A simple method for the solid phase synthesis of oligodeoxynucleotides containing O^4-alkylthymine

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ABSTRACT

A route to prepare the cyanoethyl-phosphoramidite monomer of O^4-alkylthymine and a method for the routine solid-phase synthesis of oligodeoxynucleotides containing O^4-alkylthymine are described. This method has been used to make DNA sequences up to 48 bases in length. The amino function of the adenine and guanine in the sequence were protected with the phenoxyacetyl group, and that of cytosine with the isobutyryl group. The phosphodiesters were protected with the cyanoethyl group. This allowed complete deprotection of the oligomer with alkoxide ions (methanol/1,8- diazabicyclo[5.4.0]undec-7-ene (DBU) for the oligomers containing O^4-methylthymine, or ethanol/DBU for those containing O^4-ethylthymine) thus avoiding the use of ammonia which is known to attack the O^4-alkylthymine to form 5-methylcytosine. There was no detectable loss of the alkyl group to form thymine.

INTRODUCTION

O^4-alkylthymine is produced by the alkylation of DNA by the N-nitroso compounds and is now believed to play a significant role in their carcinogenic and mutagenic properties (1,2). We have previously prepared oligodeoxynucleotides containing O^4-methylthymine by solution phase phosphotriester methods(3), but as solution phase methods are impractical for the routine preparation of small amounts of oligomers for molecular biology we have developed a method for solid phase synthesis which will give essentially pure oligomers.

The starting material O^4-alkylthymidine was originally prepared by direct alkylation of thymidine by diazoalkanes (4). The yield of the methyl derivative is too poor to be practicable but the method can be used to prepare the higher homologues although also in poor yield (c 20%). Alkylation of thymidine with isopropyl bromide and silver oxide yields O^4-isopropylthymidine from which O^4-methylthymidine can be prepared by nucleophilic substitution with methoxide ions (5). This method is only suitable for the O^4-methyl derivative because the alkoxides of higher alcohols will not effectively displace the isopropyl group (6). However the 4-triazolo derivative of thymidine can be made in very high yield (7) and is readily substituted by alkoxide ions to give the desired O^4-alkylthymidine (3).

In a previous paper on the solid phase synthesis of oligomers containing O^4-alkylthymine (6) the phosphodiesters were protected with methyl groups and the commercially available N^2-isobutyryl-protected guanine, N^6-benzoyl-protected adenine, and N^4-benzoyl-protected cytosine monomers were used. Thiophenoxide ions had to be used to remove the phosphorus-protection, and a relatively long exposure to alkoxide had to be used to remove the base protection. As a result a significant amount of the O^4-alkylthymine residues in the oligomer were dealkylated leaving thymine. We have used cyanoethyl groups to protect the phosphodiester and N^2-phenoxyacetyl-protected guanine, N^6-phenoxyacetyl-protected adenine and N^4-isobutyryl-protected cytosine phosphoroamidites ('PAC amidites') which have recently become available from Pharmacia as precursors for these normal bases. These protecting groups are very easily removed and the whole oligomer can be cut from the support and deprotected by exposure to alkoxide ions at room temperature. Using these methods high yields of pure oligomers can be obtained routinely.

RESULTS AND DISCUSSION

The synthesis of O^4-alkylthymidine is shown in Scheme **1**. The 3'-OH and 5'-OH of thymidine were first protected with the t-butyldimethylsilyl group in excellent yield, and converted to the 4-triazolo derivative which can be directly substituted by alkoxide ions, [the appropriate alcohol in the presence of the strong base 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU)], to give good yields of O^4-alkylthymidine. The half life at room temperature was about 30 min for methanol or about 3 hrs for ethanol. A better leaving group, 3-nitro-1,2,4-triazole, has been used previously (3) but triazole was chosen because it is easily available and even though the substitution is slower than that of 3-nitro-1,2,4-triazole, it can be carried out under mild conditions at room temperature.

