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Synthesis and characterization of oligodeoxynucleotides containing the mutagenic base analogue 4-O-ethylthymine

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ABSTRACT

A method for the preparation of oligonucleotides containing the mutagenic base 4-O-ethylthymine is described for the first time. Use of *p*-nitrophenylethyl type base protecting groups together with phosphite-triester solid-phase methodology makes possible the rapid and efficient preparation of oligonucleotides bearing 4-O-ethylthymine, while standard base protecting groups are not compatible with the presence of this base. Possible applications of this methodology are discussed.

INTRODUCTION

The carcinogenicity of N-nitroso alkylating agents such as nitrosoureas and nitrosoamines is believed to be mediated by some products of alkylation of the nucleobases of DNA in particular 6-O-alkylguanine and 4-O-alkylthymine (1−3). Although major attention in chemical carcinogenesis was focused on 6-O-alkylguanines, the mutagenic potential of the 4-O-alkylthymines has been recently recognized (4) and seems to be related to the very inefficient repair of 4-O-alkylthymines residues in eukaryotic cells (5).

In order to study the biological and structural role of 4-O-alkylthymines in DNA, it is important to develop an efficient and rapid method to incorporate these analogues in synthetic DNA. It is well known that these compounds are difficult to be incorporated into synthetic DNA because they are both acid and base labile, and very sensitive to nucleophiles like ammonia or amines or thiols used for the deprotection of synthetic DNA (6). These problems have been recently solved for the preparation of oligonucleotides bearing some O-alkylthymines (7,8), but the proposed methods are not useful for the rest of the O-alkylthymines.

In this paper, we would like to describe for the first time a method for the preparation of oligonucleotides containing 4-O-alkylthymines that could be easily adapted for the synthesis of oligonucleotides containing any O-alkylthymine and, in general, to other nucleophile-sensitive base analogues.

RESULTS AND DISCUSSION

Synthesis of the phosphoramidite derivatives

The preparation of 4-O-ethylthymidine containing oligonucleotides requires the synthesis of the appropiate derivative. We decided to use solid-phase phosphite-triester methodology and for that reason we have prepared 5′-O-(dimethoxytrityl)-4-O-ethylthymidine N,N-diisopropyl-O-cyanoethyl phosphoramidite (*1*) (Fig. 1). Starting with DMT-T, the preparation of the 4-O-ethylthymidine derivative was done by displacement of the triazolo group of 5′-O-(dimethoxytrityl)-4-(1,2,4-triazolyl)thymidine with sodium ethoxide. Previously, the 3′-OH of the nucleoside was protected with the trimethylsilyl group using trimethylsilyl-1,2,4-triazole. As the silylating reagent produces 1,2,4-triazole as by-product the modification of the thymidine can be done immediately without isolating the protected intermediate. The 3′ silyl protecting group is also easily removed with sodium ethoxide solution used to displace the triazole group. The phosphitylation of DMT-T^{Et} was done using 2-cyanoethyl-N,N,N′,N′-tetraisopropylphosphordiamidite (9) giving compound *1*.

We have also prepared 5′-O-(dimethoxytrityl-4-(1,2,4-triazolyl)thymidine N,N-diisopropyl-O-cyanoethyl phosphoramidite (2) using previously described protocols (10). This amidite can be incorporated into synthetic DNA using standard protocols and, at the end of the synthesis, 4-O-ethylthymidine can be generated by displacement of the triazole group with ethoxide ions.

Preliminary studies using standard base protecting groups

4-O-Alkylthymidines can not be introduced in synthetic oligodeoxynucleotides using standard protocols because they react with ammonia or amines used for the deprotection giving 5-methylcytidine derivatives (7). Recently, the preparation of oligonucleotides containing some O-alkylthymines has been solved by using methoxide ions (DBU solutions in methanol) to remove the base protecting groups (7,8).

