sodium bicarbonate was added until the solution was neutralized. The organic layer was collected and washed with two 50 ml portions of 5% sodium bicarbonate and one 50 ml portion of a saturated NaCl solution. The organic layer was dried over sodium sulfate and, after filtration, the solvent removed by rotary evaporation (*in vacuo*). The resulting foam was dissolved in 5 ml of chloroform and purified by flash column chromatography on a silica gel column using chloroform/methanol (99:1). Fractions containing product were pooled and solvent removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-O^6-hydroxyethyl-O^2-nitrophenethyl-2′-deoxyxanthosine (**7**) (700 mg, 1.07 mmol; yield 73%). ^1H NMR (d_6-DMSO) δ (p.p.m.) 8.31 [s, 1.3H, *H8*],

8.17 [d, 1.6H, Ph-*H*], 6.28 [t, 1.0H, *H1′*], 4.93 [t, 0.7H, $O^6CH_2CH_2O$*H*], 4.58 [m, 3H, *H3′*, O^2-C*H*$_2$CH$_2$-Ar], 4.49 [t, 2.0H, $O^6CH_2CH_2OH$], 3.83–3.64 [m, 5.3H, *H4′*, *H5′*/*H5″*], 2.89 [m, 1.2, *H2′*], 2.31 [m, 1.1H, *H2′*], 0.88–0.84 [s&s, 19.0H, (C*H*$_3$)$_3$C(CH$_3$)$_2$Si], 0.09 [s, 5.5H, (CH$_3$)$_3$C(C*H*$_3$)$_2$Si], –0.01 [d, 5.2H, (CH$_3$)$_3$C(C*H*$_3$)$_2$Si].

Synthesis of 3′,5′-t-butyldimethylsilyl-*N*^{1},*O*^{6}-ethano-2′-deoxyxanthosine (9)

7 (700 mg, 1.07 mmol) was dried by rotary evaporation (*in vacuo*) with anhydrous pyridine and dissolved in 20 ml of anhydrous pyridine and 2,4,6-triisopropylbenzenesulphonyl chloride (4.5 equiv., 1.5 g) was added. The reaction was allowed to stir at room temperature for 2 h; an additional equivalent of 2,4,6-triisopropylbenzenesulphonyl chloride was required to get complete conversion of the starting material as measured by TLC. The reaction was quenched by the addition of 20 ml of absolute ethanol. The resulting solution was concentrated by rotary evaporation (*in vacuo*) to give an oil. The oil was dissolved in 5 ml of pyridine and 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) was added to give a final concentration of 0.5 M. The reaction was stirred for 3 h. The reaction was concentrated by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 50 ml of ethyl acetate and washed with two 50 ml portions of a 5% sodium bicarbonate solution and one 50 ml portion of a saturated NaCl solution. The organic layer was dried over sodium sulfate and, after filtration, the solvent was removed by rotary evaporation. The resulting oil was dissolved in chloroform/methanol and purified by flash column chromatography on a silica gel column using chloroform/methanol (95:5). Fractions containing product were pooled and solvent removed by rotary evaporation (*in vacuo*) to give 3′,5′-t-butyldimethylsilyl-*N*^{1},*O*^{6}-ethano-2′-deoxyxanthosine (**9**) as a foam (350 mg, 0.71 mmol; yield 67%). ^{1}H NMR (d_6-DMSO) δ (p.p.m.) 8.07 [s, 1.0H, *H8*], 6.15 [t, 1.0H, *H1′*], 4.77 [t, 1.9H, O^6-C*H*$_2$CH$_2$-N^1], 4.57 [m, 1.0H, H3′], 4.21 [t, 2.0H, O^6-C*H*$_2$C*H*$_2$-N^1], 3.85–3.61 [m, 3.2H, *H4′*, *H5′*/*H5″*], 2.72 [m, 1.1H, *H2″*], 2.29 [m, 1.1H, *H2′*], 0.89–0.85 [s&s, 19.0H, (C*H*$_3$)$_3$C(CH$_3$)$_2$Si], 0.11 [s, 6.3H, (CH$_3$)$_3$C(C*H*$_3$)$_2$Si], 0.02 [d, 6.2H, (CH$_3$)$_3$C(C*H*$_3$)$_2$Si]; MALDI-FTMS, expected for $C_{24}H_{42}N_4O_5Si_2$ 522.79, found 523 [H$^+$], 545 [Na$^+$].

