Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 939–944

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

# OLIGORIBONUCLEOTIDES CONTAINING 8-OXO-7,8-DIHYDRO-GUANOSINE AND 8-OXO-7,8-DIHYDRO-2'-O-METHYLGUANOSINE: SYNTHESIS AND BASE PAIRING PROPERTIES

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Received 26 January 1998; accepted 9 March 1998

**Abstract:**7,8-Dihydroguanosine(8-oxoG) and 7,8-dihydro-2'-O-methylguanosine(8-oxoG-Me) were chemically synthesized. Oligoribonucleotides which contain 8-oxoG or 8-oxoG-Me were synthesized. Incorporation of nucleotides opposite 8-oxoG and 8-oxoG-Me with dNTP by MMLV reverse transcriptase during cDNA synthesis was characterized . 

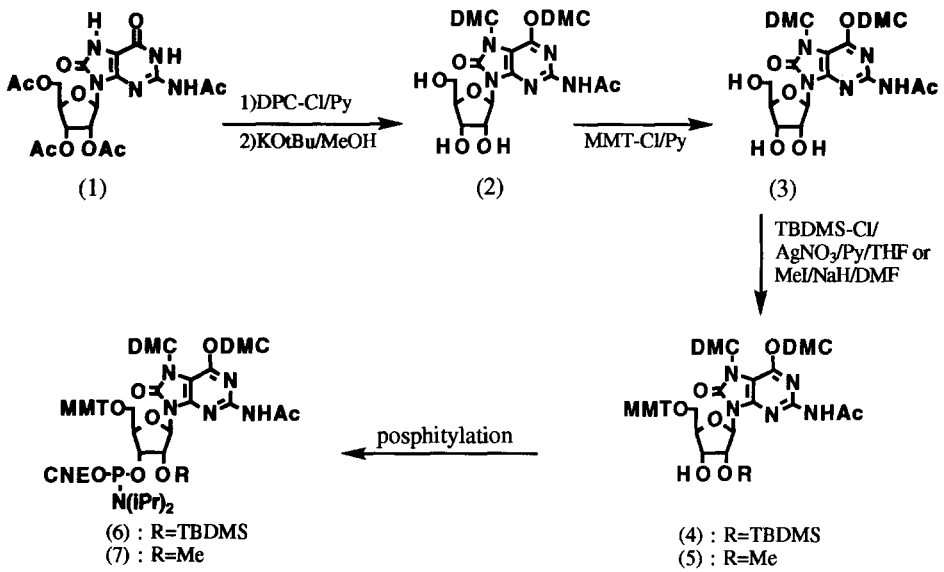
Deoxyguanosine residues in DNA are hydroxylated at the C8 position both *in vivo* and *in vitro* to form an 8-oxo-7,8-dihydroxydeoxyguanosine(8-oxodG) by oxidative stress[1-3] . The formation of 8-oxodG is considered to be a likely cause of various types of cellular DNA damage including strand breaks, abasic sites and base modifications. Some of this cellular DNA damage is suspected to play an important role in mutagenesis, carcinogenesis, aging and other degenerative diseases[1,4]. The base pairing or misreading as well as conformational change properties of the 8-oxodG was well characterized[5-9]. Oxidative base damages in RNA strands were also analyzed in the oxidoreduction of Torula yeast RNA[10], and also revealed oxidatively damaged sites using DNA primer extension assay with AMV reverse transcriptase[11]. It has been reported that 8-oxoguanosine(8-oxoG) was formed in isolated RNA bacteriophages which was photoinactivated with methylene blue and rose bengal[12].

On the other hand, it has been shown that nuclease resisting 2'-O-methylnucleotides formed stable duplexes with RNA strands, and could form triplexes with duplex DNA[13-14]. Solution and solid state studies showed that 2'-O-methyl modification of single or double-stranded RNA does not cause any significant conformational change[15-16]. In these cases, the C8 position of the 2'-O-methylnucleoside remained intact. If the 8-oxoG residue has a 2'-substituent, however, there is significant steric hindrance between the bulky oxo function of C8 and the 2'-substituent in glycosidic linkage. This structural conversion may also facilitate the incorporation of a different base opposite the lesion during transcription. We presumed that the substitution of the 2'-OH of the 8-oxoG by the methyl group(Me) may affect the conformation of the residue and may induce the incorporation of other dNTPs at the opposite to 8-oxoG-Me. In other to investigate the above presumption, we prepared 8-oxo-7,8-dihydroguanosine(8-oxoG) and 8-oxo-7,8-dihydro-2'-O-methylguanosine(8-oxoG-Me) phosphoroamidites and also synthesized 30-mer oligonucleotides containing 8-oxoG or 8-oxoG-Me.

