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Novel synthesis of O^6-alkylguanine containing oligodeoxyribonucleotides as substrates for the human DNA repair protein, O^6-methylguanine DNA methyltransferase (MGMT)

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ABSTRACT

The human DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) dealkylates mutagenic O^6-alkylguanine lesions within DNA in an irreversible reaction which results in inactivation of the protein. MGMT also provides resistance of tumours to alkylating agents used in cancer chemotherapy and its inactivation is therefore of particular clinical importance. We describe a post-DNA synthesis strategy which exploits the novel, modified base 2-amino-6-methylsulfonylpurine and allows access for the first time to a wide variety of oligodeoxyribonucleotides (ODNs) containing O^6-alkylguanines. One such ODN containing O^6-(4-bromothenyl)guanine is the most potent inactivator described to date with an IC_{50} of 0.1 nM.

INTRODUCTION

The catalytic properties of certain DNA repair proteins can be investigated using chemically synthesized oligodeoxyribonucleotide (ODN) substrates. This has previously required the production of phosphoramidites containing appropriately protected bases in order to generate ODNs of defined sequence and containing single, or multiple, lesions of known structure. That each putative substrate requires the synthesis of a specific phosphoramidite has inhibited comprehensive studies in this area: the majority of work is undertaken using a very limited number of commercially available precursors. One DNA repair protein for which the availability of a wide variety of ODN substrates would be of considerable benefit is O^6-methylguanine-DNA methyltransferase (MGMT) (1). MGMT protects normal cells against the toxic and mutagenic effects of endogenous and environmental alkylating agents by repairing toxic O^6-alkylguanine lesions in DNA which can otherwise mispair with thymine during replication (2). Repair involves the transfer of the alkyl group to the active site cysteine in a stoichiometric direct damage reversal pathway in which MGMT is inactivated (3). MGMT also protects tumour cells against the action of chemotherapeutic alkylating agents such as temozolomide and BCNU (carmustine). This has generated interest in compounds that inactivate MGMT and thereby sensitize tumour cells to killing by these agents (4,5). Substrates of MGMT are O^6-alkylguanines of extremely diverse structure (6–16). To study the repair of O^6-alkylguanine-containing DNA, the use of a single phosphoramidite that allows the efficient post-synthesis modification of ODNs to produce such defined lesions was considered a potential advance. We have therefore established a protocol based on a methylsulfonyl leaving group that provides a facile and efficient route to a wide variety of potential MGMT substrates that hitherto were unavailable using existing post-DNA synthesis chemistries. Here we present the synthesis and post-DNA modification of ODNs containing the 2′-deoxyribonucleoside of 2-amino-6-methylsulfonylpurine. We generate examples of ODNs containing biologically relevant guanine residues and characterize their interaction with human MGMT.

We chose O^6-methylguanine (O^6-MeG) and O^6-(2-hydroxyethyl)guanine (O^6-HOEtG) (Figure 1) because they are generated in DNA by environmental carcinogens and certain cancer chemotherapeutic agents (17). We also examined O^6-benzylguanine (O^6-BnG) (18,19) and O^6-(4-bromothenyl)guanine [O^6-(4-BTG)] because, as free bases, they are currently in clinical trials (18–20) as MGMT

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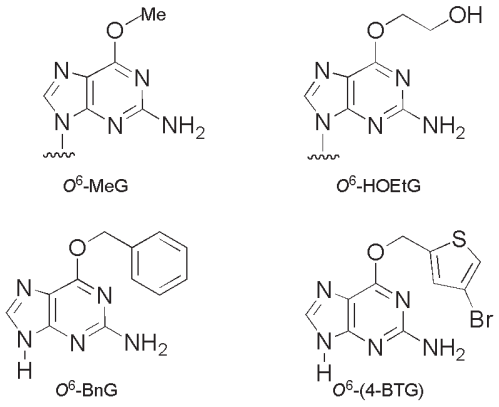


Figure 1. O^6-Alkylguanine lesions in DNA; O^6-methylguanine (O^6-MeG) and O^6-(2-hydroxyethyl)guanine (O^6-HOEtG) and potent inactivators of human MGMT; O^6-benzylguanine(O^6-BnG) and O^6-(4-bromothenyl)guanine [O^6-(4-BTG)].

inactivating agents (see above). In this context we show that a novel 16mer ODN containing O^6-(4-bromothenyl)guanine displays a picomolar IC_{50} value against human MGMT which is lower than any inactivator of the protein tested to date.