Synthesis of *N*^{1},*O*^{6}-ethano-2′-deoxyxanthosine (10)

9 (350 mg, 0.71 mmol) was dissolved in 2 ml of anhydrous tetrahydrofuran. One milliliter of a 1 M solution of tetrabutylammonium fluoride in tetrahydrofuran was added and the reaction stirred at room temperature for 45 min. The solvent was removed by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 2 ml of chloroform/methanol (95:5) and loaded onto a silica gel column. The column was washed with two column volumes of chloroform/methanol (95:5) and product eluted with chloroform/methanol (80:20). Fractions containing product were pooled and solvent removed by rotary evaporation (*in vacuo*) to give *N*^{1},*O*^{6}-ethano-2′-deoxyxanthosine (**10**) as a white solid (160 mg, 0.54 mmol; yield 77%). UV_{max} 246 in CH_3OH; R_f 0.24 in $CHCl_3$/CH_3OH (80:20); ^{1}H NMR (d_6-DMSO) δ (p.p.m.) 8.11 [s, 0.93H, *H8*], 6.16 [t, 1.0H, *H1′*], 5.30 [d, 0.21H, 3′-O*H*], 4.93 [t, 0.19H, 5′-O*H*], 4.77 [t, 1.9H, O^6-C*H*$_2$CH$_2$-N^1], 4.35 [m, 1.0H, *H3′*], 4.21 [t, 2.1H, O^6-CH$_2$C*H*$_2$-N^1], 3.84 [m, 1.0H, *H4′*] 3.59–3.46 [m, 2.1H, *H5′*/*H5″*], 2.23 [m, 1.2H, *H2′*]; MALDI-FTMS, expected for $C_{12}H_{14}N_4O_5$ 317.0856 [Na$^+$], found 317.0859 [Na$^+$].

Synthesis of *N*^{1},*O*^{6}-ethano-5′-*O*-dimethoxytrityl-2′-deoxyxanthosine (11)

10 (160 mg, 0.54 mmol) was dried by rotary evaporation (*in vacuo*) with anhydrous pyridine and dissolved in 5 ml of anhydrous pyridine. Dimethoxytrityl chloride (1.1 equiv., 200 mg) was added. The reaction was stirred overnight at room temperature; an additional 100 mg dimethoxytrityl chloride and 10 mg dimethylaminopyridine were required to get complete conversion of the starting material as measured by TLC. The reaction was quenched by addition of 0.5 ml of 95% ethanol and stirred for 5 min. The reaction mixture was concentrated by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 25 ml of ethyl acetate and the organic layer washed with two 25 ml portions of a 5% sodium bicarbonate solution and one 25 ml portion of a saturated NaCl solution. The organic layer was dried over sodium sulfate and, after filtration, the solvent was removed by rotary evaporation. The resulting foam was dissolved in 1.5 ml of chloroform/methanol/triethylamine (95:4.5:0.5) and purified by flash column chromatography on a silica gel column using chloroform/methanol/triethylamine (95:4.5:0.5). Fractions containing product were pooled and solvent removed by rotary evaporation (*in vacuo*) to give *N*^{1},*O*^{6}-ethano-5′-*O*-dimethoxytrityl-2′-deoxyxanthosine (**11**) as a white foam (190 mg, 0.32 mmol; yield 60%). ^{1}H NMR ($CDCl_3$) δ (p.p.m.) 8.96 [s, 1.0H, *H8*], 7.42–6.76 [m, 14.8H, Ar-*H*], 6.25 [t, 1.0H, *H1′*], 4.83 [t, 2.2H, O^6-C*H*$_2$CH$_2$-N^1], 4.56 [m, 1.1H, *H3′*], 4.36 [t, 2.3H, O^6-CH$_2$C*H*$_2$-N^1], 4.07 [m, 1.1H, *H4′*] 3.88–3.71 [m, 8.1H, *H5′*/*H5″*/Ar-OC*H*$_3$], 2.66 [m, 1.1H, *H2′*], 2.37 [m, 1.1H, *H2′*].

Synthesis of *N*^{1},*O*^{6}-ethano-5′-*O*-dimethoxytrityl-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite-2′-deoxyxanthosine (12)

11 (172 mg; 0.29 mmol) was dissolved in 4 ml of anhydrous dichloromethane. To the stirred solution 500 µl of diisopropylethylamine was added, followed by drop-wise addition of 150 µl of 2-cyanoethyl diisopropylchlorophosphoramidite. The reaction was allowed to proceed for 1 h, at which time the reaction was judged complete by TLC (chloroform/ethyl acetate/triethylamine 45:45:10). The reaction was quenched with 1 ml of anhydrous methanol and the solvents were removed by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 1 ml of chloroform and purified by flash column chromatography on silica gel using chloroform/ethyl acetate/triethylamine (45:45:10). Fractions containing product were pooled and the solvent removed by rotary evaporation (*in vacuo*). The resulting oil was dissolved in 1 ml of ethyl acetate and the product precipitated from rapidly stirring petroleum ether at –40°C. The resulting precipitate was collected by centrifugation and dried under vacuum to give *N*^{1},*O*^{6}-ethano-5′-*O*-dimethoxytrityl-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite-2′-deoxyxanthosine (**12**) as a white powder (190 mg, 0.24 mmol; yield 84%). ^{31}P NMR (CD_3OD) δ (p.p.m.) 157.50, 157.23.

