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Oligodeoxynucleotides Containing O^2-alkylthymine: Synthesis and Characterization

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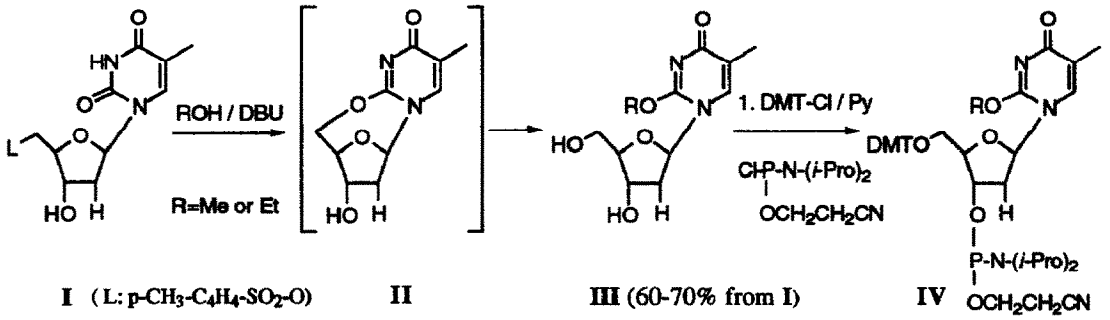
Abstract: *simple procedures for preparation of O^2-alkylthymidines and of their phosphoramidite monomers (IV) are described. These monomers have been successfully incorporated into DNA oligomers. The measurements of the melting temperature (Tm) of DNA duplexes show that O^2-methylthymine preferentially pairs with guanine rather than with adenine.*

Alkylation of DNA is believed to play an important role in carcinogenesis by alkylating agents. The greatest attention has been focussed on O^6-alkylguanine and O^4-alkylthymine and we have previously published methods for chemical synthesis of DNA containing 6-substituted guanine and 4-substituted thymine, including O^4-alkylthymine[1-3]. O^2-alkylthymine is also produced in DNA after exposure to alkylnitrosoureas[4]. In the case of N-ethyl-N-nitrosourea, O^2-ethylthymine is produced in nearly equimolar quantity to O^6-ethylguanine[5] and has a very long biological half life. But little is known of its biological properties and there are even conflicting views on its miscoding properties[6-8]. Here we wish to report an efficient method for chemical synthesis of O^2-alkylthymidine and oligodeoxynucleotide containing it, and also present some properties of this modified DNA.

Chemical synthesis

Alkylation of thymidine with diazoalkane has been used to prepare O^2-alkylthymidine[6] and O^2-alkyl-5'-dimethoxytrityl(DMT)-thymidine[8,9] for use in oligodeoxynucleotide synthesis. This alkylation is non-selective and produces O^4-, N^3- as well as O^2-alkylated derivatives. Alkylation of 5'-DMT-thymidine with diazomethane gave 6% O^2-, 87% N^3- and 7% O^4-methylated products; diazoethane gave 20% O^2- , 60% N^3- and 20% O^4-ethylated products; diazobutane gave 22% O^2-, 56% N^3- and 22% O^4-butylated products[9]. Thus this reaction is limited by the low yield of O^2-alkylated product, in particular, of O^2-methylthymine and the time-consuming separation procedure. However, Brown et al. have shown that 5'-deoxy-5'-iodo-2',3'-O-isopropylidene-uridine when treated with silver acetate cyclized to 2',3'-O-isopropylidene-O^2,5'-cyclouridine[10]. The latter could be converted into 2',3'-O-isopropylidene-O^2-methyluridine by treatment with triethylamine in methanol[10]. Watanabe et al[11] improved the method to prepare O^2,5'-cyclo-pyrimidine by using 5'-O-*p*-toluenesulphonyl pyrimidine nucleosides as starting materials. We have adapted these methods to prepare O^2-methylthymidine and O^2-ethylthymidine (scheme 1). The 5'-OH group was replaced with a good leaving group, such as the *p*-toluenesulphonyl[12] or iodo[13] group.The resulting 5'-derivative (I) in alcohol (methanol or ethanol) was converted into the corresponding O^2-alkylthymidine (III) in presence of DBU (1,8-diazabicyclo-



