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Synthesis and Characterization of DNA Containing O^6-Carboxymethylguanine

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Abstract—O^6-Carboxymethylguanine was formed in DNA treated with *N*-nitrosoglycocholic acid and believed to be implicated in human gastrointestinal and colorectal tumour. An efficient method is presented here for synthesis of oligodeoxynucleotides containing O^6-carboxymethylguanine at pre-determined positions. The synthetic protocol also allows for production of oligomers containing O^6-aminocarbonylmethylguanine. These guanine-modified oligomers have been fully characterized and could provide a useful tool for biological studies of these modified bases.

Introduction

Chemical alkylation of DNA bases is believed to play an important role in the induction of cancer. For instance the formation of O^6-methylguanine or O^4-methylthymine has a causative role in carcinogenesis by *N*-nitroso compounds.[1] A recent work showed that *N*-nitrosoglycocholic acid (NOGC), a nitrosated bile acid conjugate, reacted with DNA to give rise to several base-adducts including O^6-methylguanine and O^6-carboxymethylguanine (O^6-CMG).[2] It has been suggested in a recent paper[3] that O^6-methylguanine observed as a product of the reaction of NOGC and DNA may rise through decarboxylation of O^6-CMG, however the mechanism remains to be clarified. Recently studies of the cytotoxicity of azaserine in human cells[4] showed that it produced DNA containing carboxymethylated bases (including O^6-CMG) and O^6-methylguanine and that the carboxymethylated bases are the more significant contributor to azaserine lethality in human cells.[4] O^6-Methylguanine is well established as a toxic and promutagenic lesion and can be repaired by O^6-alkylguanine-DNA alkyltransferase (MGMT). However, O^6-CMG is not a substrate for MGMT.[2,4] The O^6-CMG analogue, S^6-carboxymethylthioguanine in a synthetic DNA was not recognized by proteins of post-replication DNA mismatch repair system.[4] However, it is not yet known whether O^6-CMG in DNA can be recognized by other DNA repair systems since DNA containing O^6-CMG was not available. To understand the mechanism of the toxicity of O^6-CMG towards human cells, the availability of DNA containing O^6-CMG at pre-determined positions would be of great use. In the past decade, we have developed various chemical methods to prepare DNA containing modified bases at predetermined positions and successfully used these modified DNA for repair assays and other biological studies.[5] Here we wish to report an efficient method for chemical preparation of oligodeoxynucleotides containing O^6-CMG.

Results and Discussion

Preparation of N2-phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine (4) and its phosphoramidite monomer (5)

The synthetic route is shown in Scheme 1 and primarily based on the preparation of O^6-methoxycarbonylmethyl-2′-deoxyguanosine [i.e. O^6-carboxymethyl-2′-deoxyguanosine, methyl ester].[6] However, acetyl groups instead of methoxyacetyl groups[6] were used in our synthesis for the protection of the hydroxyl groups of the nucleoside, and our choice of protecting groups is probably more cost-efficient. Therefore 3′,5′-diacetyl-N2-phenylacetyl-2′-deoxyguanosine (**1**) was first converted into 3′,5′-diacetyl-N2-phenylacetyl-6-mesitylenesulphonyl-2′-deoxyguanosine (**2**). **2** has been previously prepared, but not isolated.[7] In this work, the intermediate (**2**) was first purified and then converted into its 6-quinuclidinium salt, which in turn was replaced by methyl glycolate. The step for the purification of **2** did provide a better yield of the product **3**. The acetyl groups protecting the sugar of the nucleoside were selectively removed by triethylamine in methanol. This treatment did not remove the phenylacetyl group protecting the N2-amino group of the guanine and the desired product (**4**) was obtained by simple evaporation of the reagents and crystallisation from methanol. This procedure is preferred over the alternative deprotection using aqueous NaOH for a short period,[7] because the de-protection with triethylamine in methanol

Keywords: O^6-carboxymethylguanine; O^6-methylguanine; modified guanine; DNA synthesis.