Oligodeoxyribonucleotide synthesis, purification and characterization

Three oligodeoxyribonucleotides, NC-test (d-AC^{e}**X**GT), NC4 (d-CAGACTGCCG^{e}**X**CGCTGCAGGT) and NC1 (d-ACCT-GCAGCGCCGGCAGTCTG), were prepared on an Applied Biosystems model 392 DNA synthesizer using standard phosphoramidite chemistry. Phosphoramidite solutions of *N*-acetyl-protected 2′-deoxycytidine, *N*-phenoxyacetyl-protected 2′-deoxyadenosine and *N*-(4-isopropyl-phenoxy-acetyl)-protected 2′-deoxyguanosine were 0.1 M in concentration. A 0.15 M solution of **12** was used with an extended coupling period of 120 s. Coupling efficiency of **12** was >97% as measured by the trityl cation. The 5′-terminal dimethoxytrityl group was removed by the synthesizer and the oligomer-derivatized support transferred to a screw cap glass vial. The support was treated with 500 µl of concentrated ammonium hydroxide for 2 h at 37°C. The supernatant was removed from the support and the support washed with two 400 µl aliquots of 50% aqueous acetonitrile. The combined supernatant and washings were evaporated to dryness under vacuum at 37°C. Oligodeoxyribonucleotides were purified by strong anion exchange (SAX) HPLC on a Rainin Dynamax II column (0.46 × 15 cm) using a linear gradient of 0.0–0.8 M ammonium sulfate in a buffer that contained 1 mM ammonium acetate, pH 6.2, in 20% acetonitrile at a flow rate 0.6 ml/min. The column was monitored at 290 nm for preparative runs. The oligomers were desalted on a C-18 SEP PAK cartridge. The SEP PAK was pre-equilibrated by washing with 10 ml aliquots of acetonitrile, 50% aqueous acetonitrile and 2% acetonitrile in 50 mM sodium phosphate, pH 5.8 (buffer A). The oligodeoxyribonucleotide solution was diluted in buffer A to a final acetonitrile concentration of 3% and the solution applied to the cartridge. The cartridge was washed with 10 ml of water and the oligomer was eluted with 3 ml of 50% aqueous acetonitrile. Oligomers containing ^{e}**X** were subjected to enzymatic digestion with a combination of SVP and SAP by dissolving 0.1 A_{260} units in 10 µl of a solution containing 10 mM Tris, pH 8.1, 2 mM magnesium chloride, 1 µl of SVP and 0.5 µl of SAP. After 16 h at 37°C digests were analyzed by C-18 reversed phase HPLC on a Rainin Microsorb-C-18 column (0.46 × 15 cm) using a gradient of 2–3% acetonitrile over 12 min followed by a gradient of 3–20% acetonitrile over 8 min in buffer A.

Crosslinking experiments

Oligomer NC4 (4×10^{-10} mol) was radiolabeled in 9.5 µl of a solution containing 70 mM Tris, pH 7.6, 10 mM magnesium chloride, 5 mM dithiothreitol (DTT) and 63 µM [γ-^{32}P]ATP (sp. act. 17 µCi/mmol). The reaction was initiated by addition of 0.5 µl (5 U) of T4 PNK and incubated at 37°C for 16 h. End-labeling reactions were heated to 65°C for 20 min in order to inactivate T4 PNK. Excess radiolabeled ATP was not removed. Radiolabeled NC4 (5 µl) was added to a 20 µM solution of cold duplex (45 µl) containing a 10% excess of NC1 to produce a 22 µM NC1/NC4* solution in crosslinking buffer (20 mM Tris, pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM DTT and 10% glycerol). Annealing was carried out by placing samples in a heat block at 95°C for 5 min, which was then allowed to gradually cool to room temperature and finally placed on ice for 5 min.

Crosslinking was initiated by addition of 4 µl of the 22 µM NC1/NC4* solution (final concentration 4 µM) to a reaction mixture containing 50 µM human AGT (a gift from Phil Potter) in crosslinking buffer. The reaction was incubated at 37°C and quenched by addition of 6× SDS loading buffer, followed by heating at 100°C for 5 min. The reaction volume was increased 10-fold for crosslinking time courses and the reaction mixture was centrifuged at 14 000 r.p.m. in a micro-centrifuge prior to removal at each time point. Inactivation of hAGT was accomplished by heating the sample to 95°C for 2 min and then equilibrating at 37°C prior to addition of radiolabeled duplex or by incubating hAGT with 100 µM O^6-benzylguanine [i.e. 1 µl of 2 mM O^6-benzylguanine dissolved in dimethylsulfoxide (DMSO)] prior to addition of the radiolabeled duplex. O^6-Benzylguanine was prepared as previously described (18). In order to control for the effect that DMSO has on crosslinking efficiency, a mock inactivation sample was adjusted to 10% DMSO prior to addition of hAGT.