MATERIALS AND METHODS

Chemical synthesis

*6-Thio-2′-deoxyguanosine (**1**).* According to method described by Kung and Jones (21), sodium hydrosulfide hydrate (16.2 g, 289 mmol) was placed in a round bottom flask and flame-dried with Bunsen burner until no more water was evolved. The remaining orange solid was cooled to room temperature under vacuum, and dry DMF (180 ml) was added to the flask under an Ar atmosphere. The flask was placed in a sonicator and the solid crushed by sonication overnight. The majority of sodium hydrosulfide dissolved to give a dark green solution. 2′-Deoxyguanosine monohydrate (1.5 g, 5.3 mmol) was dried by evaporation of dry pyridine (3 × 30 ml), suspended in dry pyridine (120 ml) under Ar and cooled in an ice bath. To this was added dropwise trifluoroacetic anhydride (7.0 ml, 49.6 mmol) and the mixture was stirred for 1 h to afford a dark orange solution. To this was added the sodium hydrosulfide DMF solution in portions whilst the mixture was stirred at 0°C under Ar. The ice bath was removed and the mixture then stirred overnight at room temperature. The reaction mixture was then poured into saturated aqueous ammonium bicarbonate solution (300 ml), stirred for 6 h, the solvent removed under reduced pressure and the residual oil evaporated at 80°C under vacuum. To the remaining solid was added methanol (300 ml) and the white powder which formed was removed by filtration. The filtrate was evaporated to a solid, and 0.1 M triethylammonium acetate added. A black fine precipitate which formed was removed by filtration, the filtrate evaporated, and the residue recrystallized from methanol/water = 50/50 (100 ml) to give **1** (1.5 g, quantitative) as a light purple-coloured powder. Analytical data were identical to that described elsewhere (21).

*9-[3′, 5′- Bis(tert-butyldimethylsilyl)-2′-deoxyribofuranosyl]-2-amino-6-methylsulfanylpurine (**2**).* To a solution of 6-thio-2′-deoxyguanosine (2.4 g, 8.47 mmol) in concentrated aqueous ammonia (20 ml) was added iodomethane (1.0 ml, 16.1 mmol), and the mixture stirred for 1 h. The solvent was removed under reduced pressure, the residue dried under vacuum, then by coevaporation with dry pyridine (2 × 30 ml). The residue was dissolved in dry DMF (20 ml), and stirred with imidazole (5.8 g, 85.2 mmol) and *tert*-butyldimethylchlorosilane (3.8 g, 25.2 mmol) for 3 h at room temperature. The reaction mixture was concentrated and diluted with ethyl acetate (100 ml). The organic layer was washed with water (50 ml) and brine (50 ml), dried (Na_2SO_4) and evaporated. Purification by silica column chromatography (hexane/EtOAc = 4/1 to 3/1) afforded **2** as a white foam (3.9 g, 88%). R_f (hexane/EtOAc=3/1) = 0.50; ^{1}H NMR ($CDCl_3$, δ): 7.91 (1H, s, H8), 6.34–6.29 (1H, m, H1′), 4.92 (2H, bs, NH_2), 4.60–4.55 (1H, m, H3′), 4.00–3.96 (1H, m, H4′), 3.83–3.71 (2H, m, H5′), 2.63 (3H, s, MeS), 2.63–2.53 (1H, m, H2′), 2.39–2.30 (1H, m, H2′), 0.91 (9H, s), 0.90 (9H, s), 0.10 (6H, s), 0.074 (3H, s), 0.069 (3H, s); ES^+MS (*m*/*z*): 526.2704 ($[M+H]^+$, calc. for $C_{23}H_{44}N_5O_3Si_2S$ = 526.2703).

*9-[3′, 5′- Bis(tert-butyldimethylsilyl)-2′-deoxyribofuranosyl]-2-amino-6-methylsulfonylpurine (**3**).* To a solution of **2** (2.0 g, 3.80 mmol) in ethanol/water (2:1, 60 ml) was added magnesium monoperphthalate solution in water (80%, 5.2 g, 8.41 mmol in 20 ml) dropwise at room temperature, and the mixture stirred for 3 h. The solvent was removed under reduced pressure, and EtOAc (100 ml) and saturated aqueous sodium bicarbonate (30 ml) were added to the residue. The organic layer was separated, washed sequentially with water (20 ml) and brine (20 ml), dried (Na_2SO_4) and evaporated. Purification by silica column chromatography (hexane/EtOAc = 1/4) gave **3** as a white foam (1.3 g, 61%). R_f (hexane/EtOAc = 1/4) = 0.24; ^{1}H NMR ($CDCl_3$, δ): 8.24 (1H, s, H8), 6.36 (1H, t, J = 6.6 Hz, H1′), 5.50 (2H, bs, NH_2), 4.60–4.55 (1H, m, H3′), 4.03–3.99 (1H, m, H4′), 3.82 (1H, dd, J = 3.7, 11.0 Hz, H5′), 3.75 (1H, dd, J = 3.7, 11.0 Hz, H5′), 3.42 (3H, s, $MeSO_2$), 2.60–2.50 (1H, m, H2′), 2.39 (1H, ddd, J = 3.7, 6.0, 13.0 Hz, H2′), 0.91 (9H, s), 0.89 (9H, s), 0.10 (6H, s), 0.079 (3H, s), 0.073 (3H, s); ES^+MS (*m*/*z*): 558.2599 ($[M+H]^+$, calc. for $C_{23}H_{44}N_5O_5Si_2S$ = 558.2602).

