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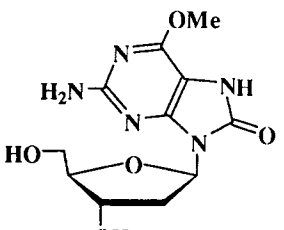
Synthesis of 8-Oxo-7,8-Dihydro-6-*O*-Methyl-2'-Deoxyguanosine and its use as a Probe to Study DNA-Base Excision by MutY Enzyme

Chamakura V. Varaprasad, Nickolai Bulychev, Arthur P. Grollman and Francis Johnson*

Department of Pharmacological Sciences, School of Medicine, Health Sciences Center
State University of New York at Stony Brook, Stony Brook NY 11794-8651

Abstract: A 23mer oligomer containing 8-oxo-7,8-dihydro-6-*O*-methyl-2'-deoxyguanosine (**1**) has been synthesized from 2'-deoxyguanosine. The activity of MutY protein toward this and a related oligomer containing 8-methoxy-dG has been studied.

Oxidative stress to DNA has emerged as one of the most important events that leads to a number of human diseases including cancer. It is known that DNA is constantly under attack by reactive oxygen species generated during normal cell metabolism. Cellular defense mechanisms against such damage include antioxidants such as uric acid, ascorbic acid and ß-carotene and a number of enzymes including superoxide dismutase, catalase and glutathione peroxidase. Despite these defenses, damage still occurs and is characterized by a number of oxidized pyrimidine and purine bases of which 8-oxo-7,8-dihydro-2'-deoxyguanosine **2**[1](8-oxo-dG) has emerged as one of the most significant. An important mechanism of repair of such potentially mutagenic lesions is the base - excision pathway. In *E. Coli* for example the damaged base **2** is efficiently repaired by a triad of proteins derived from genes designated as *mutM, mutY* and *mutT* . The enzymatic excision of damaged bases is arguably dependent on the recognition of the oxidative lesions and the binding efficiency of the specific enzymes to the DNA. In a previous paper[2] we have outlined a mechanism



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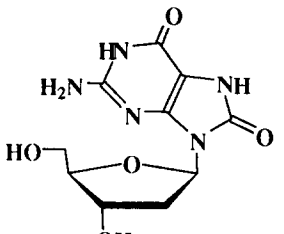

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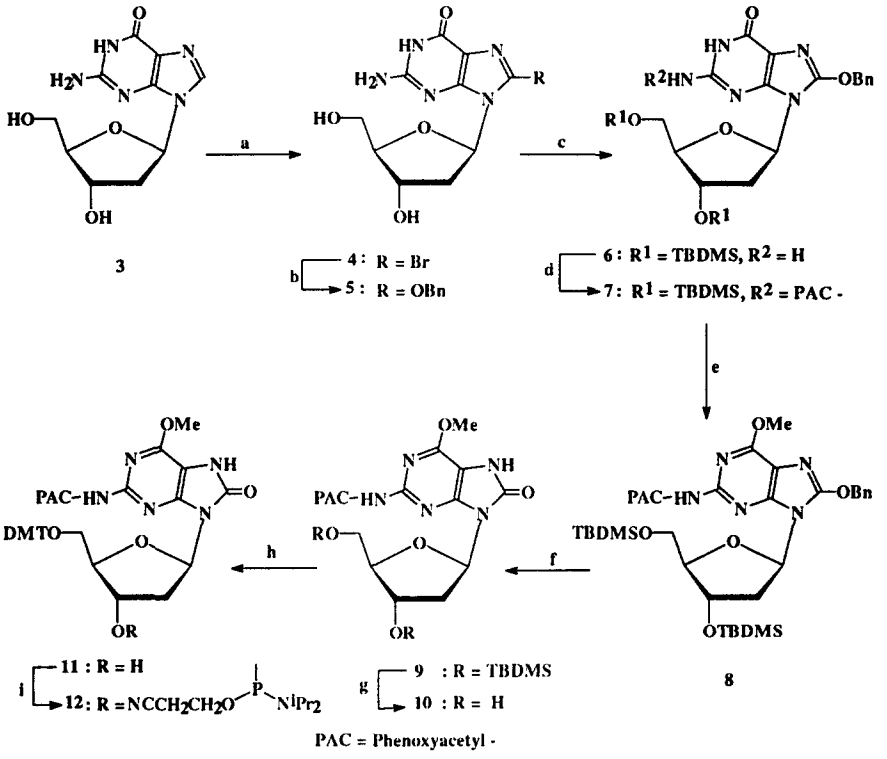
by which MutM protein effects base excision in the case of 8-oxo-dG. In continuing studies on these enzymes we have synthesized the title compound and incorporated it into a pyrimidine rich oligomer [5'-CTCTCCCTTCXCTCCTTTCCTCT- 3', (**I**); where X = 8-oxo-7,8-dihydro-6-*O*-methyl-2'-deoxyguanosine (**1**)] with the objective a) of examining the mechanism by which MutY protein effects glycosidic bond cleavage of the normal base (dA), in the mismatch pair dA:8-oxo-dG, and b) to understand the enzymatic recognition aspects of this type of damage. This letter describes the details of the synthesis of the above