RESULTS

Preparation of N^1,O^6-ethano-2′-deoxyxanthosine

The synthetic route used to prepare the 5′-dimethoxytrityl-3′-cyanoethylphosphoramidite of ^{e}**X** is shown in Figure 3. The 5′- and 3′-hydroxyl groups of commercially available 2′-deoxy-guanosine were protected with t-butyldimethylsilyl groups (17). A one-pot double displacement procedure (19) allowed the introduction of a hydroxyethyl group at the O^6 position and nitrous acid-mediated deamination (20) of O^6-hydroxyethyl-guanine gave the protected O^6-hydroxyethylxanthosine, **4**. The direct cyclization of **4** to produce the ethano bridged compound resulted in low (<2%) yields. To increase overall yields a step-wise approach was pursued. The newly introduced hydroxyethyl group was protected using dimethoxytritylchloride to give **5**, followed by protection of the carbonyl O^2 with a nitrophenethyl group via the Mitsunobo reaction (21) to give **6**. The DMT group was then removed with acid to give **7**. Activation of the exposed hydroxyl was carried out by converting it to a benzene sulphonyl derivative, **8**. Attempts to purify **8** were problematical but *in situ* treatment of **8** (no purification) with 0.5 M DBU resulted in production of the desired ^{e}**X** derivative. The ethano bridge presumably results via an intramolecular cyclization in which N^1 displacement of the sulphonyl derivative is initiated by base-catalyzed removal of the NPE protecting group. The protected nucleoside was converted to the phosphoramidite of N^1,O^6-ethano-2′-deoxyxanthosine, **12**, using conventional strategies.

Preparation of oligodeoxyribonucleotides containing ^{e}X

Oligodeoxyribonucleotides NC4 and NC-test, each containing a single ^{e}**X**, were prepared as described in Materials and Methods. Initial attempts to deprotect oligodeoxyribo-nucleotides containing ^{e}**X** using concentrated NH_4OH at elevated temperatures (65°C for 3 h) resulted in complete degradation of ^{e}**X**. In order to determine the exact nature of the degradation an aliquot of **10** was treated with concentrated NH_4OH at 65°C for 3 h and analyzed by reverse phase HPLC and mass spectrometry. Three degradation products whose retention times differed from **10** were observed. The two major species (50 and 40%) were identified from their molecular

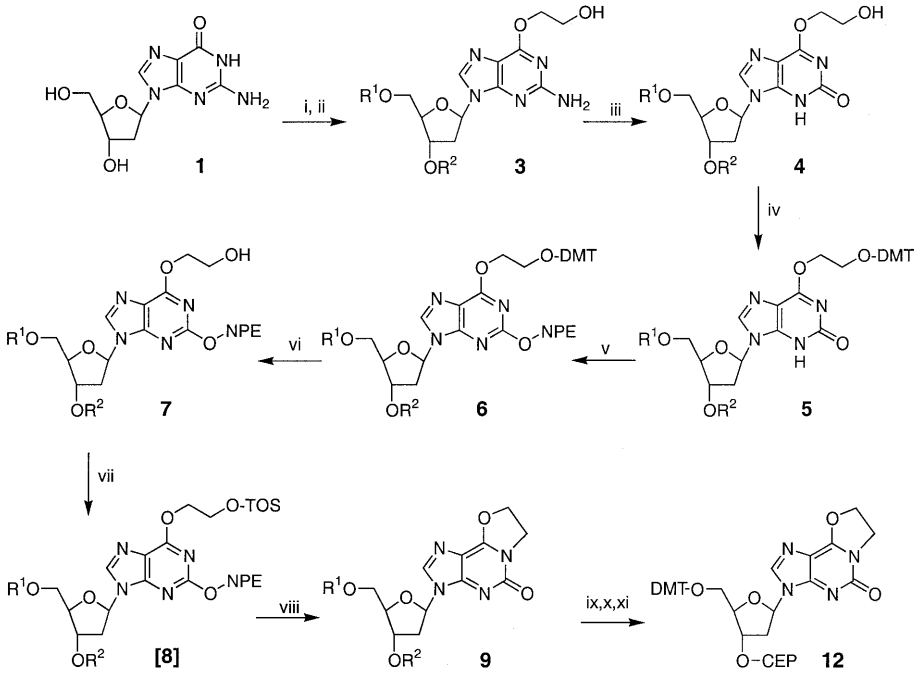

Figure 3. Synthetic route used to prepare the nucleoside phosphoramidite of N^1,O^6-ethano-2′-deoxyxanthosine: (i) TBDMSiCl, imidazole, dimethylformamide, 1 h; (ii) trifluoroacetic anhydride, pyridine, 0°C, followed by Na/ethylene glycol; (iii) $NaNO_3$, aqueous acetic acid, acetone, 2 h with agitation; (iv) 4,4′-dimethoxytritylchloride (DMTrCl), pyridine; (v) *p*-nitrophenylethanol (NPE), triphenylphosphine, diethyl azodicarboxylate, dioxane; (vi) 2% dichloroacetic acid/CH_2Cl_2, followed by $NaHCO_3$ neutralization; (vii) 2,4,6-triisopropylbenzenesulfonylchloride, pyridine; (viii) 0.1 M DBU in pyridine; (ix) 0.5 M t-butylammonium fluoride in tetrahydrofuran; (x) DMTrCl, pyridine; (xi) *N*,*N*-diisopropylethylamine, 2-cyanoethyl diisopropylchlorophosphoramidite, CH_2Cl_2. R^1, R^2, TBDMSi; DMT, dimethoxytrityl; NPE, *p*-nitrophenylethane; TOS, 2,4,6-triisopropylbenzenesulfonyl; CEP, cyanoethyl diisopropylphosphoramidite.

weight as the nucleoside and free base which correspond to ammonia displacement of the O^6-ethyl portion of the ethano bridge (data not shown). Efforts to identify the third species were unsuccessful. This type of degradation of O^6-alkylguanine analogs has been noted in the literature (22) and can be easily circumvented by using nucleoside phosphoramidites with protecting groups that can be removed under milder conditions (e.g. acetyl, PAC and iPR-PAC groups).