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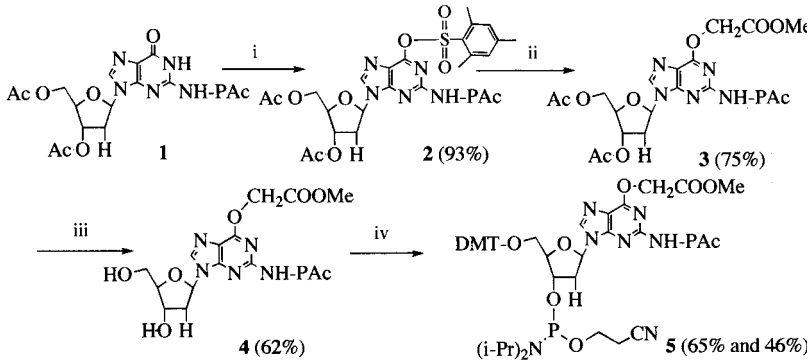


Scheme 1. i. 2-Mestiylsulphonyl chloride. ii. (a) Quinuclidine, (b) Methyl glycolate and DBU. iii. 0.5 M Triethylamine in MeOH. iv. (a) DMT-Cl, (b) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

is chemically specific and there is less the risk of removing the phenylacetyl group on the base. The product (**4**) was then reacted with DMT-Cl and phosphitylated by standard protocols[7b] to give the required monomer (compound **5**).

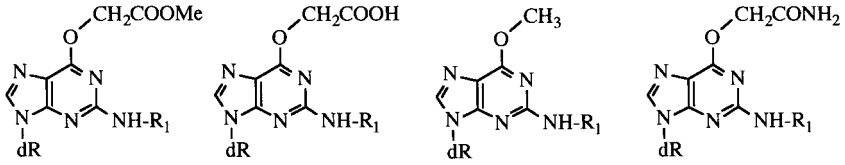
Synthesis and deprotection of oligomers containing O^6-CMG

Oligomers containing O^6-CMG were synthesized by an ABI 391 DNA synthesizer with the manufacturer's standard protocol except the modified monomer (**5**) was added manually and the coupling time for the modified monomer increased to 3 min to ensure a better coupling yield. Based upon the measurement of DMT-cleaving solution, the coupling yield of the modified base was no different from those of unmodified monomers.

In the conventional synthesis of DNA, an isobutyryl group is generally used for the protection of the N2-amino of guanine, however it has been documented that an acyl group at the N2-position of O^6-substituted guanine is much more resistant to removal by ammonia than that at N2-position of unmodified guanine.[7,8] For this reason base-labile groups such as the phenylacetyl group[9] have been used for N2 protection of O^6-alkylguanine[7] and therefore this was chosen for the protection of O^6-CMG in this synthesis. Prior to synthesis of DNA containing O^6-CMG, we examined deprotection of **4** with various deprotecting agents including 0.05 M K_2CO_3 in methanol, concentrated ammonia and 0.5 M aqueous NaOH.[10] Table 1 shows the distribution of nucleoside derivatives after overnight treatment of **4** at room temperature with each of these agents. With K_2CO_3 in methanol,[11] the deprotection was not complete and it gave rise to O^6-methyldeoxyguanosine (**8**), an unwanted product. Further exposure to this deprotecting agent would lead to formation of even greater amounts of O^6-methyldeoxyguanosine. Deprotection with concentrated ammonia produced mainly an amide, N2-phenylacetyl-O^6-aminocarbonylmethyldeoxyguanosine (**9b**).[12] The conversion of the ester (**4**) into the amide form (**9**) was rapid while the removal of phenylacetyl group from **4** by ammonia was very slow due largely to the poor solubility of the resultant amide (**9b**) in the aqueous ammonia. However both amides (**9a** and **9b**) could be readily and quantitatively converted into their carboxylate forms (**7**) by the treatment with aqueous alkaline solution. Deprotection of the amides with aqueous NaOH gave the desired product, O^6-carboxymethyl deoxyguanosine (**7a**) in very good yields.

We then examined the deprotection step at the oligomer level. A pentamer containing O^6-CMG (i.e. CGXAT, where X is O^6-CMG) was deprotected in 0.5 M aqueous NaOH overnight at room temperature and more than 90% of the product eluted as a single peak on HPLC. This product, after purification, was enzymatically digested and

Table 1. Distribution of nucleosides when **4** was treated with deprotecting agents[10]



6a R_1=H **7a** R_1=H **8a** R_1=H **9a** R_1=H

6b R_1=PAc **7b** R_1=PAc **8b** R_1=PAc **9b** R_1=PAc

dR = 2'-deoxyribosyl; PAc = Phenylacetyl

	6a+6b	7a+7b	8a+8b	9a+9b
K_2CO_3/MeOH	45+5	30+15	<5	
NH_3				10+90
0.5 M NaOH		>95		