Following this rational, we tried first to prepare oligonucleotides containing 4-O-ethylthymidine using the standard cyanoethylphosphoramidites and DBU solution in ethanol for the deprotection of the amide protecting groups of the bases. The

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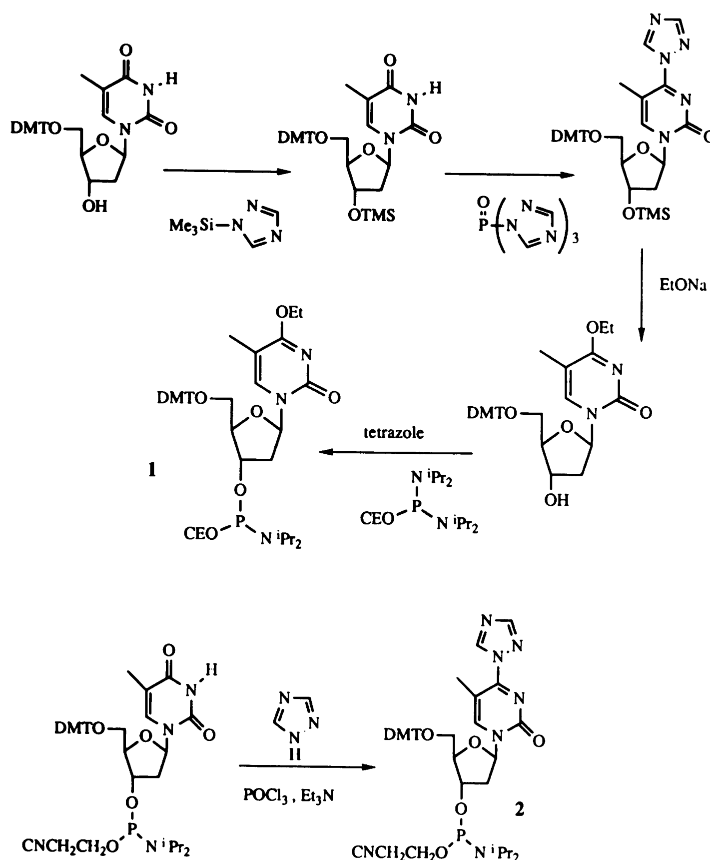

Figure 1: Preparation of phosphoramidite derivatives used for the synthesis of 4-O-ethylthymidine containing oligodeoxynucleotides.

oligonucleotide-resin AAGT^{Et}CTG-Resin was treated with a 0.1 M solution of DBU in ethanol at room temperature for a period of time from 1 to 3 days. When we analyzed the reaction crudes by HPLC we found in all cases a very complex mixture arising mainly from incomplete deprotection of the amino groups of the bases as seen by enzymatic digestion of the different peaks. We have checked that the rate of deprotection of protected nucleosides (dC^{bz}, dG^{ibu}, dA^{bz}) by DBU is much slower in ethanol than in methanol. This fact is due to the lower nucleophilicity of ethoxide ions compared to methoxide ions and indicates that it is not possible to obtain 4-O-ethylthymine oligonucleotides by the deprotection of the bases with ethanolic solutions of DBU.

We also considered the possibility to convert 4-O-methyl- into 4-O-ethylthymine containing oligonucleotide. First we treated 4-O-methylthymidine with an ethanolic solution of DBU and with a solution of sodium ethoxide. We found total conversion of 4-O-methylthymidine to 4-O-ethylthymidine with a 2 hours treatment of 4-O-methylthymidine with a solution of sodium ethoxide. No reaction was observed using an ethanolic solution of DBU. Based in these results, we prepared the oligonucleotide 5′AAGT^{Me}CTG 3′ (where T^{Me} stands for 4-O-methylthymine) and the oligonucleotide was treated with a variety of ethoxide solutions (see table 1). The best results were observed when potassium ethoxide alone and sodium ethoxide with 18-crown-6 ether were used. In longer treatments we have observed a similar HPLC profile but a significant decrease of the total amount of oligonucleotide. That means that in the best conditions we can only expect a maximum 50% conversion to the desired oligonucleotide. That could be enough for some biological experiments but it is not sufficient to prepare oligonucleotides

Table 1: Studies of the transformation of 4-O-methylthymine to 4-O-ethylthymine inside of the oligonucleotide 5′-d(AAGT^{Me}CTG) 3′.