*9-[3′, 5′-O-Bis(tert-butyldimethylsilyl)-2′-deoxyribofuranosyl]-N^2-[(dimethylamino)methylidene]-2-amino-6-methylsulfonylpurine (**4**).* Compound **3** (1.3 g, 2.12 mmol) was dissolved in freshly distilled methanol (10 ml), activated 3 Å molecular sieves were added and the mixture stirred for 30 min under Ar. *N, N*-Dimethylformamide dimethylacetal (2.3 ml, 17.3 mmol) was then added, the mixture stirred overnight, then filtered through celite and evaporated. The residue was purified by silica column chromatography ($CHCl_3$/MeOH = 99/1) to give **4** as a white foam (866 mg, 61%). R_f ($CHCl_3$/MeOH=98/2) = 0.50; ^{1}H NMR ($CDCl_3$, δ): 8.79 (1H, s, amidine), 8.41 (1H, s, H8), 6.63 (1H, t, J = 6.4 Hz, H1′), 4.56–4.51 (1H, m, H3′), 4.00–3.97 (1H, m, H4′), 3.85 (1H, dd, J = 3.2, 11.1 Hz, H5′), 3.76 (1H, dd, J = 3.2, 11.1 Hz, H5′), 3.46 (3H, s, $MeSO_2$), 3.20 (3H, s, $-NMe_2$), 3.19 (3H, s, $-NMe_2$), 2.49–2.32 (2H, m, H2′), 0.90 (9H, s), 0.89 (9H, s), 0.093 (3H, s), 0.090 (3H, s), 0.07 (6H, s); ES^+MS (*m*/*z*): 613.3042 ($[M+H]^+$, calc. for $C_{26}H_{49}N_6O_5Si_2S$ = 613.3024).

*9-(2′-Deoxyribofuranosyl)-N^2-[(dimethylamino)methylidene]-2-amino-6-methylsulfonylpurine (**5**).* To a solution of compound **4** (4.2 g, 6.85 mmol) in anhydrous THF (40 ml) was added triethylamine trihydrofluoride (2.5 ml, 15.3 mmol) and the mixture stirred overnight at room temperature under Ar. The reaction mixture was then diluted with dichloromethane (100 ml) and the precipitate formed was collected by filtration and washed with dichloromethane (100 ml) to give **5** as a white powder (2.1 g, 80%). This product was used for the next reaction without further purification. R_f ($CHCl_3$/MeOH = 4/1) = 0.50; ^{1}H NMR (*d6*-DMSO, δ): 8.75 (1H, s, amidine), 8.72 (1H, s, H8), 6.47 (1H, t, J = 6.7 Hz, H1′), 5.41 (1H, d, J = 4.3 Hz, 3′-OH), 5.06 (1H, t, J = 5.5 Hz, 5′-OH),4.49–4.44 (1H, m, H3′), 3.94–3.90 (1H, m, H4′), 3.68–3.55 (2H, m, H5′), 3.55 (3H, s, $MeSO_2$), 3.23 (3H, s, –NMe_2), 3.11 (3H, s, –NMe_2), 2.80–2.70 (1H, m, H2′), 2.42–2.33 (1H, m, H2′); ES$^+$MS (*m*/*z*): 385.1311 ([M+H]$^+$, calc. for $C_{14}H_{21}N_6O_5S$ = 385.1294).

*9-(5′-O-Dimethoxytrityl-2′-deoxyribofuranosyl)-N^2-[(dimethylamino)methylidene]-2-amino-6-methylsulfonylpurine (**6**).* To a solution of **5** (1.9 g, 2.49 mmol) in dry DMF (50 ml) was added diisopropylethylamine (1.3 ml, 7.46 mmol) and dimethoxytrityl chloride (2.0 g, 5.90 mmol). The reaction mixture was stirred under Ar for 4 h at room temperature. Methanol (2 ml) was then added and the solvent evaporated. The residue was dissolved in ethyl acetate (100 ml) and washed sequentially with saturated aqueous sodium bicarbonate (25 ml), water (25 ml) and brine (25 ml). The organic layer was dried (Na_2SO_4), filtered and evaporated and the residue purified by silica column chromatography ($CHCl_3$ with 1% Et_3N to $CHCl_3$/MeOH = 97/3 with 1% Et_3N) to give **6** as a white foam (3.1 g, 91 %). R_f ($CHCl_3$/MeOH = 97/3) = 0.15; ^{1}H NMR ($CDCl_3$, δ) = 8.76 (1H, s, amidine), 8.22 (1H, s, H8), 7.40–7.16 (9H, m, DMTr), 6.82–6.76 (4H, m, DMTr), 6.61 (1H, t, J = 6.4 Hz, H1′), 4.63–4.58 (1H, m, H3′), 4.15–4.10 (1H, m, H4′), 3.77 (6H, s, 2× MeO), 3.44 (3H, s, $MeSO_2$), 3.38 (2H, t, J = 4.0 Hz, H5′), 3.18 (3H, s, –NMe_2), 3.17 (3H, s, –NMe_2), 2.59 (2H, t, J = 5.8 Hz, H2′); ES$^+$MS (*m*/*z*) = 687.2582 ([M+H]$^+$, calc. for $C_{35}H_{39}N_6O_7S$ = 687.2601).