Oligodeoxyribonucleotides prepared in this manner were cleaved from the support and deprotected using concentrated NH_4OH at 37°C for 2 h, purified using strong anion exchange chromatography and shown to be >98% pure as measured by analytical reverse phase HPLC and denaturing PAGE. Figure 4 shows the nucleoside composition of oligodeoxyribonucleotides containing ^{e}**X** following digestion of the oligodeoxyribonucleotides with SVP and SAP followed by analysis by reverse phase HPLC on a C18 column. Co-injection of **10** with oligodeoxyribonucleotide digests confirmed that the peak labeled **d^{e}X** is the expected product, indicating that the ethano bridge survived the mild deprotection procedure (data not shown). Estimated degradation of the ethano portion of ^{e}**X** under these conditions was <2%.

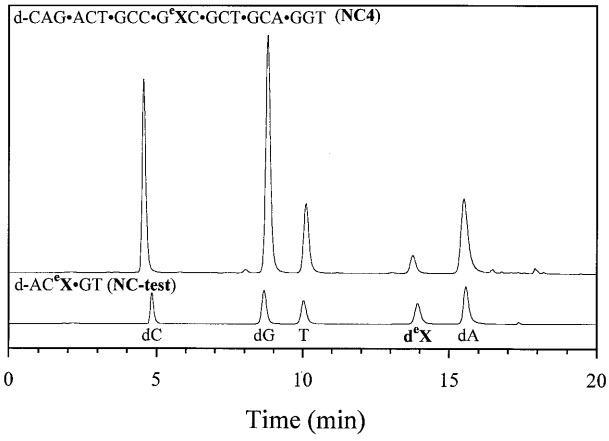

Figure 4. Reversed phase HPLC analysis of oligomers NC4 and NC-test after digestion with SVP and calf intestinal phosphatase. The oligodeoxyribonucleotide sequences are shown above the chromatogram and the nucleoside peaks are labeled at the bottom.

Formation of covalent hAGT–DNA complexes

The ability of ^{e}**X** to covalently trap hAGT in a post-repair protein–DNA complex is shown in Figure 5. Covalent crosslinking of the hAGT to radiolabeled DNA oligomers containing ^{e}**X** can be monitored by following the formation of a protein–DNA complex that is insensitive to thermal and chemical denaturation. Thus aliquots from a solution containing 4 µM oligodeoxyribonucleotide and 50 µM hAGT in crosslinking buffer were quenched with SDS gel loading buffer and boiled for 5 min at the indicated times. Prior to electrophoresis on a 12% SDS–polyacrylamide gel the samples were boiled for 5 min and following electrophoresis complexes

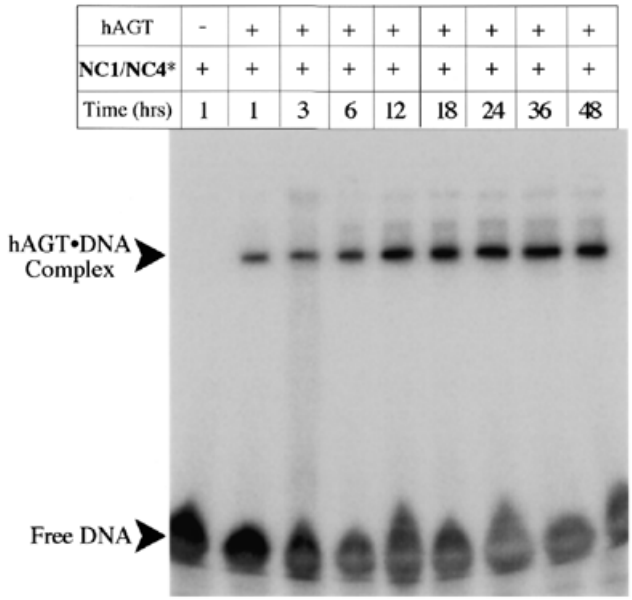

Figure 5. Autoradiograph of an SDS–polyacrylamide gel showing covalent crosslinking of N^1,O^6-ethano-2′-deoxyxanthosine-containing oligodeoxyribonucleotides to hAGT as a function of time. Radiolabeled oligodeoxyribonucleotide duplexes were incubated at 37°C in the absence of hAGT (lane 1) or in the presence of hAGT (lanes 2–8) for up to 48 h.