I (L: p-CH_3-C_4H_4-SO_2-O) II III (60-70% from I) IV

[5,4,0]undec-7-ene) in high yield. In this one-pot reaction, DBU abstracts the N^3-proton of thymidine resulting in formation of 4-enol and 2-enol ion forms. The latter form attacks the 5'-C atom to form O^2,5'-cyclothymidine (II). The appearance and disappearance of II were observed by TLC during the course of the reaction. Methoxide or ethoxide, formed from the corresponding solvent alcohol by DBU, then attacks C-2 of II giving the desired products (III, R=Me or Et). The products have been isolated, characterized and confirmed by ^1H NMR and mass spectrometry to be methylated or ethylated thymidine. The UV maximum absorption wavelength of eg. O^2-ethylthymidine (257 nm) agrees with reported data for O^2-ethylthymidine[8] and is clearly different from that of either N^3- (269 nm[14]) or O^4-ethylthymidine (281 nm[1]). O^2-alkylthymidines differ from their O^4-alkyl isomers in many respects. Big differences of mobilities were observed by TLC (eg. Rf: 0.35 and 0.55 for O^2- and O^4-methylthymidine in 15/85 CH_3OH/CH_3Cl) and on HPLC (eg. Rt: 11.5 min and 16.8 min for O^2- and O^4-methylthymidine, see the legend of fig. 1 for the conditions).

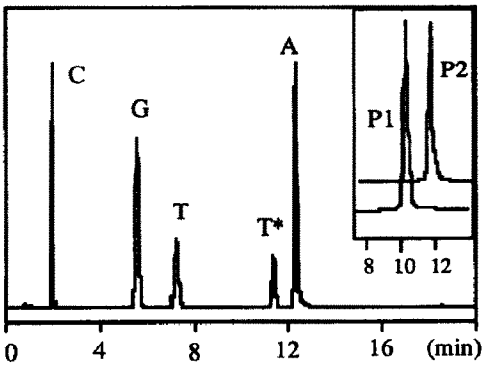


Fig.1 The reversed phase HPLC profile of the nucleosides from enzymatic digestion of a synthetic 12 mer AGC GAA T*TC GCT (T*=O^2-methylthymidine) conditions: Waters Nova-Pak C18 cartridge, 3 mL/min. The column was eluted for 8 min with 1.25% acetonitrile in 50 mM KH_2PO_4 (pH 4.5), then acetonitrile was increased to 12.5% over 14 min. The figure inset is the ion exchange chromatographic profiles of the modified 12 mer (P1, Rt=10 min) and the control oligomer containing thymine (P2, Rt=12 min). Conditions: Pharmacia mono Q HR 5/5 column; 0.8 mL/min. The column was eluted with A (0.4 M NaCl, pH 12) for first 2 min, then B (1.2 M NaCl, pH 12) was increased to 20% over 3 min, then to 40% for the following 20 min.

The resulting O^2-alkylthymidines (**III**, R=Me and Et) were converted into the 5'-DMT derivatives, then into the corresponding phosphoramidites by the standard procedures. These monomers were incorporated into DNA as described before[1]. The coupling yield measured by the amount of released DMT cation was satisfactory. After synthesis, DBU dissolved in the corresponding alcohol[2] was employed at room temperature to cleave the oligomer from the support and remove all protecting groups (phenoxyacetyl on G and A, isobutyryl on C and cyanoethyl on phosphate). The synthetic oligomer containing O^2-methylthymine is well separable from the control oligomer containing thymine in ion exchange liquid chromatography (Fig.1 inset). The earlier elution of O^2-methylthymine oligomer is predictable[15] and confirms that the modified thymine lacks an imino proton on the N^3-position. The right base composition was confirmed by HPLC analysis of nucleosides from enzymatic digestion of the modified DNA (Fig. 1).

The above synthetic strategy, coupled with an effective separation[15], could be a general method for production of highly pure DNA containing O^2-alkylthymine base residues.