Reagent*	time	Composition (%)		
		- -T^{Me}- -	- -T^{Et}- -	- -T- -
0.1M EtONa	20 hr	59	27	14
0.1M EtOK	12 hr	40	51	9
0.1M EtONa / 18-Crown-6	12 hr	45	55	0
0.1M EtOK / 18-Crown-6	12 hr	91	9	0

* in Ethanol/pyridine (1:1)
- -T^{Me}- -, - -T^{Et}- -, and - -T- - represent oligonucleotides containing T^{Me}, T^{Et}, and T respectively

for structural studies. In conclusion we have found that the standard amide type protecting groups are not convenient for an efficient preparation of oligonucleotides containing 4-O-ethylthymine and for that reason a new scheme of protecting groups had to be developed.

Use of *p*-nitrophenylethyl type protecting groups

A protecting group suitable for the preparation of oligonucleotides containing 4-O-ethylthymidine must fulfil the condition that ethoxide ions are the only nucleophile present during the deprotection. We have selected the *p*-nitrophenylethyl (NPE) type groups developed by Pfleiderer et al. (11−14). This protecting group has been used as amino protecting group with the phosphate-triester approach (12), but, so far, the preparation and use in oligonucleotide synthesis of NPE-protected nucleoside phosphoramidites has not yet been developed (15). We have prepared 5′-O-(dimethoxytrityl)-NPE protected (A^{NPEOC}, C^{NPEOC}, G^{NPEOC,NPE}) nucleosides (Fig. 2) following previously

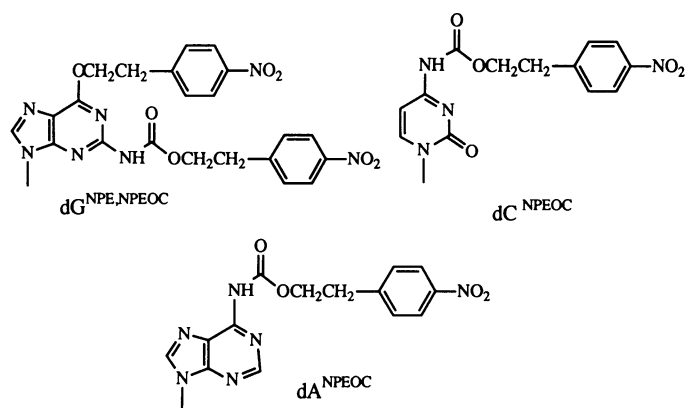

Figure 2: NPE-protected nucleosides.

described methods (11). The preparation of the N,N-diisopropyl-O-2-cyanoethyl phosphoramidites of these compounds was carried out following published procedures (9).

In order to find the optimal deprotection conditions to be used in our case we have treated NPE-protected nucleosides (dA^{NPEOC}, dC^{NPEOC} and $dG^{NPEOC,\ NPE}$) with different solutions of DBU in non-protic solvents (dioxane, pyridine) and a protic solvent (ethanol/dioxane (1:1)). As it can be seen in table 2, $dG^{NPEOC,\ NPE}$ is more easily deprotected than dA^{NPEOC} and dC^{NPEOC}. In aprotic solvents (pyridine and dioxane) the deprotection is faster than in ethanol/dioxane. Aproximately, between 2 and 4 hours are needed to deprotect NPE-nucleosides in a non-protic solution of DBU and at least 32 hrs using a DBU solution in ethanol/dioxane (1:1).

The assembly of the oligonucleotides indicated in table 3 were done either in a home-made manual synthesizer or in a commercial automatic synthesizer. Coupling yields with the NPE-protected amidites were similar to those obtained using commercial amidites. For the introduction of 4-O-ethylthymidine in the oligonucleotide A we have compared the use of the amidite of 4-O-ethylthymidine (compound *1*) and the amidite of 4-(1,2,4-triazolyl)thymidine (compound *2*). We have treated $TTC^{NPEOC}T^{Et}C^{NPEOC}TT$-resin with 0.5 M DBU solutions in pyridine and ethanol/tetrahydrofuran (1:1) and $TTC^{NPEOC}T^{tri}C^{NPEOC}TT$-resin with a 0.5 M DBU solution in ethanol/tetrahydrofuran (1:1). We found that the treatment with 0.5 M DBU in pyridine could not cleave the succinyl type oligonucleotide-resin bond. Even after 5 days only 30−40% of the expected amount of oligonucleotide was released from the resin. On the other hand, the HPLC chromatograms of the products obtained from the DBU treatments were very similar and, as it can be seen in figure 3, consisted of a major peak that had a correct composition after HPLC analysis of enzymatic digestions and a minor peak that did not have 4-O-ethylthymidine but in which 4-O-ethylthymine was dealkylated to thymine.