oligomer containing the lesion **1**[3,4] and the preliminary results of the activity of MutY enzyme toward both the above oligomer and an identical oligomer containing 8-methoxy-dG[11] in place of **1**.

The synthesis (Scheme-1) of the target phosphoramidite **12** needed for the incorporation of compound **1** began with 2'-deoxyguanosine **3**. The method of synthesis initially utilizes the literature procedure for the preparation of 8-benzyloxy-dG **5**[5]. Deoxyguanosine was treated with Br_2 in water to obtain 8-bromo-2'-deoxyguanosine **4** which upon reaction with sodium benzyloxide (3.2 eq, freshly generated *in situ* from sodium in hot (65°C) benzyl alcohol) in a mixture of benzyl alcohol and DMSO (1:1) afforded 8-benzyloxy-dG **5** (overall yield from 2'-deoxyguanosine, 60%). Compound **5** was then converted into the N^2-protected intermediate **7** via the *bis*-TBDMS ether **6** . This was accomplished by treating diol **5** with TBDMS-Cl (3.0 eq) in DMF in presence of freshly sublimed imidazole (6.0 eq) for 4h at 24°C. Compound **6** then reacted smoothly

Scheme-I



Scheme-I: a) Br_2/ H_2O; b) NaOBn, BnOH-DMSO (1:1), 65°C, 24h; c) TBDMS-Cl (3.0eq), Imidazole (6.0eq), DMF, 24°C, 4h; d) Phenoxyacetyl chloride (1.5eq), Py., 24°C, 16h; e) DEAD, Ph_3P, anhy. MeOH (50.0 eq), dioxane, 24°C, 16h ; f) H_2 (50.0 psi), Pd/C (10%), 24°C, 1h; g) TBAF (4.0eq, 1.0M), 24°C, 2h; h) DMT-Cl (1.1eq), DMAP (0.1eq), Py., 24°C, 15min. ; i) Cl-PN^iPr_2(OCH_2CH_2CN) (1.2 eq), NEt_3 (4.0 eq), CH_2Cl_2, 30min. 24°C, quantitative.

with phenoxyacetyl chloride[6] in pyridine giving intermediate **7** in high yield (71% from compound **5**). The conversion of the C6 keto group of compound **7** to the corresponding methyl ether **8** proved difficult. However application of the Mitsunobu[7] method was reasonably successful. Thus the treatment of compound **7** with DEAD (1.5 eq), Ph_3P(1.5 eq) and anhydrous MeOH (50.0 eq) in anhydrous dioxane for 16h at 24°C

resulted in the required 6-*O*-methyl ether **8**[8] in moderate yield (54.5%). The ^{1}H NMR of the compound **8** showed a sharp singlet at 4.05ppm (3H) and this together with the absence of the amidic proton at position 1 clearly indicated the presence of the 6-*O*-methyl group.

Hydrogenation (50 psi H_2) of **8** in the presence of a 10% Pd/C catalyst for 1h at 24°C led to the 8-oxo derivative **9** in excellent yield (96%). Further treatment of compound **9** with 1M tetrabutylammonium fluoride (4.0 eq) in THF then afforded the N^2-protected monomeric diol **10** in high yield (90%) after silica gel chromatography. Monomer **10** was first protected as its DMT-ether **11** (96% yield) by treatment with DMT-Cl (1.1 eq) in the presence of a catalytic amount (0.1 eq) of DMAP in pyridine at 24°C for 15min. Thereafter the target phosphoramidite **12** was obtained from the alcohol **11** by allowing it to react with the 2-cyanoethyl N,N-diisopropyl chlorophosphoramidite(1.2 eq) in presence of triethylamine (4.0 eq) using a procedure[9] developed in these laboratories. The phosphoramidite **12** thus obtained was sufficiently pure for use in oligomer synthesis.