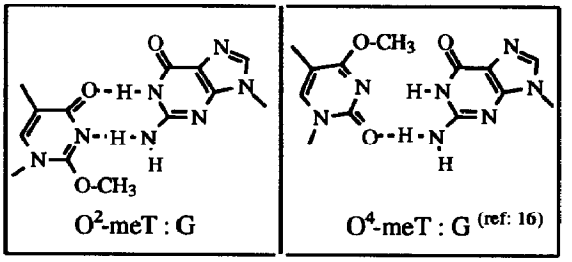
Base-pairing properties of DNA duplexes containing O^2- and O^4-methylthymine

In order to evaluate effects of alkylation at the O^2-position of the thymine on the DNA structure, the melting temperature (Tm) was measured of oligomers containing O^2-methylthymine base-paired with either adenine or guanine in the complementary strand (Table 1). The Tm values show that O^2-methylthymine can form a much better base-pair with guanine than with adenine, with the Tms of DNA cantaining these pairs differing by 13.7°C. O^2-methylthymine is also better in its base-pairing with guanine than O^4-methylthymine. These observations could be explained by the following base-pairing models (Fig.2). The O^2-meT:A pair possibly retains Watson-Crick alignment, like O^4-me-T:G pair[16], but the lack of a proton on N^3 of the O^2-meT allows only one H-bond to be formed. Furthermore it is possible that the presence of the O^2-methyl group prevents close approach of the two bases. Both factors may contribute to the low Tm value. By contrast, DNA containing O^2-me-T:G has a high Tm value and this may reflect the formation of a "wobble" pair since the formation of a pair with Watson-Crick alignment may be impossible[6]. Thus neither the O^2-meT:G pair nor O^2-meT:A pair is ideal for DNA polymerase and this may be the reason why the presence of O^2-ethylT in the template DNA blocks DNA synthesis[8].

Table 1. The melting temperature (Tm, °C) of DNA duplexes containing O^2-methylthymine or O^4-methylthymine [17]

5'-CAG GAA TXC GC 3'-GTC CTT AYG CG			
X : Y	Tm	X : Y	Tm
T : A	58.7	T : G	51.1
O^2-meT : A	39.1	O^2-meT : G	52.8
O^4-meT : A	39.3	O^4-meT : G	46.5

Fig. 2. Postulated models for base-pairing of O^2-methylthymine and O^4-methylthymine with guanine



EXPERIMENTAL

Chemicals and instruments: All chemicals were from either Aldrich or Sigma and used directly without further purification unless stated otherwise. Syntheses of oligomers were carried out by ABI 391 DNA synthesizer (Applied Biosystems), with PAC amidite monomers (Pharmacia). General methods such as purification with Nensorb Prep cartridges (Du Pont) or ion exchange liquid chromatography on a Mono Q HR 5/5 column (Pharmacia), nucleoside composition analysis by reversed phase HPLC and melting curve measurement by CARY 3 (Varian) were carried out as described before[1,2].

Synthesis of O^2-alkylthymidines (III) : Compound I (10 mmoles), prepared with isolated yield of 70% by reaction of thymidine with *p*-toluenesulphonyl chloride according to the published procedure[12], was suspended in 100 ml of alcohol (methanol or ethanol previously dried overnight with molecular sieve 4A). DBU (22 mmoles) was added. The solution was boiled with reflux for 3 hr or 10 hr respectively for methanol or ethanol. The reaction was monitored by TLC on silica plates ($CH_3OH/CHCl_3$, 15/85, v/v). After all starting material (Rf: 0.8) and the intermediate, compound II (Rf: 0.23) had been converted into compound (III) (Rf: 0.38 for methylation and Rf: 0.55 for ethylation), the reaction solution was cooled to room temperature and neutralized with Dowex-50 (pyridinium form). The filtrate was collected and the resin washed with 2 x 30 ml of the corresponding alcohol. The combined filtrates were concentrated into a small volume and co-evaporated