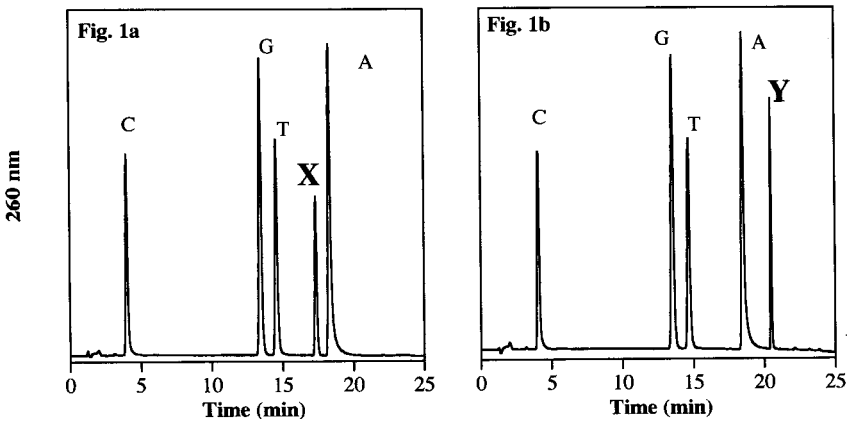


Figure 1. Reverse phase HPLC profiles of the nucleosides from enzymatic digestion of synthetic pentamers: (a) CGXAT, X=O^6-carboxymethyl-2′-deoxyguanosine; (b) CGYAT, Y=O^6-aminocarbonylmethyl-2′-deoxyguanosine. See Ref. 13 for the analysis conditions.

found to have O^6-CMG nucleoside and four standard nucleosides (Fig. 1a), confirming the pentamer has the right composition. It is worth noting that the alkaline deprotection did not remove the carboxymethyl group from the 6-position of the guanine. A possible explanation is that the alkaline solution first hydrolyses the ester and resultant negatively charged carboxylate anion prevents further nucleophilic attack by hydroxide on the 6-position of the guanine.

Deprotection of synthetic oligomers is commonly done by using concentrated ammonia. As mentioned above, deprotection of the modified nucleoside (**4**) was limited by its poor solubility in ammonia. However the oligomer was soluble in aqueous ammonia, but removal of the phenylacetyl group on N2-position of O^6-methoxycarbonylmethylguanine was still slow. The result is consistent with previous reports[7,8] that acyl groups in O^6-alkylguanine are more resistant to ammonia deprotection. Complete removal by aqueous ammonia of the protecting groups from the oligomer was achieved by incubation for 5 days at room temperature. However this had converted the modified oligomer into the oligomer containing O^6-aminocarbonylmethylguanine (Fig. 1b). This may provide a simple method to produce oligomers containing O^6-aminocarbonylmethylguanine. O^6-Aminocarbonylmethylguanine in oligomers could be converted readily into O^6-CMG by alkaline treatment, and Fig. 2 shows a typical time course of such a conversion.

As shown above, deprotection with aqueous alkali satisfactorily produced oligomer containing O^6-CMG. This result is agreed with our early finding[14] that aqueous alkaline solution can be used as a simple deprotecting agent when base-labile groups such as the phenoxyacetyl group are used to protect guanine and adenine and the isobutyryl group to protect cytosine. A possible risk with the alkaline solution is that it might transform N6-acyladenine and N4-acylcytosine into hypoxanthine and uracil respectively. We have examined this possibility by deprotection of a synthetic 12 mer [CGC XAG CTC GCG (X: O^6-CMG)] with 0.5 M aqueous NaOH for 1 day, 2 days or 3 days,[15] and found that all deprotection methods produced the desired oligomer and that there was no substantial difference between these procedures. Form our data, deprotection with 0.5 M aqueous NaOH for 2 days should be sufficient and without the risk to damage the bases of the oligomers. With this protocol for deprotection, longer oligomers (such as 34 mer) containing O^6-CMG were also successfully prepared. These successful results probably depended upon our use of base-labile groups for the protection of the amino groups of all naturally occurring base monomers (from Glen research) as the

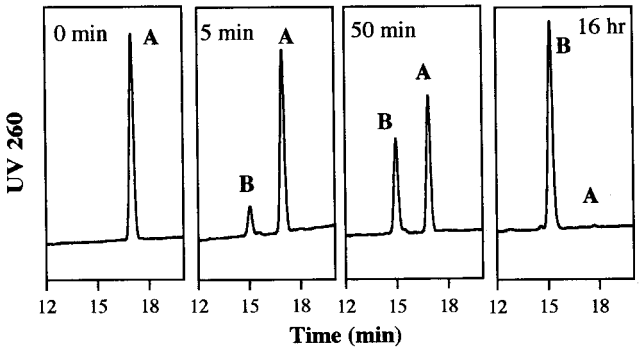


Figure 2. The time course of conversion of pentamer **A** [CGYAT, Y=O^6-aminocarbonylmethylguanine) into pentamer **B** [CGXAT, X=O^6-carboxymethylguanine]: Oligomer **A** was dissolved in 0.5 M aqueous NaOH. The solution at the specified time was analyzed by reversed phase HPLC (for conditions, see Ref. 19).