*9-(5′-O-Dimethoxytrityl-2′-deoxyribofuranosyl)-N^2-[(dimethylamino)methylidene]-2-amino-6-methylsulfonylpurine 3′-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite (**7**).* To a solution of **6** (385 mg, 0.56 mmol) in anhydrous dichloromethane (10 ml) was added diisopropylethylamine (390 µl, 2.24 mmol) under an Ar atmosphere and the mixture cooled in an ice bath. 2-Cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (250 µl, 1.12 mmol) was then added dropwise and the reaction stirred for 30 min at 0°C, then for 2 h at room temperature. The reaction mixture was diluted with EtOAc (50 ml) and washed sequentially with saturated aqueous sodium bicarbonate (10 ml), water (10 ml) and brine (10 ml). The organic layer was dried (Na_2SO_4), filtered and evaporated and the residue purified by column chromatography (hexane/acetone = 2/1 to hexane/acetone = 1/1 to $CHCl_3$/acetone = 4/1 to $CHCl_3$/acetone = 2/1 to $CHCl_3$/MeOH = 98/2, all eluents contained 1% Et_3N) to give **7** as a white foam (293 mg, 59%). R_f (hexane/acetone = 1/1) = 0.33; ^{1}H NMR ($CDCl_3$, δ) = 8.79 (1H, s, amidine), 8.26 (1H, d, J = 11.0 Hz, H8), 7.41–7.18 (9H, m, DMTr), 6.82–6.77 (4H, m, DMTr), 6.64 (1H, t, J = 6.7 Hz, H1′), 4.70–4.63 (1H, m, H3′), 4.27–4.23 (1H, m, H4′), 3.88–3.30 (6H, m, OCH_2CH_2CN and H5′), 3.771 (3H, s, MeO), 3.768 (3H, s, MeO), 3.45 (3H, s, $MeSO_2$), 3.20 (3H, s, –NMe_2), 3.19 (3H, s, –NMe_2), 2.66–2.57 (3H, m, [(Me_2CH)N]$_2$ and H2′), 2.43 (1H, t, J = 6.9 Hz, H2′), 1.28–1.05 (12H, m, [(Me_2CH)N]$_2$); ^{31}P NMR ($CDCl_3$, δ) = 149.18; ES$^+$MS (*m*/*z*) = 887.3710 ([M+H]$^+$, calc. for $C_{44}H_{56}N_8O_8PS$ = 887.3679).

Oligodeoxyribonucleotide synthesis

ODNs were synthesised using standard protocols on an Applied Biosystems 394 automated synthesiser using mild/fast deprotection phosphoramidites, reagents and columns (acetyl dC, *t*-butylphenoxyacetyl dG and dA). All syntheses were performed on a 1 µmol scale using 0.1 M solutions of normal phosphoramidites and a 0.15 M solutions of the modified phosphoramidite **7**. Following DNA synthesis, the CPG-bound protected ODN (approximately one-third of sample) was treated with 500 µl of alcohol/dry acetonitrile/dry DBU (9:9:2, v/v/v) and incubated at 37°C for 2 days. Concentrated aqueous ammonia solution (33%, 1 ml) was then added and the mixture shaken for 3 days at room temperature. For water insoluble alcohols (4-bromothenyl, benzyl and $DMTrOCH_2CH_2OH$), the aqueous layer was removed with a pipette and the organic layer extracted with 500 µl of water and the water layers combined. The ODN solutions were then concentrated to residual oils (∼100 µl) and CH_2Cl_2 (1 ml) added. The suspension was then briefly vortexed, centrifuged and the liquid decanted leaving an ODN pellet. The pellet was dissolved in water (500 µl) and purified by reversed phase high-performance liquid chromatography (HPLC) (Hichrom ACE C18 4.6 × 250 mm column) using the following conditions: buffer A = 5% CH_3CN in 0.1 M aqueous triethylammonium bicarbonate pH 7.0, buffer B = CH_3CN, linear gradient of 0–20% B over 30 min, flow rate 1 ml/min, monitored at 260 nm. Typical retention times for the ODNs are shown in Table 1. Following HPLC purification, samples were evaporated and redissolved in water (500 µl) purified using a spin column (MILLIPORE Microcon® 3000 molecular weight cut off) and characterized by MALDI ms (Table 1). (The hydroxyethyl-modified ODN was detritylated with 20% aqueous acetic acid for 30 min then evaporated and redissolved in water prior to spin column purification). Following analysis, ODN solutions were evaporated and stored as 100 µM solutions in dry dimethyl sulfoxide at −20°C. Determined extinction coefficients at 260 nm for the modified bases in water at pH 7 were as follows; 3.4 for O^6-methylguanine and O^6-hydroxyethylguanine, 3.6 for O^6-benzylguanine and 5.5 for O^6-(bromothenyl)guanine. These

Table 1. Physical data of 16mer ODNs (5′-AACAGCCATATXGCCC)

Modification (X)	HPLC[a] retention time (min)	MALDI ms data Calculated	Found
G	23.4	4835.1	4836
O^6-MeG	24.9	4849.2	4851
O^6-BnG	25.9	4925.3	4927
O^6-hoEtG	23.1	4881.2	4881
O^6-(4-BTG)	27.0	5010.2	5011

[a]HPLC conditions are described in Materials and Methods.

values were used to determine the extinction coefficients of the modified ODNs as described elsewhere (22).