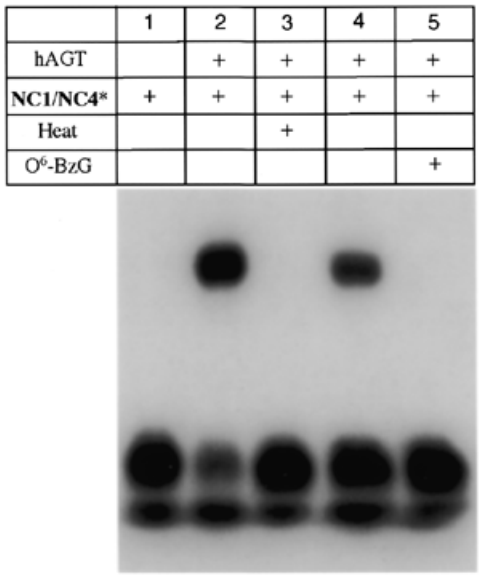

Figure 6. Autoradiograph of an SDS–polyacrylamide gel showing covalent crosslinking of N^1,O^6-ethano-2′-deoxyxanthosine-containing oligodeoxyribonucleotides to hAGT. Radiolabeled oligodeoxyribonucleotide duplexes (4 μM) were incubated for 50 h at 37°C in the absence of hAGT (lane 1), in the presence of 50 μM hAGT (lane 2), in the presence of heat-inactivated hAGT (lane 3), in the presence of hAGT pretreated with 10% DMSO (lane 4) and in the presence of hAGT pretreated with 10% DMSO and 100 μM O^6-benzylguanine.

were visualized by autoradiography. Lane 1 shows the electrophoretic mobility of the radiolabeled DNA duplex NC1/NC4* in the absence of protein. On SDS–polyacrylamide gels the oligodeoxyribonucleotide NC4* runs near the gel front and appears as a poorly resolved band. Unincorporated radiolabeled ATP is responsible for a slightly faster moving band (see Fig. 6 for clearer resolution of the bands); this material does not participate in crosslinking. Lane 2 shows formation of a slowly migrating radiolabeled species that is insensitive to denaturation. Visualization of the gel by staining with Coomassie Blue showed a new protein-containing band that runs coincident with the radioactive species (data not shown), consistent with a covalent protein–DNA complex.

It was observed over these prolonged incubations that a precipitate of free hAGT formed. This precipitation depleted the reaction mixture of soluble hAGT within 24 h and led to the apparent leveling off of crosslinking seen in Figure 5, lanes 2–8. Complete conversion of radiolabeled NC4 to the protein–DNA complex can be achieved by addition of more hAGT (data not shown). We also note that while precipitated protein was cleared from the solution by centrifugation the covalent complex formed remained soluble even after 48 h at 37°C.

Figure 6 shows the effect of thermal and chemical inactivation of hAGT on formation of the protein–DNA complex. Lanes 1 and 2 show the electrophoretic mobility of the ^{e}**X**-containing oligodeoxyribonucleotide with and without hAGT. Thermal denaturation (90°C for 2 min) of hAGT prior to incubation with the oligodeoxyribonucleotide abolishes formation of the covalent complex (lane 3), as does incubation with O^6-benzylguanine (18), a mechanism-based inhibitor of hAGT (lane5). Lane 4 is a mock inactivation of hAGT by DMSO (the solvent used to add the O^6-benzylguanine). Crosslinking could also be abolished if NC4 was treated with concentrated NH_4OH for 3 h at 65°C prior to annealing and incubation with hAGT (data not shown), presumably due to degradation of the ethano bridge.

DISCUSSION

In the past several years the proposed model for the recognition and repair of alkylated guanine by AGT has evolved. Early models, based in part on the crystal structure of the C-terminal domain of the *E.coli* Ada protein, postulated a conformational change that would bring the buried active site cysteine into proximity to an alkylated base in an unperturbed helix (23). The realization that some DNA-modifying proteins (e.g. cytosine 5-methyltransferases and DNA glycosylases) carry out the chemical modification of DNA by extruding the target base from the double helix into a buried active site led to the suggestion that AGT may use this mechanism. Recent structures of hAGT in the unalkylated and alkylated forms (24,25) and models of *E.coli* (26) and human AGT (24) bound to duplex DNA suggest that base flipping could result in productive repair complexes. Furthermore, NMR studies of the C-terminal domain of the Ada protein show that DNA binding does not involve significant conformational changes by the protein, an observation consistent with a base flipping mechanism (27).

In spite of the growing consensus that AGT recognition and repair of alkylated DNA involves base flipping there is little direct evidence. Efforts to produce complexes of AGT with DNA suitable for structural and biophysical characterization have been unsuccessful. Isolation of pre-repair complexes is complicated by a lack of appropriate cofactors which can be

omitted in order to trap complexes and while active site mutants which retain binding specificity for DNA containing methylated guanine have been reported (28), it is not clear how useful these mutants will be for structural work. Post-repair complexes also pose a significant technical challenge. Alkyl transfer results in a significant destabilization of the protein leading to an increased Stokes radius (29), increased proteolytic sensitivity (30) and rapid degradation in HT29 cells and cell-free extracts (31). The structure of alkylated AGT suggests that alkyltransfer causes the disruption of an extensive hydrogen bonding network, promotes helix displacement and results in dramatic destabilization of alkylated AGT (24).