After cleavage of the silyl group from resulting product by fluoride ion the desired O^4-alkylthymidine **4** can be obtained pure by column chromatography on silica gel and crystallization. The purity was checked by TLC, HPLC (Fig1), NMR, and microanalysis. Crystallization of the product was necessary to

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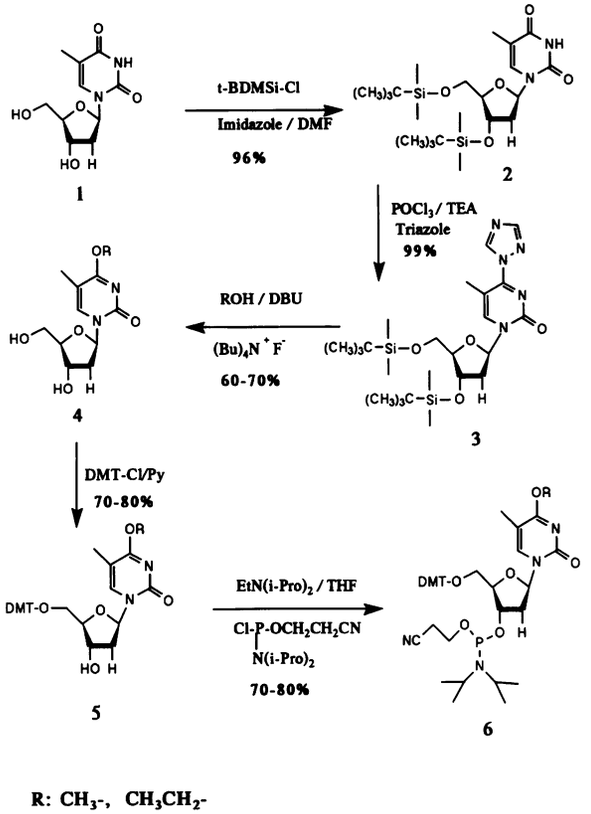
Scheme 1



R: CH_3-, CH_3CH_2-

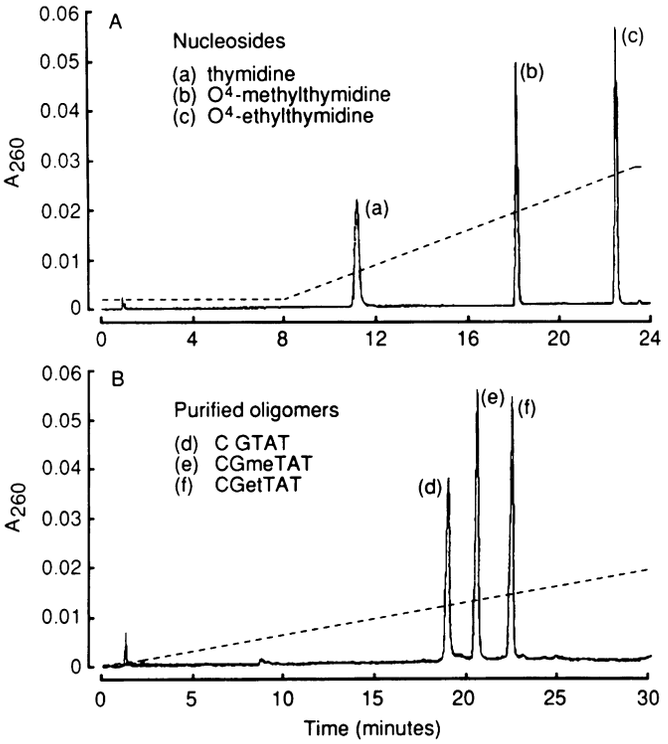


Fig. 1A. Reverse phase HPLC of thymidine, O^4-methylthymidine and O^4-ethylthymidine. Waters Nova Pak C18 cartridge; 3 ml/min; 25°C. The column was eluted for 8 min with 1.25% acetonitrile in 50 mM aqueous KH_2PO_4 (PH 4.5), then the concentration of acetonitrile was increased to 12.5% over 14 min. Fig.1B. Reverse phase HPLC of synthetic oligomers (purified 5 mers CGTAT, CG*me*TAT and CG*et*TAT). Gradient: 0–11% acetonitrile in 50 mM aqueous KH_2PO_4 (pH6.3); 3 ml/min; 45°C.

remove all minor impurities, especially thymidine. It is important to remove thymidine completely at this stage because it is difficult to separate thymidine from O^4-alkylthymidine later in the preparation of the alkylthymidine monomer.