The synthetic routes for the 8-oxoG and 8-oxoG-Me building blocks are illustrated in Scheme 1. The starting compound **1** was prepared from guanosine according to the described procedure by Roelen et al.[17]. Dimethyl carbamoyl(DMC) group was used to protect the exocyclic amino and lactam functions of 8-oxoG. The DMC was

0960-894X/98/$19.00 
*PII:* S0960-894X(98)00150-4

chosen as it offers also O6-protection of deoxyguanosine that is compatible with the oligodeoxynucleotide synthesis[18]. Compound **1**(5 g, 10.67 mmol) was treated DMC-Cl(1.1 mL, 12 mmol) and diisopropylethylamine (3.13 mL, 18 mmol) in dry pyridine(100 mL) at room temperature for 5h. Pyridine was evaporated in vacuo and the residue was precipitated in n-hexane, and the crude mixture was purified by column chromatography on silica-gel, eluting with gradient of 0-4% methanol in methylene chloride. Deacetylation of compound **1** with 0.1M solution of potassium *tert*-buthoxide in methanol for 30 min gave 2',3',5'-free guanosine derivative(**2**) in 80% yield. $^1$H NMR analysis of the obtained product revealed the presence of the two DMC groups[19]. Tritylation of **2**(4.2 g, 7.36 mmol) with methoxytritylchloride(3.42 g, 11.04 mmol) and triethylamine(2.31 mL, 16.56 mmol) in dry pyridine gave compound **3** in 72% yield. The tritylation allowed efficient chromatographic separation for 2'-hydroxyl protection of guanosine with silyl or methyl group.




**Scheme 1**

The guanosine derivative **3**(2.5 g, 2.97 mmol) was dissolved in THF(10 mL). To this silver nitrate(0.76g, 4.45mmol) and pyridine(0.89 mL, 10.99 mmol) were added and the solution was stirred until the silver nitrate was completely dissolved. *tert*-Butyldimethylsilyl(TBDMS)chloride(0.76 g, 5.05 mmol)[20] was added and the reaction was stirred at room temperature for 6h. The different mobilities of the 2'- and 3'-silylated isomers were shown on TLC. After work-up, the 2'-silylated isomer was separated in 53% yield by column chromatography on silica-gel and the separate was precipitated in petroleum ether.

2'-O-Methylation of **3** was performed with methyl iodide[21]. Compound **3**(3 g, 3.56 mmol) was dissolved in DMF(20 mL) and the reaction was cooled to -50°C. To this sodium hydride(0.14 g, 5.8 mmol) was added and the mixture was stirred for 40 min. Several portions of methyl iodide (4 g, 28.5 mmol) was added to the reaction mixture. After 5h, the reaction was allowed to cool to -15°C, ammonium chloride was added. $^1$H NMR analysis of the crude compound **5** showed the presence of 2'-O-methyl and 3'-O-methyl isomers in a ratio of 60:40, and the 2'-O-methyl isomer was separated from this mixture in 55% yield[22].

Phosphitylation of the protected nucleosides was accomplished by treatment with 2-cyanoethyl-N,N-

diisopropylaminochlorophosphine, 2,4,6-collidine and N-methylimidazole in THF[23]. After purification by column chromatography, the phosphoramidite units were precipitated in toluene-petroleum ether at -20°C. Phosphoramidites of 8-oxo-7,8-dihydroguanosine(8-oxoG) **6** and 8-oxo-7,8-dihydro-2'-O-methylguanosine(8-oxoG-Me) **7** were used in the solid support synthesis of the oligoribonucleotides[5'-UCC AUU UUC AXA AUU GGG UGU CGA CAU AGC-3'(X=G, :ORNS-1, X=8-oxoG:ORNS-2, X=8-oxoG-Me: ORNs-3)] using an automated synthesizer(Applied Biosystem DNA Synthesizer, model 391). Standard protocol of Trityl-Off oligoribonucleotides synthesis carried out with 1μmole of controlled pore glass(CPG) support with phosphoramidite unites **6,7** and commercially available ribonucleoside phosphoramidites as the incoming ribonucleoside synthons.