The strategy pursued by our laboratory to prepare AGT–DNA complexes has been to use modified nucleosides to trap covalent complexes. To that end we previously demonstrated that it was possible to crosslink hAGT to a synthetic oligodeoxyribonucleotide containing a thiol-derivatized guanine (32). The N^6-thioethyl derivative of 2,6-diaminopurine was shown to produce a covalent disulfide crosslink between the hAGT active site cysteine and the modified nucleoside. Unfortunately, the crosslinking reaction was inefficient and required the use of very large excesses of protein to achieve low levels of crosslinking.

The ^{e}**X** analog described here was designed to circumvent many of the limitations of the earlier thiol-based crosslinker and is based on the observation that DNA treated with CENUs produced covalently trapped AGT–DNA complexes. Early work done by Brent and co-workers (11,13–15) elucidated the mechanism by which a CENU-derived intermediate could serve as a substrate for and crosslink AGT to DNA. This intermediate is the reactive N^1,O^6-ethanoguanine (^{e}**G**), which results from intramolecular cyclization of the original alkylation product, O^6-chlorethylguanine. AGT can recognize ^{e}**G** in a repair reaction that involves nucleophilic attack by the active site cysteine on the O^6-methylene group to form a covalent complex between AGT and DNA via an N^1-ethylcysteine crosslink (11).

Unfortunately, ^{e}**G** is not well suited to the preparation of homogeneous site-specific AGT–DNA complexes. *In situ* generation of ^{e}**G** by treatment with CENU results in the formation of a variety of alkylation products (33) and the ^{e}**G** adduct itself undergoes both hydrolysis (16) and interstrand crosslink formation (12), significantly dampening our enthusiasm for its utility as a useful crosslinker. Rather, we set out to design a nucleoside analog that maintained the major groove structure of ^{e}**G**, in order to facilitate recognition and crosslinking with AGT, but which removed the positive charge in order to ameliorate the reactivity of ^{e}**G**. The most straightforward and least perturbing route to preparing such a nucleoside analog was to replace the exocyclic amino group of ^{e}**G** with a carbonyl oxygen. The deamination of guanine to xanthine allows the normally protonated N^3 of xanthine to lose its proton and undergo a tautomeric shift in which the positive charge at N^1 can be ameliorated.

The strategy presented here for the preparation of ^{e}**X** was one of several under consideration. The availability of 2′-deoxyguanosine and a method for introduction of the ethano portion of the molecule were the principal advantages to starting with 2′-deoxyguanosine rather than 2′-deoxyxanthosine. A convenient procedure for the introduction of a variety of alkyl groups at the O^6 position of deoxyguanosine had been reported (19) and appeared to be adaptable to the introduction of a hydroxyethyl group. This one-pot double displacement procedure relies upon trifluoroacetic anhydride to activate the O^6, which when carried out in pyridine results in *in situ* generation of the pyridinium derivative. Quenching the reaction with an alcohol in the presence of a base, a solution of sodium dissolved in ethylene glycol in this case, results in the displacement of pyridine to produce the desired O^6-alkyl derivative. A large excess of freshly prepared ethylene glycol/sodium solution was required to prevent the decreases in yield noted in the original description of this procedure (19). With the introduction of the 2-carbon bridge the conversion to a deoxyxanthosine derivative was pursued. Deamination of 2′-deoxyguanosine using nitrous acid has been reported (20) and was used to convert O^6-hydroxyethyl-dG **3** to the 2′-deoxyxanthosine derivative **4**. Attempts at deamination using *n*-pentylnitrite as reported by Steinbrecher *et al.* (34) for O^6-nitrophenethyl-protected dG were unsuccessful.

A direct cyclization of **4** to produce ^{e}**X** was initially envisioned, however, in a non-productive competing reaction the O^2 carbonyl oxygen reacted with the benzenesulphonyl chlorides used to activate the hydroxyethyl group. This competing reaction decreased the yield of the desired cyclized compound and necessitated masking the O^2 position. The nitrophenethyl group is a convenient protecting group that has been used to protect the exocyclic oxygens of guanine (35), xanthine (19) and uric acid (36) and could be easily introduced via the Mitsunobo reaction (21). Thus the hydroxyethyl group was first masked with dimethoxytrityl chloride, the O^2 protected with an NPE group and finally the DMT was removed to afford the desired O^2-protected hydroxyethyl derivative, **7**. The exposed hydroxyl group could then be selectively activated using 2,4,6-triisopropylbenzenesulphonyl chloride to produce the tosylate, **8**. This tosylate was poorly behaved during purification attempts and had to be converted to the desired ethano compound without purification. Thus after quenching the tosylation reaction and evaporation of solvents, the resulting oil was dissolved in pyridine and, following the addition of a strong base, DBU, the desired O^6-CH_2CH_2-N^1 bridge-containing compound was produced. Cyclization presumably occurs following base-catalyzed β elimination of the NPE group from the O^2 position, which in turn increases the nucleophilicity of N^1.