The oligomers were made using a Cruachem DNA synthesizer without changing the routine programme. The O^4-alkylthymine monomer **6** was added manually as before(8) routinely giving more than 98% coupling yield. In order that the oligomer could be deprotected easily, 'PAC amidites' were used in the synthesis. These 'PAC' monomers can also be used with advantage to make oligomers containing O^6-alkylguanine and if the phenylacetyl group is used to protect the amino function of the O^6-alkylguanine the oligomer can be completely deprotected by overnight exposure to concentrated ammonia at room temperature without detectable formation of 2,6-diaminopurine (Fig 3).

Because O^4-alkylthymidine is susceptible to attack by nucleophiles and base (3,6) preliminary studies were carried out before the deprotection and purification. In agreement with previous work (3,6) the half life at room temperature in concentrated ammonia of O^4-methylthymidine and of O^4-methylthymine in the 5 mer (CG*me*TAT), was found to be approximately 10 hrs and 35 hrs respectively with 5-methylcytosine being the main product. This nucleophilic attack precludes use of concentrated ammonia for deprotection of oligomers containing O^4-methylthymine, and therefore alkoxide ions (the respective alcohol with DBU) were used for deprotection. Full deprotection must cleave the oligomer from the support and also remove the protecting groups from the bases and phosphodiester links. We used anion exchange chromatography to follow the cleavage of a synthetic dimer TpT from its support. The half life was less then 40 min in DBU: methanol (1:9 v/v). Pharmacia claim that the PAC groups protecting the bases are removed completely by DBU: ethanol (1:9 v/v) at room temperature for 24 hrs (9). We found that 36 hrs in DBU: methanol (1:9 v/v) was adequate even for the deprotection of 48 mers providing that a lipophilic counter ion (cetyl trimethylammonium) was present to keep the partially deprotected oligomer in solution (10). After deprotection the DBU must be removed because it is a very strong base and the OH^- ion produced when water is added to it will substitute for the alkyl group of O^4-alkylthymine forming thymine in the oligomer. To remove the DBU and the cetyl trimethylammonium ions the solution was neutralized with acetic acid then immediately passed through Dowex 50-Na^+ ion exchange resin. The yields from deprotection were very satisfactory (Table 1).

The resulting oligomer was most easily purified with Nensorb Prep columns or OPC cartridges to give reasonably pure oligomers (Fig 2a and Fig 3) suitable for most research. More pure oligomers can be obtained by chromatography (Fig 2b). The two chromatographic systems most commonly used for the purification of synthetic oligomers are anion exchange using 'mono Q' columns (Pharmacia) and reverse phase HPLC. Mono Q is not suitable for the purification of oligomers containing O^4-methylthymine because elution is with an increasing concentration of NaCl at pH 12 and in this condition 2% of O^4-methylthymidine was converted to thymidine in 1 hr. However mono Q is suitable for the purification of oligomers containing O^6-methylguanine because after 16hrs under the same

Table 1: The Yield and base composition of oligomers containing O4-methylthymine or O4-ethylthymine. Yield is the amount (A260 units) of oligomer recovered from the Nensorb Prep cartridge. Purity reports the proportion of the total absorbance recovered from the Nensorb cartridge which was found to be the desired oligomer when the material was chromatographed by HPLC. Base analysis was carried out on the this HPLC purified material.