The required oligodeoxynucleotide (**I**) was synthesized following a standard phosphoramidite protocol[10]. However the concentration of the modified monomer was 2.5 fold greater than that normally used with the commercial phosphoramidites and the duration of addition was also increased by five times compared with standard monomer additions. This allowed a coupling yield of more than 96% by trityl assay at this step in the synthesis. The oligomer after synthesis was released from the resin by treatment with an aq. solution of conc. ammonia (Sp. gr. 0.90, 14.8N) in presence of ascorbic acid. The addition of an antioxidant is important otherwise the oligomer is oxidatively degraded during the alkali treatment. Surprisingly complete deprotection of N^2-phenoxyacetyl group required an extended period of time (40h). The crude 5'-DMT-oligomer thus obtained was purified over reverse phase HPLC (Bondapak C_{18} column, 7.8x300mm) with acetonitrile and aq. triethylammonium acetate (0.1M, pH 7.3) as eluants using a gradient { from 16% to 36% (30min.)} of acetonitrile. The oligomer containing the 5'-DMT was treated with a solution of acetic acid and H_2O (5:1) for 20 min. at 24°C to remove the DMT. The resulting DNA again was purified by reverse-phase HPLC using the same eluants {gradient from 9% to 30% (30min.) of acetonitrile}.

The determination of the molecular weight of the 5'-DMT-oligomer was accomplished by electrospray mass spectrometry (Calculated: 7109.061; Found: 7108.95) which confirmed (a) the incorporation of the 8-oxo-6-*O*-methyl-2'-deoxyguanosine (**1**) and (b) that no other species were present. Gel electrophoresis also indicated that the material was homogeneous. The interactions of the MutY enzyme with oligomers containing **1** and 8-methoxy-dG[11] were then studied. The preliminary data is shown below (table).

Table: Enzyme Affinity Assay Results

S. no.	DNA duplex	K_d (nM)	$k_{cat.}$ (min^{-1} x 10^3)
1.	dA: 8-Oxo-dG	5.5 ± 0.7	95.0 ± 3.5
2.	dA: 8-methoxy-dG	20.0 ± 4.1	3.2 ± 0.1
3.	dA: 6-*O*-methyl-8-oxo-dG	5.8 ± 0.8	No cleavage

These results indicate that the duplex DNA containing the mismatch dA: 6-*O*-methyl-8-oxo dG binds the enzyme in the same order as it binds damage mismatch dA:8-oxo-dG but unlike the latter case, no excision of dA occurs. On the other hand the duplex oligomer containing the mismatch dA:8-methoxy-dG[11] binds the enzyme poorly and the cleavage of the dA residue is very inefficient. These data[12] suggest a) that in the dG residue, opposite the base that is being lysed (dA) a carboxamide or urea-like residue must be present in the imidazole ring to serve as a damage - recognition element for the enzyme (the presence of an sp^3 hybridized oxygen at the 8 position of dG is insufficient for enzyme complexation) and surprisingly b) that to achieve enzymatic excision of the normal base i.e. dA in the mismatch dA:8-oxo-dG, an oxo group must also be present at the 6 position of the guanine base. Further studies on the mechanism of base excision by MutY protein are in progress.

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3. The compound **1** (92% yield) was obtained from its PAC-derivative **10** by its treatment with ethanolic methyl amine (33%) for 4h at 24°C. Interestingly no displacement of the 6-*O*-methyl group was observed when the monomer **10** treated with methyl amine or ammonia under the conditions of DNA synthesis in contrast to such substitution which occurs with 6-*O*-methyl dG.
4. Analytical data of Compound **1**: ^{1}H NMR (DMSO-d_6, 250MHz) PPM:10.85(br s, 1H, NH), 6.19 (s, 2H, NH_2), 6.09 (t, J=7.26,1'-H), 5.13 (1H, 3'-OH), 4.86 (1H, 5'-OH), 4.35 (m,1H, 4'-H), 3.88(s, 3H, -OCH_3), 3.76 (m,1H, 3'-H), 3.63-3.39 (m, 2H, 5-CH_2), 3.0 (m, 1H, 2'-H), 1.93 (m,1H, 2'-H); ^{13}C NMR(DMSO-d_6, 62.50 MHz) PPM: 158.23, 153.10, 152.13, 151.20, 97.22, 87.39, 81.21, 71.41, 62.41, 53.03, 35.58. EIMS: m/z 297 [(M^+) 8.26%], 181[(Heterocyclic base+1H)$^+$ 100%], 165(78.5%). Crystallization from MeOH - Ethyl acetate mixture gave white crystals (Found: C, 44.13; H, 5.33; N, 23.04. $C_{11}H_{15}N_5O_5$ 0.2 H_2O requires C, 43.91; H, 5.16; N, 23.28 %). M. p. 191°C.
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12. For a complete discussion of the biological data see Bulychev, N. ; Varaprasad, V. C. ; Johnson, F. ; Grollman, A. P. *J. Biol. Chem.*(submitted for publication).

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