MGMT assays

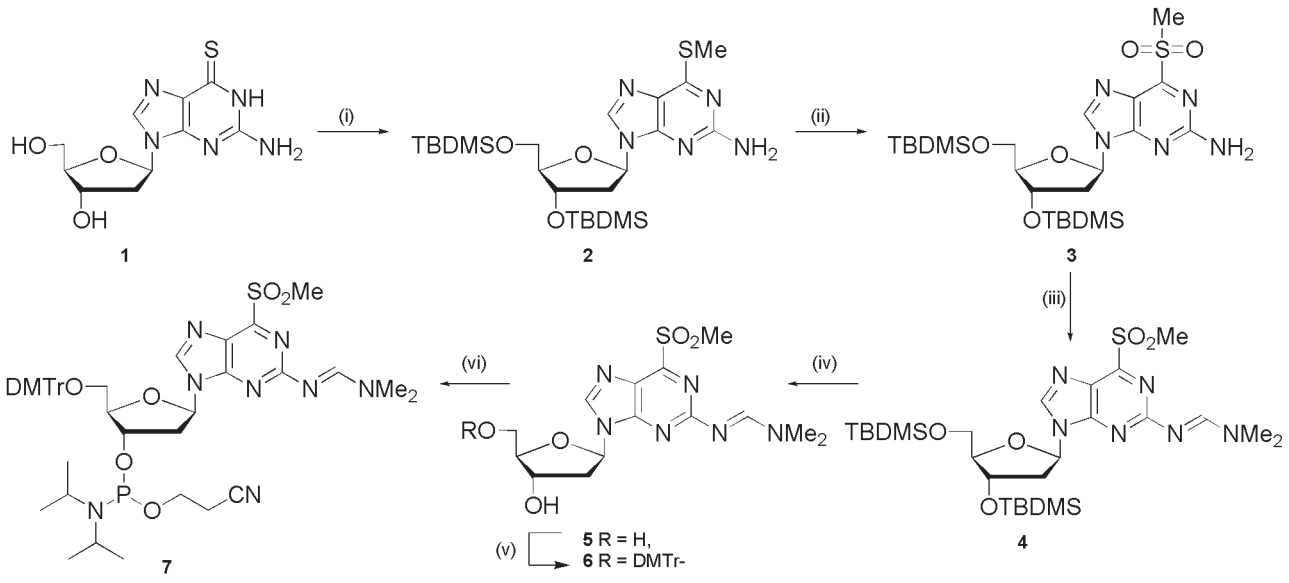
Recombinant human MGMT was overexpressed in *E.coli* strain UC978 which is deleted for the endogenous alkyltranferases *ada* and *ogt* (23) Cell-free extracts were prepared by sonication in buffer I (50 mM Tris–HCl, 1 mM EDTA and 3 mM DTT, pH 8.3) containing phenylmethlysulfonyl fluoride and leupeptin, and centrifugation at 4°C for 10 min in a refrigerated microcentrifuge. The amounts of MGMT present in the crude sonicates were determined following incubation with *N*-nitroso-*N*-[^{3}H]methylurea-methylated calf thymus substrate DNA for 60 min at 37°C as described elsewhere (24). To assess the ability of the ODNs to inactivate MGMT, aliquots (88 fmol) of MGMT were incubated in buffer I (50 mM Tris–HCl, 1 mM EDTA and 3 mM DTT, pH 8.3) with incrementally increasing amounts of ODNs at 37°C for 60 min. Excess [^{3}H]-methylurea-methylated calf thymus substrate DNA was then added and incubation continued for a further hour, and samples processed as described. A pilot experiment was firstly carried out using a very wide range of concentrations to establish the range to use for each ODN. IC_{50} values were determined from the lines of best fit to the dose-response curves (Figure 3) and represent the amount of inactivator required to reduce the MGMT activity to 50%. The data used in the dose–response curves were the mean of triplicate measurements. To ensure that the pre-incubation had been carried out for sufficient time for the initial reaction to go to completion, fixed amounts of ODNs that did not achieve complete inactivation of MGMT in the titration assay were incubated with 88 fmol of MGMT at 37°C for various times up to 4 h then processed as above.

RESULTS AND DISCUSSION

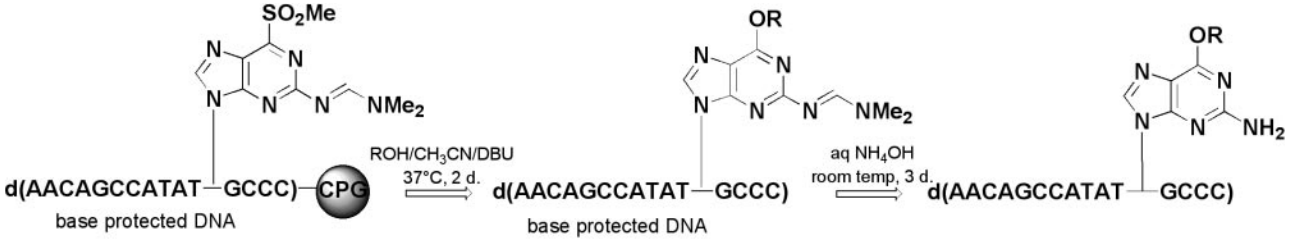
The post-synthetic modification of DNA involves the incorporation of a reactive nucleoside analogue into the synthetic oligomer via an appropriate phosphoramidite precursor (25–30). The reactive nucleoside contains a suitable leaving group at the desired position on its heterocyclic base which may be displaced by nucleophiles following DNA synthesis. The analogue must display a complete stability to the reagents employed in the coupling cycle during standard DNA synthesis but possess a sufficiently high reactivity to allow the subsequent efficient and selective transformation to the desired modified derivative.