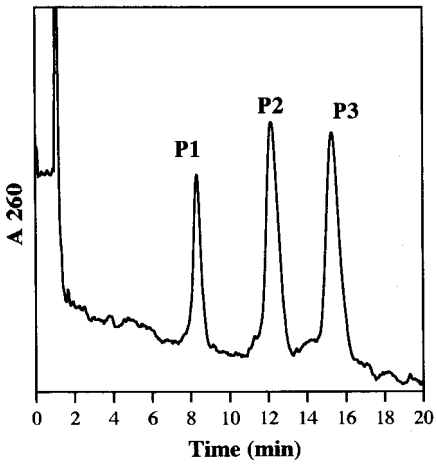


Figure 3. Liquid chromatogram on a NucleoPac column at pH 12 of synthetic 12 mers with the sequence CGC XAG CTG GCG, where X is O^6-methylguanine in **P1**; X is O^6-carboxymethylguanine in **P2**; X is guanine in **P3** respectively. These identical oligomers with one base being different were well separated (for conditions, see Ref. 20).

base-labile groups were removed very rapidly and resultant adenine and cytosine in the oligomer are more stable towards the alkaline solution.

Characterization of oligomers containing O^6-CMG

Oligomers containing O^6-CMG, synthesized and deprotected as above, were recovered with a Nensorb cartridge (from Dupont) and then purified with liquid chromatography. Short oligomers (e.g. pentamers) were purified with reversed phase HPLC[19] which can effectively separate a pentamer containing O^6-CMG from the pentamer containing O^6-aminocarbonylmethylguanine (see Fig. 2). For oligomers of medium or longer length (e.g. 12 mer or 34 mer), anion exchange liquid chromatography is preferred.[16] An eluent of pH 12 gave a good separation of a 12 mer containing O^6-CMG from the same 12 mer containing O^6-methylguanine or the parent containing guanine (Fig. 3). This separation of the O^6-CMG oligomer from the O^6-methylguanine oligomer could be ascribed to the fact that the former has an extra charge from the carboxylate anion.[16] At pH 12 the oligomer containing O^6-CMG has the same number of charges as its parent containing guanine. However the later elution of the parent oligomer may be due to its relatively high hydrophobicity compared to the O^6-CMG oligomer as we noted before that oligomers containing thioguanine (which is believed to be more hydrophobic than guanine) were always eluted later that their parents.[16] These good separations should be very useful for monitoring possible de-carboxylation and further demethylation of O^6-CMG in DNA repair processes.

Purified modified oligomers were enzymatically digested and analyzed by reversed phase HPLC in which the peak for modified nucleoside was observed and co-eluted with authentic sample. These procedures establish a method to produce oligomers containing the desired modified guanines.

In this paper, an efficient method is reported for chemical synthesis of oligomers containing O^6-carboxymethylguanine. Deprotection of modified guanines with various agents were thoroughly examined at the both nucleoside and oligomer levels. Treatment of the synthetic oligomers with aqueous alkali gave the desired modified oligomers while treatment with concentrated ammonia gave oligomers containing O^6-aminocarbonylmethylguanine. The latter could be easily converted to O^6-carboxymethylguanine. These guanine-modified oligomers have been fully characterized and are now being used for biological studies.

Experimental

Chemicals and general methods

Syntheses of oligodeoxynucleotides were carried out by ABI 391 DNA synthesizer (Applied Biosystems), using Ultramild monomers (from Glen research) and supports in which the amino group of dA is protected with the phenoxyacetyl group, that of dG protected with the iso-propylphenoxyacetyl group and that of dC protected with the acetyl group. All other chemicals were from either Aldrich or Sigma and used directly without further purification unless stated otherwise. General methods such as purification with Nensorb Prep cartridges (Du Pont) or Fast protein liquid chromatography (FPLC) on a Mono Q 5/5 column (Pharmacia),[16] nucleoside composition analysis by reversed phase HPLC were carried as described before.[17]