twice with toluene. The residue was either dissolved in alcohol/acetone (10/90, v/v) and left in an open flask in a sealed tank containing n-pentane (diffusion crystallization) to give a crystalline product (O^2-methylthymidine) or purified with silica gel column (for O^2-ethylthymidine) followed by diffusion crystallization. The overall isolated yield was 60-70% from I. O^2-methylthymidine had mp 159-160°C; [anal: calcd for $C_{11}H_{16}N_2O_5$ C: 51.56, H: 6.25, N:10.93, found C: 51.50, H: 6.16, N:10.64]. ^1H NMR data: 1.78 (3H, s, 5-CH_3), 2.14 (2H, m, 2'-H and 2"-H), 3.56 (2H, m, 5'-H), 3.77 (1H, m, 4'-H), 3.86 (3H, s, O^2-CH_3) 4.23 (1H, m, 3'-H), 5.06 (1H, t, 5-OH, exchangeable), 5.28 (1H, d, 3'-OH, exchangeable), 6.08 (1H, t, 1'-H) and 7.81 (1H, s, 6-H). UV λ_{max} 256.6 nm (ε= 11.07 x 10^3), λ_{min} = 236.5 and 215.7 nm. FAB-MS 257 (M + H+, 31.3%) 141(methylated base + 2H+, 100%). O^2-ethylthymidine had mp 153-155°C. [anal: calcd for $C_{12}H_{18}N_2O_5$, C: 53.33, H: 6.67, N: 10.37, found: C: 53.21, H: 6.49, N: 10.29], ^1H NMR data: 1.29 (3H, t, $\underline{CH_3}$ of O^2-$CH_2\underline{CH_3}$),1.78 (3H, s, 5-CH_3), 2.15 (2H, m, 2'-H and 2"-H), 3.60 (2H, m, 5'-H), 3.78 (1H, m, 4'-H), 4.22 (1H, m, 3'-H), 4.32 (2H, q, $\underline{CH_2}$ of O^2-$\underline{CH_2}CH_3$), 5.11 (1H, t, 5-OH, exchangeable), 5.34 (1H, d, 3'-OH, exchangeable), 6.08 (1H, t, 1'-H) and 7.80 (1H, s, 6-H). λ_{max}=257.0 nm (ε= 10.4 x10^3), λ_{min}=236.3 and 216.5 nm. FAB-MS 271(M + H+, 100%).

Synthesis of O^2-alkylthymidine phosphoramidite monomers (IV): O^2-methyl- and O^2-ethyl-thymidines were converted into 5'-DMT derivatives, then into phosphoramidites in the standard procedure as used for O^4-alkylthymine derivatives[1]; These products have been characterized. ^1H NMR (in $DMSO_6$) for O^2-methyl-5'-DMT-thymidine: 1.47 (3H, s, 5-CH_3), 2.29 (2H, m, 2'-H and 2"-H), 3.24 (2H, m, 5'-H), 3.72 (6H, s, 4' and 4"-OCH_3 of DMT), 3.86 (3H, s, 2-OCH_3), 3.91 (1H, m, 4'-H), 4.32 (1H, m, 3'-H), 5.39 (1H, d, 3'-OH, exchangeable), 6.12 (1H, t, 1'-H), 6.86-7.35 (13H, m, aromatic-H of DMT) and 7.62 (1H, s, 6-H). ^{31}P NMR (in $CDCl_3$): 148.04 and 148.36 for the ethylated nucleotide.

Synthesis, deprotection and purification of oligodeoxynucleotides containing O^2-alkylthymine: General procedures for the automated synthesis, deprotection and purification of modified oligomers were employed as before[1]. Briefly, after synthesis the CPG-support bearing the fully protected oligomer containing O^2-methyl- or O^2-ethyl-thymine was put into an Eppendorf tube and treated with 10% DBU in corresponding alcohol (methanol or ethanol) for 2 days at 25°C, then the solution was neutralized with acetic acid and immediately passed through Dowex-50 (Na^+ form) column. The collected UV-absorptive fractions was purified by Nensorb Prep cartridge and further with ion exchange chromatography.

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