Post-DNA/RNA synthesis chemistry that has been employed successfully for the preparation of oligonucleotides containing 6-modified purine nucleosides has utilized leaving groups such as phenoxide (23,26) and methylsulfoxide, methylsulfonyl and chloride (27,29,30). However, none of these examples afford DNA containing a 2-amino-6-substituted purine and in all cases, the leaving group of the modified base is displaced by an efficient nucleophile such as a thiol or an amine. The preparation of DNA containing O^6-alkylguanines using post-DNA synthesis is considerably more challenging due to the much lower electrophilicity at C6 of 6-substituted derivatives of 2-aminopurine versus analogous 6-substituted purines. Previously, DNA containing 2-amino-6-modified purines (26) has been prepared via post-DNA synthesis using ODNs containing S^6-(2,4-dinitrophenyl)thioguanine (30). However, this chemistry has only been used for the preparation of O^6-methylguanine-containing DNA, which involves displacement of the thiolate leaving group by methanol, i.e. a relatively nucleophilic alcohol (26). Furthermore, the 2,4-dinitrophenylthio group is not ideal in this context since it has two electrophilic sites and thus can also undergo nucleophilic addition to the 2,4-dinitrobenzene moiety leading to the formation of thioguanine (26,27). Of interest to us was the need for DNA containing O^6-modified guanines bearing alkyl groups more complex than methyl that are not accessible by these existing methods. O^6-Sulfonate esters have been employed previously to prepare O^6-alkylguanine (13) and O^6-alkyl-2′-deoxyguanosine analogues (31) using a two-step process that involves the initial displacement of the leaving group by a tertiary amine, which in turn is displaced by an alcohol. However, we expected that these derivatives would be too reactive for use during DNA synthesis (32) and as an alternative we considered the use of either 6-sulfoxy- or preferably the more reactive 6-sulfonyl-2-aminopurine-containing ODNs. Based on the known trends in leaving group ability during nucleophilic aromatic substitution reactions (S_NAr) reactions (33), we expected such analogues to display a reactivity intermediate between that of the analogous sulfonate esters and the 2,4-dinitrothiophenol derivative described by Xu (27). Although the 2′-deoxyribonucleoside of 2-amino-6-methylsulfonylpurine has not been prepared previously, the high reactivity of 6-methylsulfonylpurine 2′-deoxyriboside (34) and of oligonucleotides containing 6-methylsulfonylpurine (29,30) has been described elsewhere. Thus we envisaged a post-DNA synthesis strategy in which DNA containing 2-amino-6-methylthiopurine-2′-deoxyriboside could be oxidized using MMPP to the corresponding sulfone. Previously DNA (29) containing 6-methylsulfoxypurine and RNA (30) containing 6-methylsulfonylpurine has been obtained following MMPP oxidation of a synthetic ODN containing 6-methylthiopurine. Thus, initially we prepared a 12mer ODN containing 2-amino-6-methylthiopurine using the phenoxyacetyl-protected phosphoramidite monomer described previously (8). Following DNA synthesis, the modified ODN was then treated with two equivalents of MMPP. Oxidation to the corresponding sulfoxide occurred cleanly within 3 h [as evidenced by HPLC (Supplementary Figure 1) and characterization of a new M+16 peak by MALDI ms]. However, the corresponding sulfone-modified DNA was not formed even after prolonged treatment with excess MMPP (50 equiv., 1 week) (data not shown). Furthermore, displacement of the methylsulfoxy group within the modified ODN by alcohols was extremely inefficient (data not shown).

As an alternative we considered that the sulfone-modified base could be incorporated into DNA directly using an appropriately protected phosphoramidite. Consequently we prepared the novel sulfone phosphoramidite **7** in six steps from 2′-deoxythioguanosine (Scheme 1). Initially 2′-deoxythioguanosine was alkylated using MeI under basic conditions and then protected on the 3′ and 5′ hydroxyl groups using TBDMSCl and the product **2** purified by silica column chromatography. In principle, the protection of the sugar of the modified nucleoside is not required during the synthesis of **7**.



Scheme 1. Synthesis of sulfone phosphoramidite. Reagents and conditions: (i) NH_4OH, MeI then TBDMSCl, imidazole, DMF, 88%; (ii) MMPP, aqueous EtOH, 61%; (iii) *N*,*N*-dimethylformamide dimethylacetal, DMF, 61%; (iv) $Et_3N.HF$, DMF, 80%; (v) DMTrCl, pyridine, 91%; (vi) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, $(i\text{-}Pr_2N)_2EtN$, CH_2Cl_2, 59%.



Scheme 2. Post-DNA synthesis modification of ODNs to incorporate O^6-alkylguanine analogues (R = methyl, benzyl, 4-bromothenyl, $DMTrOCH_2CH_2$–).