Oligomer	Scale (μ moles)	Yield (A_{260})	Purity (%)	Base Composition C	G	A	T	meT	etT
AGCGAATTCGCT	0.2	9.8	88	3.00	3.36	2.80	3.24		
AGCGAAmeTTCGCT	1.0	38	87	3.00	3.29	2.90	1.93	1.09	
AGCGAAetTTCGCT	1.0	46	85	3.00	2.96	2.95	1.93		1.05
CGTAT	0.2	4.6	>90						
CGmeTAT	0.2	4.0	>90						
CGetTAT	0.2	4.5	>90						

conditions no hydrolysis products of O^6-methyldeoxyguanosine were found.

After purification of synthetic oligomers with reverse phase HPLC samples were enzymically digested to nucleosides and the amount of each nucleoside measured as before (8). The expected ratios of bases are obtained (Table1), which confirms that the above procedures of synthesis and purification are satisfactory and that there is no significant substitution of O^4-alkylthymidine by nucleophile.

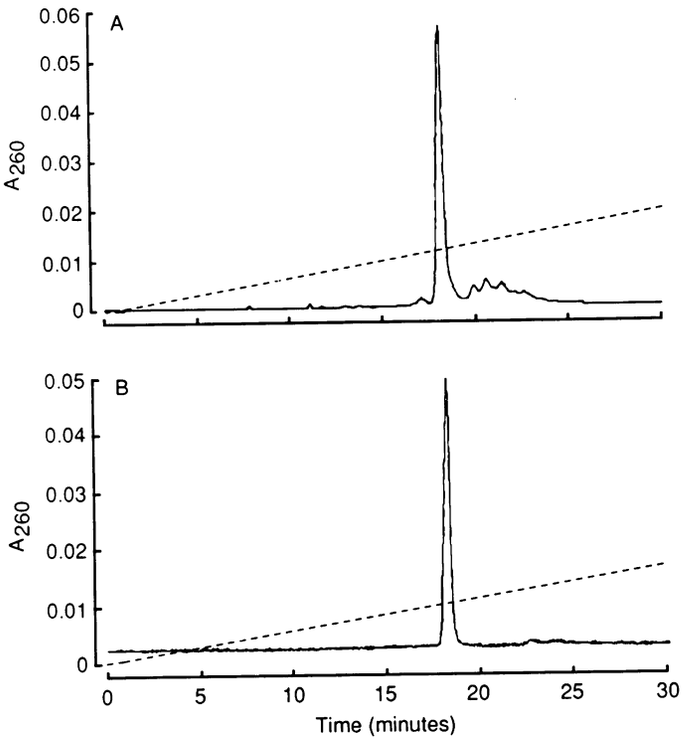


Fig. 2 Reverse phase HPLC of synthetic oligomer of AGCGAA*me*TTCGCT. Gradient: 0–11% acetonitrile in 50 mM aqueous KH_2PO_4 (pH6.3); 3 ml/min; 45°C. (A) crude synthetic oligomer after Nensorb Prep cartridge purification. (B) After purification with reverse phase HPLC.

EXPERIMENTAL

Chemicals, enzymes and general methods:

The cpg-linked monomers and the chemicals used on the synthesizer were obtained from Cruachem (Glasgow, Scotland) and the monomers of 2-cyanoethylphosphoroamidites protected with phenoxyacetyl on the amino functions of adenine and guanine, and with isobutyryl on the amino function of cytosine ('PAC amidites') were from Pharmacia. All other chemicals were from either Aldrich or BDH. The water content of anhydrous solvents was checked by Karl Fischer titration. Reverse phase HPLC and short column chromatography was carried out as before (8). All Rfs refer to TLC on Merck Kieselgel 60 F_{254} aluminium backed TLC sheets developed with $CH_3OH/CHCl_3$, 5:95 (Solvent A); $CH_3OH/CHCl_3$, 10:90 (Solvent B); n-pentane/diethyl ether, 50:50 (Solvent C); diethyl ether (Solvent D); CH_2Cl_2/EtOAc/diisopropylethylamine, 85:15:1 (Solvent E). ^1H NMR spectra were obtained with a Varian VXR-400 with tetramethylsilane as internal standard and DMSO-d_6 as solvent and D_2O exchanges were carried out to assign exchangeable protons.