3′,5′-Diacetyl-N2-phenylacetyl-6-mesitylenesulphonyl-2′-deoxyguanosine (2). 3′,5′-Diacetyl-N2-phenyl acetyl-2′-deoxyguanosine (3 g, 6.4 mmol), prepared as described before[7] was dissolved in dichloromethane (100 mL), to which triethylamine (3 mL), dimethylaminopyridine (30 mg) and 2-mesitylenesulphonyl chloride (1.5 g, 6.8 mmol) were added sequentially. TLC (10% CH_3OH/$CHCl_3$) was used to monitor the reaction. After 30 min, about two thirds of the starting material (R_f=0.55) was converted to a new spot (R_f=0.85). Then 0.5 g of 2-mesitylenesulphonyl chloride was supplemented and the solution was left stirred for further a 30 min by which period the starting material was almost consumed. Then the solution was concentrated to a small volume and applied to a silica gel column and eluted with chloroform. The fractions containing the product were pooled together and concentrated to a small volume for crystallization. The crystals formed were washed with diethylether and collected by filtration and dried to give crystals of 3.9 g (yield: 93%). UV λ_{max}=279 nm. ^{1}H NMR Data (in DMSO-d_6): 2.14 (3H, s, acetyl at 5′), 2.21 (3H, s, CH_3 at 4-position of mesitylsulphonyl), 2.29 (3H, s, acetyl at 3′), 2.62 (6H, s, 2×CH_3 at 2 and 6 positions of mesitylsulphonyl), 2.63–3.11 (2H, m, 2′-H and 2″-H), 3.93 (2H, s, CH_2 of phenylacetyl), 4.31–4.38 (3H, m, 4′-H and 5′-H), 5.44 (1H, d, 3′-H), 6.36 (1H, t, 1′-H), 7.36–7.47 (7H, m, C_6H_5 of phenylacetyl and 2×H at 2 and 5 positions of mesitylsulphonyl), 8.48 (1H, s, 8-H) and 12.10 (1, s, N2–H, ex).

3′,5′-Diacetyl-N2-phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine (3). Compound **2** (2.9 g, 4.46 mmol) was dissolved in acetonitrile (20 mL, pre-dried over molecular sieves), to which quinuclidine (0.77 g, 6 mmol) was added. The solution was stirred at room temperature.

After 30 min, TLC (10% $CH_3OH/CHCl_3$) showed all of the starting material was converted into a new spot with its R_f being near zero. Then, methyl glycolate (2 mL, 25 mmol) and 1,8-diazabicyclo(5,4,0)undec-7-ene [DBU] (0.6 mL, 3.9 mmol) were added. After 2 h, about 80% of the intermediate was converted into another new spot (R_f=0.8). Then additional methyl glycolate (0.5 mL) and DBU (0.3 mL) were added and the solution was left stirred overnight. Then the solution was concentrated to a small volume and diluted with ethyl acetate (100 mL), washed with saturated aqueous NaCl (3×100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to an oily residue. The residue was co-evaporated with toluene and purified by silica gel column chromatography. The desired product was eluted with chloroform, collected and dried to give the title compound **3** (in foam, 1.8 g, 75%). UV λ_{max}=267 nm; ν_{max}(KBr) 3446 (br) (N–H), 1743, 1616, 1456, 1234 cm^{-1}; ^1H NMR Data (in DMSO-d_6): 1.98 (3H, s, acetyl at 5′), 2.08 (3H, s, acetyl at 3′), 2.56–3.18 (2H, m, 2′-H and 2″-H), 3.67 (3H, s, OCH_3), 3.79 (2H, s, CH_2 of phenylacetyl), 4.20–4.31 (3H, m, 4′-H and 5′-H), 5.19 (2H, s, CH_2 of O^6-CH_2O), 5.40 (1H, m, 3′-H), 6.34 (1H, t, 1′-H), 7.20–7.35 (5H, m, C_6H_5), 8.48 (1H, s, 8-H) and 10.67 (1H, s, N2–H, ex). MS *m/z* (FAB positive ion) 542 (45, MH^+), 342 (100, MH^+−sugar fragment), 224 (25, MH^+−sugar fragment−Pac group); HRMS (FAB positive ion): MH^+, found 542.1906. $C_{25}H_{28}O_9N_5$ requires 542.1887.