After the synthetic cycles were completed, the CPG support was treated with 25% ethanolic ammonia at 55°C for 15h. The oligonucleotide solution was removed from CPG support and the solvent was evaporated in vacuo. For desilyation of the oligonucleotides, fluoride ion treatment was employed. Neat triethylamine trihydrofluoride (1 mL)[24] was added to the residue and the mixture was vortexed throughly, and then shacked at ambient temperature for 24h. The mixture was quenched with sterilized water(200 μL) and was added 1-butanol(10 mL) and then chilled at -20°C over 1h. The tube was centrifuged at 3000 r.p.m. for 5 min and the buthanol decant to collected the precipitated oligonucleotide. For further purification, 20% polyacrylamide gel electrophoresis (PAGE)) was performed. About 15 ODU of pure oligonucleotide were obtained from each oligonucleotides by PAGE. The purities of the oligonucleotides were analyzed by PAGE. The PAGE showed that better coupling and deblocking efficiences were obtained when the 8 -oxoG and 8-oxoG-Me phosphoramidite units were

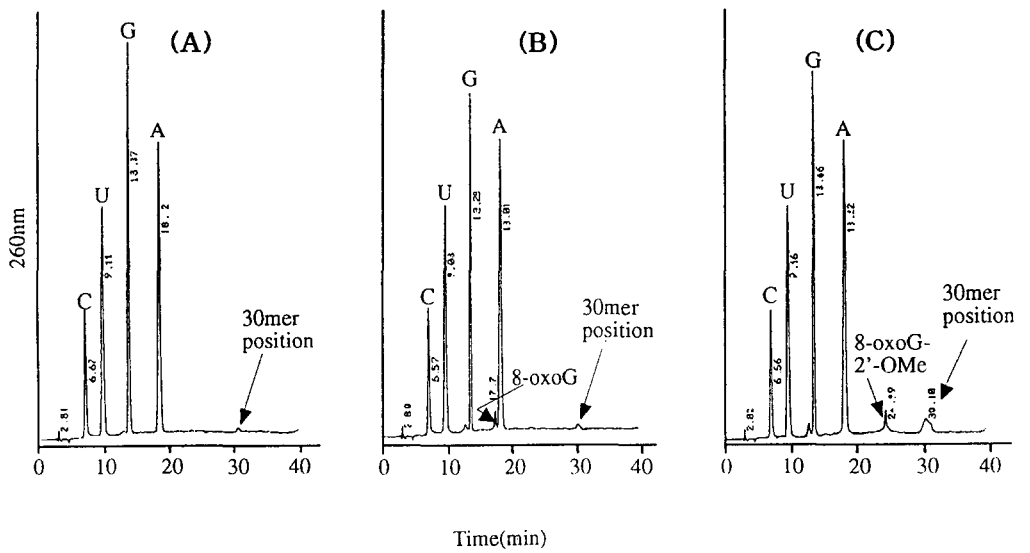



Figure 1. Reverse phase HPLC of the enzymatic digestion products of ORNs-1: (A), ORNs-2 : (B), ORNs-3 : (C). 0.5 OD($A_{260nm}$) of ORNs-1, -2 or -3 was incubated in a 100 μL solutions of 10 μL of 1 M Tris-HCl(pH 7.5), 1 μL of 1 M $MgCl_2$, 10 μL of phosphodiesterase I (0.26 mg/mL) and 5 μL of Alkaline phosphatase(0.15 u/mL) at 37°C for 1h. HPLC conditions : column : Bio-sil C18HL 250x4.6mm(BioRad) ; buffers : A = 0.1 M TEAA(pH7.0), B = 0.1 M TEAA(pH7.0)/$CH_3CN$, 50/50 ; gradient : 0-1 min 100%A, 1-25min 25% B, then 25-35 min 50% B; Detection : 260 nm ; flowrate : 1 mL/min ; column temperature : 35°C.