Chemical synthesis of O^4-alkylthymine monomers

3',5'-O-Di-t-butyldimethylsilylthymidine (Compound **2**): Thymidine (12.1gm, 50mmol) and imidazole (10.2 gm, 150mmol) were dissolved in 100 ml of dimethylformamide and t-butyldimethyl-silyl chloride (22.5 gm,150 mmol) added to the stirred solution at room temperature. After 3 hrs, all starting material had been changed to a compound with higher R_f (0.8, Solvent A; 0.54, Solvent C). The solution was concentrated into a small volume, then partitioned between 200 ml of diethyl ether and 200 ml of saturated aqueous NaCl. The organic layer was

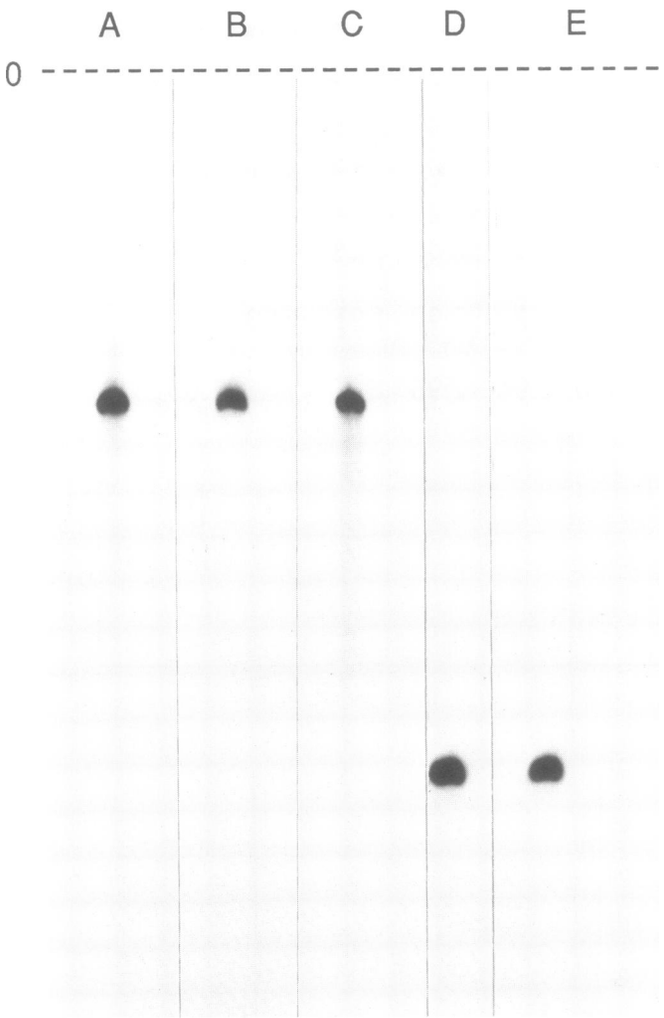


Fig. 3 Electrophoretic analysis of longer synthetic oligomers containing O^4-methylthymidine and O^6-methylguanine (after Nensorb or OPC cartridge purification). The electrophoresis was carried out on a 20% (v/v) acrylamide: N,N'-methylenebisacrylamide gel in tris:EDTA:borate (0.093M: 0.089M: 2.5mM) buffer pH 8.3 containing 8M urea under a potential of 50 volts/cm.

washed twice with 200 ml of saturated aqueous NaCl and dried over anhydrous Na_2SO_4. The filtrate was evaporated to give a white solid (22.5 gm , 96% yield). ^{1}H NMR data: 0.07(12H, 2 s, 3' and 5'-Si(CH_3)$_2$-R), 0.87 (18H, 2 s, 3' and 5'-Si(R)$_2$-(CH_3)$_3$),1.76 (3H, s, 5-CH_3), 2.05 (1H, m, 2'-H), 2.18 (1H, m, 2"-H), 3.72 (2H, m, 5'-H), 3.76 (1H, m, 4'-H), 4.34 (1H, m, 3'-H), 6.15 (1H, t, 1'-H) and 7.42 (1H, s, 6-H).