N2-Phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine (4). Compound **3** (1.7 g, 3.15 mmol) was dissolved in 25 mL of 0.4 M triethylamine in methanol. TLC (10% $CH_3OH/CHCl_3$) followed the deacylation course. The acetyl groups of the starting material **3** (R_f=0.8) were gradually removed to form three new spots (R_f=0.73 and R_f=0.62 for the 3′-deacetylated and 5′-deacetylated products, and R_f=0.35 for the 3′,5′-dideacetylated product). Overnight, all the starting material was converted into a single spot (R_f=0.35). The solution was concentrated to a small volume, then co-evaporated with absolute alcohol (once) and toluene (twice) to give a white foam. The residue was crystallized from methanol to give white crystals (650 mg from first crop and 250 mg from second crop, yield: 62.5%). Mp 129–131°C; [found: C, 54.47; H, 5.18; N, 14.78. $C_{21}H_{23}O_7N_5$ requires C, 55.14; H, 5.04; N, 15.32]; UV λmax=269 nm; ν_{max}(KBr) 3420–3354 (br) (O–H, N–H), 1705,1620, 1450, 1234 cm^{-1}; ^1H NMR Data (in DMSO-d_6): 2.30–2.70 (2H, m, 2′-H and 2″-H), 3.57 (2H, m, 5′-H), 3.64 (3H, s, OCH_3), 3.80 (2H, s, CH_2 of phenylacetyl), 3.84 (1H, m, 4′-H), 4.41 (1H, d, 3′-H), 4.91 (1H, t, 5′-OH, ex), 5.18 (2H, s, CH_2 of O^6-CH_2O), 5.32 (1H, d, 3′-OH, ex), 6.34 (1H, t, 1′-H), 7.23–7.33 (5H, m, C_6H_5), 8.51 (1H, s, 8-H) and 10.67 (1H, s, N2–H, ex). MS *m/z* (FAB positive ion) 458 (47, MH^+), 342 (100, MH^+−sugar fragment), 224 (30, MH^+−sugar fragment−Pac group); HRMS (FAB positive ion): MH^+, found 458.1692. $C_{21}H_{24}O_7N_5$ requires 458.1676.

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-N2-phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylamino)-phosphoramidite (5). Compound **4** was converted to its DMT derivative by a standard procedure[18]. In brief: 450 mg of the protected nucleoside **4** was dissolved in 10 mL of anhydrous pyridine and the solution was cooled in an ice-bath. 10 mg of dimethylaminopyridine and 340 mg of dimethoxytrityl chloride (DMT-Cl) were added. TLC (ethyl ether, then 5% $CH_3OH/CHCl_3$) was used to monitor the reaction, and when required, additional amount of DMT-Cl was added to complete the reaction. The reaction was then quenched by addition of 0.5 mL of methanol. After work-up, the product was separated by silica gel column chromatography (0–1.5% methanol in chloroform) and dried to give a foam. The foam was precipitated with toluene in cold pentane and dried to give a slightly yellowish powder (480 mg, 65%). The DMT-derivative was then converted into the title compound **5** by a standard protocol described before.[18] In brief: the above product 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-N2-phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine was dissolved in 5 mL of dried THF and 0.6 mL of *N*,*N*-diisopropylethylamine. Under stirring, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.2 mL) was added. The mixture was stirred for half a hour at room temperature by which period the starting material was almost completely converted to two higher R_f products as checked by TLC (dichloromethane/ethyl acetate/diisopropylethylamine 75:25:2). The mixture was diluted with 30 mL of ethyl acetate and washed with saturated $NaHCO_3$ (3×30 mL). The organic phase was dried over Na_2SO_4 and evaporated to a small volume, then co-evaporated with toluene to an oily residue. The residue was purified by chromatography on silica gel eluting with dichloromethane/ethyl acetate/diisopropylethylamine (75:25:2). The fractions containing the desired products were combined, evaporated and precipitated with toluene into cold pentane, and freeze-dried with benzene to give a while powder (120 mg, 46%).

Modified nucleosides: 6, 7, 8 and 9

Compound **6a** [O^6-methoxycarbonylmethyl-2′-deoxyguanosine] was prepared by a similar procedure as reported before[6] except that in our synthesis acetyl groups instead of methoxyacetyl groups[6] were employed for the protection. In brief: 3′,5′-diacetyl-2′-deoxyguanosine, prepared as described before[7a], was converted into 3′,5′,-diacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine by reacting the starting material with 2-mesitylsulphonyl chloride and then with methyl glycolate/DBU in a similar protocol to that used to prepare compound **3** (see above). Then the resultant product 3′,5′,-diacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine (200 mg) was dissolved in 5 mL of 1 M triethylamine in methanol. After 20 min at room temperature, besides the starting material (R_f=0.75), there were three UV spots (R_f=0.7, 0.6 and 0.3) as shown by TLC (10% $CH_3OH/CHCl_3$). The de-acylation was complete overnight as TLC showed a single UV spot (R_f=0.3). Thus, the reaction solution was evaporated under reduced pressure and lyophilized with benzene to give a white powder **6a**. The UV and NMR data of the product **6a** are in agreement with those published[6] for O^6-methoxycarbonylmethyl-2′-deoxyguanosine. Compound **6b** [N2-phenylacetyl-O^6-methoxycarbonylmethyl-2′-deoxyguanosine] is the same compound as named **4**.