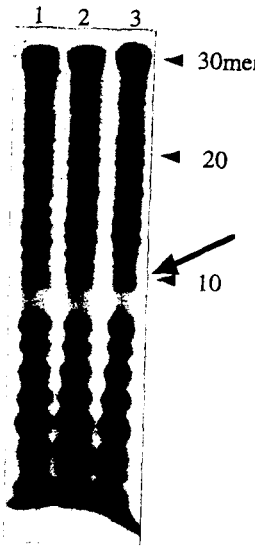



Figure 2. Autogadiography of a polyacrylamide gel of alkali treatment of the modified or unmodified oligoribonucleotides. Lane 1 : $^{32}$P-ORNs-1, Lane 2 : $^{32}$P-ORNs-2, Lane 3 : $^{32}$P-ORNs-3. 20000 cpm/μL of $^{32}$P-ORNs-1, -2 or -3 was incubated in a solutions of 50mM of $NaHCO_3$(pH 9.0) and 1mM EDTA at 90°C for 10 sec. Blow arrow of the lane 3 indicated the 8-oxoG-Me position.

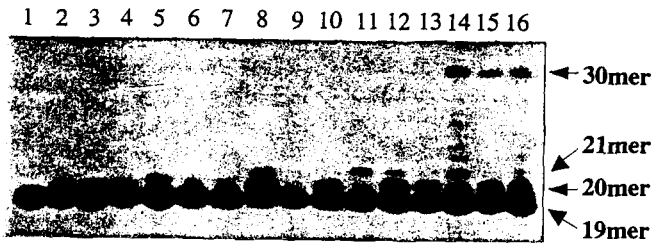



Figure 3. Autoradiogram of a polyacrylamide gel showing incorporation of dNTPs into the primer at the site opposite to **X** ; the normal template ORNs-1(**X**=G, lane 2,5,8,11,14), modified template ORNs-2(**X**=8-oxoG, lane 3,6,9,12,15) or ORNs-ORNs-3(**X**=8-oxoG-Me, lane 4,7,10,13,16) and the primer[5'-$^{32}$P-d(GCT ATG TCG ACA CCC AAT T)3', lane 1] were incubated with MMLV-RT in the presence of dATP(lane 2-4), dGTP(lane 5-7), dCTP(lane 8-10), TTP(lane 11-13) and dNTPs(lane 14-16). 19mer indicates unextended primer and 20mer, 21mer and 30mer indicate extended primers.

employed to the synthesis of oligoribonucleotides at predetermine positions.

The presence of 8-oxoG and 8-oxoG-Me in synthetic oligoribonucleotides was verified by digestion with a combination of phosphodiesterase I and alkaline phosphatase, followed by separation of the component nucleosides by reverse phase HPLC analysis(Fig. 1). The quantitation of the digestion products from the fragments, ORNs-2 and ORNs-3, showed that they were present in expected molar ratios. The molar ratios of the nucleosides from the digestion of ORNs-2 and ORNs-3 were as follows: C/U/G/8-oxoG/A = 4.06/7.10/1.89/1.00/4.95 and C/U/G/8-oxoG-Me/A = 4.16/7.14/1.91/1.00/4.93. More direct proof for the presence of 8-oxoG or 8-oxoG-Me in the ORNs-2 or ORNs-3 was obtained from the partial digestion with alkali treatment(Fig. 2). The hydrolized products were loaded on denaturated PAGE. The bands of ORNs-2 and ORNs-3 showed distinctive migrations from the ORNs-1. Especially the disappeared 19th band of ORNs-3 indicated the presence of 8-oxoG-Me.