3',5'-O-Di-t-butyldimethylsilyl-4-(1,2,4-triazolo)thymidine (Compound **3**): 1,2,4-Triazole (25.6 gm, 370 mmol) was suspended in 300 ml of anhydrous CH_3CN at 0°C. 8 ml of $POCl_3$, then 60 ml of triethylamine was added slowly. After an hour, compound **2** (11.75 gm, 25 mmol) in 100 ml of CH_3CN was added over 30 min. Then the solution was stirred for16 hrs at room temperature and all the starting material converted into a new compound with lower R_f (0.05 Solvent C, 0.43 Solvent D). The reaction mixture was filtered, diluted with ethyl acetate (600 ml) and washed with saturated aqueous $NaHCO_3$ (500 ml), then twice with 500 ml of saturated aqueous NaCl. The organic layer was dried over anhydrous Na_2SO_4 and the solvent evaporated. The residue was dried by repeated evaporation of a toluene solution to give a slightly-yellow solid (12.6 gm, 99%). ^{1}H NMR data: 0.09 (12H, 2 s, 3' and 5'-Si(CH_3)$_2$-R), 0.88 (18H, 2 s, 3' and 5'-Si(R)$_2$-(CH_3)$_3$), 2.23 (1H, m, 2'-H), 2.40 (3H, s, 5-CH_3), 2.46 (1H, m, 2"-H), 3.83 (2H, m, 5'-H), 3.98 (1H, m, 4'-H), 4.38 (1H, m, 3'-H), 6.11 (1H, t, 1'-H), 8.22 (1H, s, 6-H) and 8.37−9.32 (2H, 2 s, triazolyl-H).

O^4-Alkylthymidine (Compound **4**): Compound **3** (5.11 gm, 10mmol) was dissolved in 40 ml of CH_3CN at 0°C and 3 ml of alcohol (MeOH or EtOH) and 2.5 ml of DBU was added to the stirred solution. After 30 min, it was allowed to warm to room temperature and stirring continued for 3 hrs (for MeOH) or overnight (for EtOH). The solution was neutralized by addition of 25 ml of 0.5 M aqueous KH_2PO_4 (pH 6.5) and the product extracted into 100 ml of $CHCl_3$. This was washed with 3×100 ml of saturated aqueous NaCl then dried over anhydrous Na_2SO_4. The filtrate was concentrated and tetrabutylammonium fluoride in tetrahydrofuran (0.15 M, 30 ml) added. After 1 hr all silyl groups had been removed to give a compound with lower Rf (0.3, Solvent B) and a small amount (less than 10%) of thymidine (Rf 0.15, Solvent B). The solution was concentrated and co-evaporated with toluene to give an oil, which was purified by silica gel column . The desired product was eluted with 2% CH_3OH in $CHCl_3$. The fractions containing pure product were pooled together, concentrated and dried by repeated evaporation of a toluene solution to give a dry solid. This product was redissolved in the minimum quantity of 5% CH_3OH in EtOAc for crystallisation (60−70% overall yield from compound **1**). O^4-methylthymidne had mp 172−174°C (170−172°C(3)) [anal: calcd for $C_{11}H_{16}N_2O_5$ C:51.6, H: 6.3, N:10.9, found C 51.54 , H: 6.41 , N: 10.88]. ^{1}H NMR data: 1.87 (3H, s, 5-CH_3), 1.99 (1H, m, 2'-H), 2.16 (1H, m, 2"-H), 3.58−3.62 (2H, m, 5'-H), 3.79 (1H, m, 4'-H), 3.83 (3H, s, 4-OCH_3) 4.21 (1H, m, 3'-H), 5.08 (1H,t, 5-OH, exchangeable), 5.24 (1H, d, 3'-OH, exchangeable), 6.13 (1H, t, 1'-H) and 8.01 (1H, s, 6-H). UV λ_{max} 281 nm (ϵ = 6.3×10^3), λ_{min} = 242 nm. O^4-Ethylthymidine had mp 178−180°C. [anal: calcd for $C_{12}H_{18}N_2O_5$, C: 53.3, H: 6.67, N: 10.37, found: C: 53.07, H: 6.79, N: 10.42], ^{1}H NMR data: 1.30 (3H, t, CH_3 of O^4-ethyl),1.89 (3H, s, 5-CH_3), 2.03 (1H, m, 2'-H), 2.19 (1H, m, 2"-H), 3.57−3.63 (2H, m, 5'-H), 3.82 (1H, m, 4'-H), 4.23 (1H, m, 3'-H), 4.33 (2H, q, CH_2 of O^4-ethyl), 5.03 (1H,t, 5-OH, exchangeable), 5.20 (1H, d, 3'-OH, exchangeable), 6.14 (1H, t, 1'-H) and 8.00 (1H, s, 6-H). λ_{max}=281 nm (ϵ= 6.2×10^3), λ_{min}=242 nm.