Compound **7a** [O^6-carboxymethyl-2′-deoxyguanosine] was prepared either by hydrolysis of **6a** (R_m=7.2 min, in

HPLC[21]) with 4 mM $Ca(OH)_2$ as reported before[6] or by enzymatic de-esterification with esterase [EC 3.1.1.1 from Porcine liver from Sigma] in 0.55 M phosphate buffer (pH 8.0). Both protocols gave the same compound (R_m=4 min) as analyzed by HPLC[21] Compound **7b** [N2-phenylacetyl-O^6-carboxymethyl-2′-deoxyguanosine, R_m=8.2 min in HPLC[21]) was produced by the same enzymatic de-esterification of **4** (R_m=12 min in HPLC[21]).

Compound **8a** [O^6-methyl-2′-deoxyguanosine] and **8b** [N2-phenylacetyl-O^6-methyl-2′-deoxy guanosine] were prepared as reported before.[7]

Compound **9a** [O^6-aminocarbonylmethyl-2′-deoxyguanosine] and compound **9b** [N2-phenylacetyl-O^6-aminocarbonylmethyl-2′-deoxyguanosine] were prepared by ammonolysis of **4**. Compound **4** (45 mg) was placed in 5 mL of concentrated aqueous ammonia and the solution vigorously stirred at room temperature for 10 min during which time the clear solution became a white suspension. TLC (20% CH_3OH/$CHCl_3$) showed that the starting material (R_f=0.7) were almost completely converted to a major spot (R_f=0.5, for **9b**) and a minor spot (R_f=0.3 for **9a**). The suspension was spun with centrifuge. The supernatant was removed, and the white precipitate was washed with water (5 times) and dried under reduced pressure to give white powder compound **9b** (35 mg, 80%). Compound **9a** was prepared in the same way as above except that the suspension was vigorously stirred overnight to remove the phenylacetyl group from N2-position of the nucleoside. By this protocol 40 mg of **9a** (white powder) was obtained from the ammonolysis of 60 mg of **4**. Data for **9a**: R_f=0.33 (in 20% MeOH/$CHCl_3$); R_m=5.5 min (HPLC[21]); UV: λ_{max}=284 and 247 nm; ν_{max}(KBr) 3420 (br) (O–H, N–H), 1693, 1624,1245 cm^{-1}; ^{1}H NMR Data (in DMSO-d_6): 2.29–2.73 (2H, m, 2′-H and 2″-H), 3.47–3.56 (2H, m, 5′-H), 3.81 (1H, m, 4′-H), 4.34 (1H, d, 3′-H), 4.82 (2H, s, CH_2 of O^6-CH_2O), 4.99 (1H, t, 5′-OH, ex), 5.28 (1H, d, 3′-OH, ex), 6.20 (1H, t, 1′-H), 6.41 (2H, br, NH_2 at N2, ex), 7.34–7.39 (2H, br, NH_2 of $CONH_2$, ex) and 8.12 (1H, s, 8-H). MS *m/z* (FAB positive ion): 325 (35, MH^+) 209 (70 MH^+−sugar fragment), 136 (88); HRMS (FAB positive ion): MH^+, found 325.1266. $C_{12}H_{17}O_5N_6$ requires 325.1260. Data for **9b**: R_f=0.5 (in 20% MeOH/$CHCl_3$); R_m=8.7 min (HPLC[21]); UV: λ_{max}= 268 nm; ν_{max}(KBr) 3446–3225 (br) (O–H, N–H), 1695, 1579, 1251 cm^{-1}; ^{1}H NMR Data (in DMSO-d_6): 2.29–2.73 (2H, m, 2′-H and 2″-H), 3.57–3.60 (2H, m, 5′-H), 3.84 (3H, m, 4′-H and CH_2 of phenylacetyl), 4.42 (1H, d, 3′-H), 4.92 (1H, t, 5′-OH, ex), 4.94 (2H, s, CH_2 of O^6-CH_2O), 5.32 (1H, d, 3′-OH, ex), 6.33 (1H, t, 1′-H), 7.23–7.52 (7H, m, C_6H_5 and NH_2 of $CONH_2$), 8.49 (1H, s, 8-H) and 10.67 (1H, s, N2–H, ex). MS *m/z* (FAB positive ion): 443 (40, MH^+), 327 (100, MH^+−sugar fragment) 270 (30, MH^+−sugar fragment−alkyl), 209 (15, MH^+−sugar fragment−Pac group); HRMS (FAB positive ion): MH^+, found 443.1694. $C_{20}H_{23}O_6N_6$ requires 443.1679.