However, in practise we have found large variability in the purity of 2′-deoxythioguanosine that has been prepared from 2′-deoxyguanosine using the described one-pot procedure (which employs trifluoroacetic anhydride and NaSH) (21). The protection of the 2′-deoxythioguanosine following methylation provides a compound which is readily purified by silica column chromatography and as a consequence even relatively impure samples of the thionucleoside can be used in the synthesis. The oxidation of compound **2** to give the sulfone **3** was achieved using MMPP. Deprotection of N^2-isobutyryl-O^6-alkylguanine-containing ODNs under standard conditions (NH_4OH, 55°C for 6 h) can lead to the formation of 2,6-diaminopurine and guanine-containing DNA (35). However, such problems can be eliminated when the phenoxyacetyl protecting group is used since this can be removed under milder conditions (NH_4OH, room temperature) (8). For this reason we chose the dimethylformamidine moiety for protection of the amino group of **7** since it can also be removed under mild conditions but in addition, it is easy to introduce.

For reasons outlined above, we chose to prepare a 16mer ODN containing the four different O^6-alkylguanine modifications indicated. The ODN sequence chosen is shown in Scheme 2. This sequence containing O^6-benzylguanine is known to be an excellent substrate for MGMT (36). The synthesis of the modified ODNs was performed using the modified phosphoramidite **7** together with base-labile phosphoramidite reagents of the standard nucleosides (acetyl C, *t*-butylphenoxyacetyl G and A) and with final removal of the 5′-dimethoxytrityl protecting group. The phosphoramidite **7** was used in a standard coupling protocol but was employed as a 0.15 M solution in acetonitrile rather than the normal 0.1 M concentration and gave comparable coupling efficiencies to the normal phosphoramidites. Following synthesis, the CPG-bound ODN was treated with the desired alcohol together with DBU dissolved in dry acetonitrile (alcohol: acetonitrile: DBU, 9:9:2 v/v/v) for 2 days (Scheme 2). Aqueous ammonia solution was then added and after 3 days the ODNs were purified by reversed phase HPLC and characterized by MALDI ms (Table 1). All ODNs displayed masses within 4 mass units of their calculated masses. A typical HPLC trace before purification is shown in Figure 2 for the ODN containing O^6-benzylguanine.

The conditions used by Xu *et al.* (26) for the post-DNA synthesis of O^6-methylguanine-containing DNA involve the displacement of a 2,4-dinitrophenylthio leaving group using a mixture containing alcohol/DBU 9:1. However in our hands, this produced highly viscous solutions when an alcohol other than methanol was used. Furthermore, following ammonia

deprotection of the DNA, ODNs containing guanine rather than the O^6-alkylguanine analogue were obtained. The high reactivity of the methylsufonyl group toward nucleophilic displacement by alcohol/DBU was confirmed by using ^{1}H NMR. Thus in a model experiment, we monitored the time required for completion of the displacement reaction using deuterated methanol as the nucleophile (Supplementary Figure 2). Thus, whilst the reaction was complete in 10 mins at room temperature for 2-amino-6-methylsulfonylpurine-2′-deoxyriboside, the analogous reaction with S^6-(2,4-dinitrophenyl)-thio-2′-deoxyguanosine required over 2 h. These results clearly demonstrate the much higher reactivity of the methylsulfonyl leaving group toward displacement by alcohols and our approach provides a practical and convenient procedure for the generation DNA containing O^6-alkylguanines. It is interesting to note the very large disparity in reactivity of the 2-amino-6-methylsulfonylpurine in the nucleoside and the ODN, the latter requiring 2 days for complete reactivity. The very large difference in reactivity between substituted purines in nucleosides and within DNA has also been noted by others (30). This difference in reactivity underlines the need for a very good leaving group at the 6-position of the purine in order to prepare a wide variety of O^6-alkylguanines using post-DNA synthesis.

Table 2. IC_{50}values of ODNs and free base inactivators of human MGMT

Human MGMT inactivator	IC_{50} (μM)
d(AAC AGC CAT AT(O^6-MeG) GCC C)	0.0157
d(AAC AGC CAT AT(O^6-BnG) GCC C)	0.0002
d(AAC AGC CAT AT[O^6-(4-BTG)] GCC C)	0.0001
d(AAC AGC CAT AT(O^6-HOEtG) GCC C)	5.50
O^6-methylguanine (O^6-MeG)[a]	428
O^6-benzylguanine (O^6-BnG)[a]	0.18
O^6-(4-bromothenyl)guanine [O^6-(4-BTG)][a]	0.0034

IC_{50} values defined as the concentration of inactivator required to reduce the activity of MGMT to 50%.
[a]IC_{50} values determined using same assay conditions as described in Materials and Methods are taken from Ref. (37).