5'-O-(4,4'-dimethoxytriphenylmethyl)-O^4-alkylthymidine (Compound **5**): The O^4-alkylthymidine (1 mmol) was treated with dimethoxyltrityl (DMT) chloride (1.1 equivalents) in 10 ml of anhydrous pyridine overnight. The solution was evaporated to a small volume, then taken into 50 ml of $CHCl_3$, washed with 3×50 ml of saturated aqueous $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4, concentrated and co-evaporated

with toluene to give a slightly yellow foam, then purified by silica gel column chromatography with $CHCl_3$ as eluent to give the corresponding 5'-O-DMT derivative (70–80% yield in different experiments). 5'-DMT-O^4-methylthymidine: ^1H NMR data 1.54 (3H, s, 5-CH_3), 2.17 (1H, m, 2'-H), 2.27 (1H, m, 2"-H), 3.40 (2H, m, 5'-H), 3.94 (1H, m, 4'-H), 3.71 (6H, s, 4' and 4"-OCH_3 of DMT), 3.85 (3H, s, 4-OCH_3), 4.32 (1H, m, 3'-H), 5.37 (1H, d, 3'-OH, exchangeable), 6.18 (1H, t, 1'-H), 6.86–7.35 (13H, m, aromatic-H of DMT) and 7.82 (1H, s, 6-H). 5'-DMT-O^4-ethylthymidine: ^1H NMR data. 1.35 (3H, t, CH_3 of O^4-ethyl),1.50 (3H, s, 5-CH_3), 2.20 (1H, m, 2'-H), 2.42 (1H, m, 2"-H), 3.32 (2H, m, 5'-H), 3.78 (6H, s, 4' and 4"-OCH_3 of DMT), 4.02 (1H, m, 4'-H), 4.40 (1H, m, 3'-H), 4.38 (2H, q, CH_2 of O^4-ethyl), 5.39 (1H, d, 3'-OH, exchangeable), 6.28 (1H, t, 1'-H), 6.83–7.42 (13H, m, aromatic-H of DMT) and 7.83 (1H, s, 6-H).