Conversion of **9** to the nucleoside phosphoramidite and its incorporation into oligodeoxyribonucleotides was accomplished using standard procedures, with one important exception. Due to the anticipated sensitivity of ^{e}**X** to ammonia-catalyzed displacement of the O^6-alkyl moiety, protecting groups for the dA, dC and dG amidites were selected that could be removed under mild conditions. The sensitivity of O^6-alkylguanine to displacement by ammonia has been observed (22) and while there have been no such reports for alkylxanthine derivatives, it seemed likely that similar reactivity might exist. Indeed, when the NC-test sequence was prepared with traditional protecting groups (i.e. *N*-benzoyl-dC and dA) deprotection using concentrated NH_4OH at 65°C for 3 h resulted in complete degradation of ^{e}**X**. When acetyl- and phenoxyacetyl-protected monomers were substituted the preparation of oligodeoxyribonucleotides containing intact ^{e}**X** was easily accomplished.

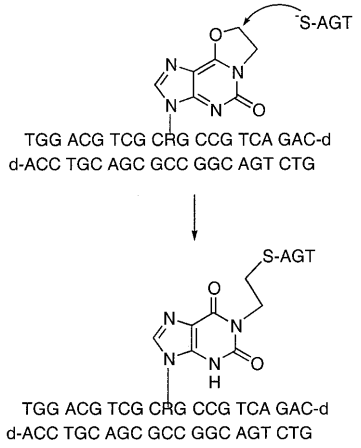

Figure 7. Scheme showing the proposed mechanism of crosslink formation between hAGT and ^{e}**X**.

Oligodeoxyribonucleotides containing ^{e}**X** produce covalent protein–DNA complexes when incubated with hAGT, presumably via the proposed mechanism shown in Figure 7. Nucleophilic attack by the active site cysteine on the O^6-methylene carbon in a repair-like reaction results in alkyl transfer and covalent capture of a protein–DNA complex. The ability of O^6-benzylguanine, a mechanism-based inhibitor of hAGT (18), to abolish crosslink formation is consistent with the proposed mechanism-based crosslinking scheme. While nucleophilic attack at the N^1-methylene carbon would be expected to produce a covalent complex indistinguishable at the current level of characterization it seems unlikely that the active site architecture would accommodate such an attack.

Though designed to mimic ^{e}**G**, there are several important differences between the crosslinking reported with ^{e}**G**-containing DNA (11) and the crosslinking described here. First, the rate of crosslink formation with ^{e}**X**-containing oligomers appears to be slower than that of ^{e}**G**-containing oligomers. Crosslinking with *in situ* generated ^{e}**G** (i.e. DNA treated with CENU) is complete within 1 h, while crosslinking with ^{e}**X** occurred more slowly (~12 h to give 50% crosslinking). While direct comparisons are complicated, it is not unreasonable to assume that the ^{e}**X** analog would be less reactive than ^{e}**G**. The presence of a positive charge on ^{e}**G** can stabilize the negative charge that forms on the leaving group in an S_N2 type displacement (i.e. the O^6 of ^{e}**G**). Removing the positive charge would then be expected to decrease the rate of crosslinking. It is also possible that substitution of oxygen for the exocyclic amino group of guanine has an effect on binding and/or recognition of the substrate, also contributing to slower crosslinking. The second difference between ^{e}**G** and ^{e}**X** is the extent of crosslink formation. Maximal crosslinking efficiencies for ^{e}**G**-containing DNA were ~50% compared to the near quantitative crosslinking efficiency achievable with oligomers containing ^{e}**X**. The inability to achieve complete crosslinking using ^{e}**G** is likely a result of several competing reactions: (i) removal of the O^6-chloroethyl group by AGT prior to cyclization; (ii) hydrolysis of ^{e}**G** producing the unreactive N^1-hydroxyethyl derivative; (iii) formation of the G-C interstrand crosslink. The third difference achieved with ^{e}**X** is the formation of only a single protein–DNA species, whereas DNA treated with CENU and incubated with hAGT resulted in the formation of multiple crosslinked protein–DNA species.

The differences observed between crosslinking with ^{e}**G** and the nucleoside analog ^{e}**X** are consistent with the hypothesis that amelioration of the positive charge results in a better behaved, albeit slower, crosslinker. The crosslinking reactions described here required only a 10-fold excess of protein over DNA and resulted in high levels of covalent protein–DNA complexes. Furthermore, the persistent solubility of the hAGT–DNA complex versus the free protein suggests that these complexes may be amenable to further biophysical characterization.

CONCLUSIONS

The modified nucleoside N^1,O^6-ethano-2′-deoxyxanthosine has been prepared, incorporated into oligodeoxyribonucleotides and used to generate covalent complexes with hAGT. Mechanism-based covalent trapping of the hAGT–DNA complex occurs with high yield and the resulting complexes should facilitate structural studies of the recognition and repair activity of this important class of DNA repair proteins. Further characterization of the covalent protein–DNA complexes involving both hAGT and the AGT domain of *E.coli* Ada protein is underway.

ACKNOWLEDGEMENTS

This work was supported by grants from the Patterson Trust and the Alexander and Margaret Stewart Trust. We thank Dr Phil Potter for the hAGT and Dr Paul Miller for space, equipment and helpful discussions regarding the synthesis of ^{e}**X**.

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