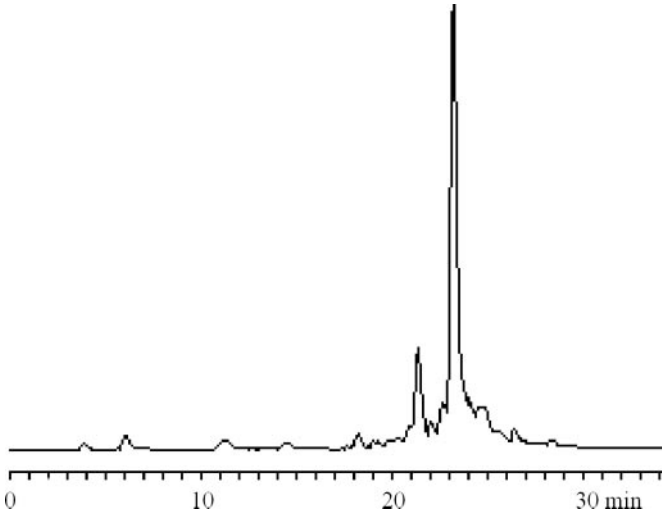


Figure 2. HPLC trace of crude DMT-ON O^6-BnG-containing 16mer ODN. For HPLC conditions see Materials and Methods.

The ability of the modified ODNs to act as inactivators of human MGMT was assessed using a standard assay (24) in which the ODNs were preincubated with the protein for 1 hour followed by the addition of an excess of DNA containing tritiated O^6-methylguanine. Residual MGMT activity remaining after the preincubation step is measured by the incorporation of the tritium label. To establish that the period of incubation of the ODNs with MGMT was appropriate for the reaction to proceed to completion, amounts of the ODNs that gave measurable, but not complete inactivation of MGMT were incubated for various times with MGMT prior to addition of radiolabelled substrate DNA as described elsewhere (24). Under these conditions the inactivation of MGMT by the ODN substrates was complete within 30 min at 37°C (data not shown). The results indicate that the O^6-MeG-, O^6-BnG- and O^6-(4-BTG)-, but not the hydroxyethyl guanine-modified ODNs, were effective inactivators of MGMT. The IC_{50} values for these ODNs were derived from dose-response curves (Figure 3) and represent the concentration of inactivator required to reduce the activity of MGMT by 50%. The respective IC_{50} values are displayed in Table 2. Under the conditions used, the O^6-BnG- and O^6-(4-BTG)-containing ODNs were ∼900 and 34 times more effective than the free bases (37). Furthermore, these

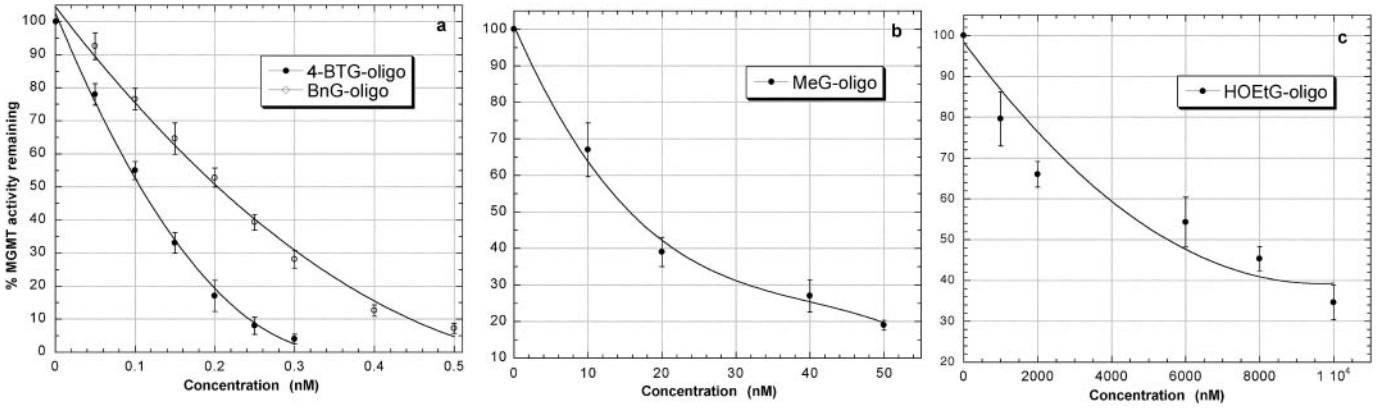


Figure 3. Dose response curves for inactivation of human MGMT by 16mer ODNs. (**a**) O^6-(4-BTG)- and O^6-BnG-containing ODNs; (**b**) O^6-MeG-containing ODN; (**c**) O^6-HOEtG-containing ODN.

ODNs were two orders of magnitude more potent than the corresponding O^6-MeG-containing ODN. The lower IC_{50} of the O^6-(4-BTG)-containing ODN compared with the O^6-BnG-containing ODN is in the same order as the IC_{50} values displayed by the respective free bases.

CONCLUSIONS

In summary we have developed an efficient and robust post-DNA synthesis methodology applicable to the preparation of a wide variety O^6-alkylguanine containing ODNs. The availability of such ODNs will enable for the first time a more comprehensive assessment of the broader substrate range of MGMT and related prokaryotic and eukaryotic alkyltransferases and alkyltransferase-related proteins (38,39). Furthermore, the highly effective inactivation of MGMT by an ODN containing O^6-(4-bromothenyl)guanine suggests that such ODNs might have therapeutic applications if problems of delivery can be addressed.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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