5'-O-(4,4'-dimethoxytriphenylmethyl)-O^4-alkylthymidine-3'-O-(2-cyanoethyl)-di-isopropylamido-phosphite (compound **6**): To a stirred solution of compound **5** (1.0 mmol) in 5 ml of tetrahydrofuran at room temperature was added diisopropyl-ethylamine (1.0 ml, 5 mmol) and 2-cyanoethyl-N,N-diiso-propyl-chlorophosphoramidite (400 µl, 1.5 mmol). After 30 min, the starting material had been converted to two main compounds with higher Rfs (0.4 and 0.6, Solvent E). The solution was deluted with 25 ml ethyl acetate and washed with 3×25 ml saturated aqueous NaCl, then dried over anhydrous Na_2SO_4. The filtrate was evaporated with 3 × 10 ml of toluene to give an oil which was dissolved in a small amount of toluene and added dropwise into 200 ml of stirred n-pentane. The resulting precipitate was dissolved in benzene and lyophilized to give a powder (70–80% yield in different experiments). ^{31}P NMR data: 149.09 and 148.44 for methylated nucleotide;149.06 and 149.43 for ethylated nucleotide.

Oligonucleotide synthesis, deprotection and purification

Oligonucleotides were synthesized on a Cruachem PS200 automatic DNA synthesizer (Cruachem Ltd., Glasgow, Scotland) using PAC amidites of the normal bases (see above). The portion of the oligonucleotide 3' to the O^4-alkylthymine was synthesized on the machine, then O^4-alkylthymine monomer added manually. In both 1.0 µm and 0.2 µm scale 10 mg of compound **6** in a 2 ml conical glass vial with septum top (Wheaton reacti-vial) was dissolved in 0.1 ml of anhydrous acetonitrile and 0.1 ml of 0.5 M tetrazole in anhydrous CH_3CN added. The bottom end of the cartridge was disconnected from the machine and the mixture of monomer and tetrazole injected from a gas tight syringe. The syringe was used to draw the solution in and out of the cartridge several times over a period of 3 min., then the cartridge was immediately reconnected to the synthesizer to complete the synthesis. The yield of each coupling reaction was assessed by measuring the amount of 5'-protecting group (DMT) released by dichloroacetic acid (8).

After synthesis the CPG-support was removed from the cartridge, put in an Eppendorf tube and 1 ml of DBU in anhydrous alcohol (1:9 v/v) (methanol for oligomers containing O^4-methylthymine, ethanol for those containing O^4-ethylthymine) and 10 mg of cetyltrimethylammonium bromide were added and left for 24–36 hrs at room temperature. Then the solution was neutralized by adding 90 µl of 50% aqueous acetic acid (1.5 equivalent to DBU) and immediately passed through a Dowex 50×8, Na^+ form, 400 mesh ion exchange column (10 ml wet volume) and the oligomer eluted with water (10 ml) and collected in1 ml fractions. The oligomers were usually found in fractions 4 to 6 by measuring UV absorption at 260 nm. These dimethoxytrityl(DMT)-containing oligonucleotides were separated from failure sequences and the DMT group finally removed from the oligonucleotides using a Nensorb Prep Cartridge (NEN Research Products, Du Pont Co., Boston, MA 02118, USA) or OPC Cartridge (Applied Biosystems, 850 Lincoln Center Dr., Foster City, California 94404 USA) according to the maker's instructions.

As an example, 40% of the product from the synthesis on 0.2 µmole scale of a 21 mer containing O^4-methylthymine gave 2.56 A_{260} units after partial purification with a Nensorb Prep Cartridge, and a one-third sample gave 2.45 A_{260} units after partial purification by OPC Cartridge. The oligomer purified on Dowex ion exchange and Nensorb Cartridge or OPC Cartridge was reasonably pure (Fig 3) and could be used directly for molecular biology. Oligonucleotides up to 12 bases in length could also be purified by C-18 reverse phase HPLC (Fig 2).

The purity of the oligomers was assessed by nucleoside analysis and by chromatography. The oligomers were hydrolysed enzymically to nucleosides separated by HPLC and the amount of each nucleoside measured by integration of the absorbance of each peak(8). The comparison of the peak areas on the chromatogram of the sample with that of a standard mixture of nucleosides gives the base ratio in the oligomer (Table 1).

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