We tested the base pairing properties of modified or unmodified guanosine with incoming dNTPs substrates during cDNA synthesis. When the normal template ORNs-1, 3'(CGAUAC AGC UGU GGG UUA AXA CUU UUA CCU)5'(X=G) and primer, 5'd($^{32}$P-GCT ATG TCG ACA CCC AAT T)3', were incubated with MMLV reverse transcriptase in the presence of each dNTP, newly generated a spot for 20mer[5'($^{32}$P-GCT ATG TCG ACA CCC AAT TC)3', in the case of the presence of dCTP in Fig. 3(lane 8)] was predominantly observed. In contrast, when the template ORNs-2(X=8-oxoG) or ORNs-3(X=8-oxoG-Me) was used, the formation of 8-oxoG:T (lane 12) and 8-oxoG-Me:dC(lane 10) or 8-oxoG-Me:T(lane 13) were observed. The ratio of incorporation of dNTP at the site opposite to X were also calculated only to the 20-mers by an image analyzer[25]. The ratio of dATP:dGTP:dCTP:TTP were 2.3:13:49:1 for G, 3.5:0.5:0.3:38 for 8-oxoG and 1.5:0.3:43:18 for 8-oxoG-Me, respectively. Although, the 30-mers of full length cDNA were also observed in the all templates in the reaction containing mixed dNTPs(lane 14, 15 or 16) and the expected formation of 8-oxoG:dA was not clearly observed in this assay, the altered glycosidic linkage of the 8-oxoG of 8-oxoG-Me might be generated abnormal the Watson-Crick base pairing. Incorporation preference of ribonucleotide opposite 8-oxoG during cDNA synthesis by reverse transcriptase was found not to parallel incorporation preference of deoxynucleotide opposite 8-oxodG during DNA synthesis by DNA polymerase reported by S. Shibutani et al[6]. They found that there was the preference for inserting dA to dC opposite 8-oxodG and the ratios of dA to dC vary greatly according to DNA polymerases.

In conclusion, the readily prepared phosphoramidite units(**6** and **7**) have been successfully utilized for the solid support synthesis of oligoribonucleotides containing 8-oxo-7,8-dihydroguanosine and 8-oxo-7,8-dihydro-2'-O-methylguanosine residues at predetermined positions. The synthetic oligoribonucleotide-30mers have been used as template for cDNA synthesis in order to study incorporation properties for base modified guanosine. MMLV reverse transcriptase tested showed preference for inserting T to C opposite 8-oxoG and showed the preference for inserting C opposite 8-oxoG-Me as G. The modification of C8 of guanosine with oxo group probably makes changes in the conformation of the gylcosidic linkage and be induced to basepair with T. On the other hand, the 8-oxoG-Me still retains anti conformation due to 2'-O-methyl group and shows similar incorporation property to the unmodified guanosine. Physicochemical and incorporation properties of the 8-oxoG or the 8-oxoG-Me with dNTP by another reverse transcriptase will be soon reported elsewhere.

**Acknowledgment.** This work was supported by a Grant from Korea Science and Engineering Foundation through Center for Biofunctional Molecules. S.K.K gratefully acknowledges the receipt of postdoctoral fellowship from the Korea Research Foundation.

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19. $^1$H NMR($CDCl_3$); δ8.02(s,1H, NH), 5.90(d, 1H, H-1'), 5.08(m, 1H, H-2'), 4.60(m, 1H, H-3'), 4.11(m, 1H., H-4'), 3.41-3.28(m, 2H, H-5'), 3.08(s, 3H, DMC), 3.15(s, 3H, DMC), 2.01(s, 3H, $CH_3$, NHAc).
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22. $^1$H NMR($CDCl_3$); δ8.12(s, 1H, NH), 7.44-7.16(m, 14H, arom.H's, MMT), 6.01(d, 1H, H-1'), 5.19(m, 1H, H-2'), 4.46(m, 1H, H-3'), 4.09(m, 1H., H-4'), 3.77(s, 3H, $OCH_3$, MMT), 3.47(s, 3H, 2'-O-$CH_3$), 3.45-3.37(m, 2H, H-5'), 3.08(s, 3H, DMC), 3.15(s, 3H, DMC), 2.01(s, 3H, $CH_3$, NHAc).
MS =m/z: 770($MH^+$, calcd for $C_{39}H_{43}N_7O_{10}$ 769.8), UV(MeOH): λmax 220.8, 259.8, 282.2nm, mp=116.5-117.5°C, $R_f$=0.54(MeOH:$CH_2Cl_2$=1:9)
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25. The ratio(%) of incorporation of dNTPs into the 3'-end of the primer can be calculated as : Ratio = (radioactivity of newly generated spots) / (radioactivity of the remaining primer + radioactivity of newly generate spots) x100.