Synthesis and purification of oligodeoxynucleotides containing O^6-carboxymethylguanine or O^6-aminocarbonylmethylguanine

Oligodeoxynucleotides containing modified bases were synthesized by an ABI DNA synthesizer with the manufacturer's standard protocol except the modified monomer was added manually and its coupling time increased to 3 min to ensure a better coupling yield. The CPG support bearing the synthesized oligomers was treated with 0.5 M aqueous NaOH (2 days at RT) for production of oligomer containing O^6-carboxymethylguanine or with concentrated ammonia (5 days at RT) for production of O^6-aminocarbonylmethylguanine respectively. The resultant oligomers were first recovered by Nensorb Prep cartridges according to the manufacturer's instruction. For further purification, short oligomers (e.g. 5 mer) was purified by reversed phase HPLC with 0.05 M KH_2PO_4 buffer at pH 6.6 and acetonitrile,[19] and longer oligomers (12 mer and 34 mer) were purified by FPLC with 0.4 to 1.2 M aqueous NaCl (pH 12) similar to that reported before.[16]

Conversion of O^6-aminocarbonylmethylguanine oligomers to O^6-carboxymethylguanine oligomers

O^6-Aminocarbonylmethylguanine oligomers prepared as above were dissolved in 0.5 M aqueous NaOH, and the conversion was monitored either by reversed phase HPLC (for 5 mer)[19] or by FPLC (for 12 mer).[16]

Nucleoside composition analysis

Oligomers, after purification, were digested by incubation with nuclease P1 in a solution of pH 4.5 overnight at 37°C, then with alkaline phosphatase in a solution of pH 8 for 2 h at 37°C. The digest was analyzed with reversed phase HPLC[13] as reported before.[14,18]

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10. The general protocol for deprotection was carried out as follows: N2-phenylacetyl-O^6-methoxycarbonylmethyldeoxyguanosine (**4**) [2 mg] was placed in an Eppendorf tube, to which a deprotecting agent (200 μL of 0.05 M K_2CO_3 in methanol, or of concentrated ammonia or of 0.5 M aqueous NaOH) was added. The reaction solution was left at room temperature overnight and analyzed by reversed phase HPLC (for conditions, see Ref. 13) and by TLC (in 20% MeOH/$CHCl_3$).
11. The producer of this agent (Glen Research, USA) recommended that the agent could completely remove the protecting groups from the oligomers made by Ultramild monomers (where guanine is protected with isopropylphenylacetyl group) within 2 h at room temperature. We also used 10% DBU in methanol for deprotection of the modified nucleoside (**4**) and found that about one third of the product was an unwanted O^6-methyldeoxyguanosine (**8a**).
12. The product, N2-phenylacetyl-O^6-aminocarbonylmethyldeoxyguanosine (**9b**) was not soluble in aqueous ammonia and therefore precipitated out. However, when the solution was vigorously stirred, the product **9b** was further deprotected to give O^6-aminocarbonylmethyldeoxyguanosine (**9a**) as the major product.
13. Analysis conditions: Column: 8NVC184μ Radial-Pac Cartridge from Waters, 1 mL/min, 260 nm. The column was eluted by buffer A (0.05 M KH_2PO_4, pH 4.5) and buffer B (consisting of 67% buffer A and 33% CH_3CN) with the following gradient: Time (buffer B): 0 min (2%), 4 min (2%), 15 min (15%), 20 min (50%), 25 min (75%).
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15. After synthesis, the CPG support bearing synthesized 12 mer [CGC XAG CTC GCG (X: modified G)] was divided into three parts. Each part was placed in an Eppendorf tube, to which 1 mL of 0.5 M aqueous NaOH was added and the solution was left at room temperature for a specified period (e.g. 1 day, 2 days or 3 days) respectively. The oligomer was recovered with Nensorb Prep purification cartridge and then analyzed by FPLC with alkaline solution (pH 12) [85% of 0.4 M NaCl and 15% of 1.2 M NaCl] (also see Ref. 16).
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19. Analysis conditions: Column: 8NVC184μ Radial-Pac Cartridge from Waters, 1 mL/min, 260 nm. The column was eluted by buffer C (0.05 M KH_2PO_4, pH 6.6) and buffer D (consisting of 67% buffer C and 33% CH_3CN) with the following gradient: Time (buffer D): 0 min (10%), 30 min (40%).
20. Analysis conditions: NucleoPac PA-100 column from Dionex; 1 mL/min, 260 nm. The column was eluted by Eluent E (0.2 M NaCl, pH 1.2) and Eluent F (1.2 M NaCl, pH 12) with the following gradient: Time (Eluent F): 0 min (15%), 5 min (15%), 20 min (18%).
21. Analysis conditions: Column: 8NVC184μ Radial-Pac Cartridge from Waters, 1 mL/min, 260 nm. The column was eluted by buffer A (0.05 M KH_2PO_4, pH 4.5) and buffer B) with the following gradient: Time (buffer B): 0 min (15%), 5 min (50%), 8 min (70%), 15 min (70%), 17 min (15%).