Afterwards, we studied the deprotection in an oligonucleotide that contained all the bases together with 4-O-ethylthymine (sequence B). The product obtained using 0.5 M DBU in ethanol/tetrahydrofuran was still not satisfactory because a large amounts of side products were observed. We found that the best results were obtained when a two-steps deprotection protocol was used. First, a 6 hrs treatment of the oligonucleotide-resin with a 0.5M solution of DBU in pyridine was used to remove the major part of the protecting groups and eliminate the olefins formed during the β-elimination process and a second treatment with a 0.5 M DBU solution in ethanol/pyridine (1:1) to liberate the oligonucleotide from the resin. Using this protocol we have prepared in a 10 μmol scale an oligonucleotide containing 4-O-ethylthymine. In the process of scaling-up we observed that the removal of the oligonucleotide from the resin was slower than in small scale and we needed to do 5 treatments of 24 hrs with the DBU solution in ethanol/pyridine. The HPLC profiles of the product obtained after deprotection and the HPLC-purified product are shown in figure 4. The desired decanucleotide was obtained in a 33% overall yield (synthesis and purification) and was characterized by HPLC analysis of the enzymatic digestion (figure 5). Structural studies with this oligonucleotide are presently under way in order to stablish the base-pairing properties of 4-O-ethylthymine.

CONCLUSIONS

The data presented here show that oligonucleotides containing 4-O-ethylthymine can be synthesized in a very efficient way using NPE-protected nucleosides and the solid-phase phosphite-triester methodology, while standard amide-type protecting groups failed in giving the correct product in good yields. The proposed protocol can be easily adapted to the synthesis of oligonucleotides containing any O-alkylthymines and other nucleophile-sensitive base analogues (for example 6-O-alkylguanines (16), 2- and 4-thiouracils (17)) and for this reason the method proposed here can be of great interest because it opens the possibility to prepare oligonucleotides containing biologically important analogues.

To our knowledge this is the first time that phosphoramidites containing NPE base protecting groups have been used for the preparation of oligonucleotides (15). We have also demonstrated that the deprotection conditions should be carefully optimized in order to avoid undesirable side reactions like base alkylation by the olefins formed during the β-elimination process. We think that some improvements in the nature of the β-eliminating groups should be done to make the deprotection step faster and perhaps avoiding the possibility of alkylation of the bases. It is also important to develop other types of linkages between the oligonucleotide and the resin in order to completely avoid the use of nucleophiles (including even ethoxide) to cleave the linkage. Work in these directions are currently being done. Finally, another aspect that we wish to point out is that using NPE protecting groups and the standard succinyl linkage between oligonucleotide and the resin one could generate by a DBU treatment in a non-protic solvent (pyridine or dioxane), an oligonucleotide completely free of protecting groups and still attached to the solid support. These kind of products could be of interest for the purification of DNA-binding proteins or the separation of specific single-stranded DNA fragments from a complex mixture.

MATERIAL AND METHODS

Abbreviations: AcOEt: ethyl acetate; DBU: 1,8-diaza-biciclo[5.4.0]undec-7-ene; bz: benzoyl; DMT: 4,4′-dimethoxytrityl; EtOH: ethanol; EtOK: potassium ethoxide; EtONa: sodium ethoxide; Et_3N: N,N,N-triethylamine; ibu:isobutiryl; MeOH: methanol; NPE: *p*-nitrophenylethyl; NPEOC: *p*-nitrophenylethoxycarbonyl; T^{Et}: 4-O-ethylthymidine; T^{Me}: 4-O-methylthymidine; T^{tri}: 4-(1,2,4-triazolyl)thymidine; THF: tetrahidrofuran.

5′-O-DMT-A^{NPEOC} (11), 5′-O-DMT-$G^{NPEOC,\ NPE}$ (14) and 5′-DMT-T^{tri}-3′-O-N,N-diisopropyl-O-cyanoethyl

Table 2: Deprotection (%) of NPEOC, NPE-protected nucleosides as estimated by TLC.

Nucleoside	0.5M DBU/dioxane			0.5M DBU/pyridine		0.5M DBU/ethanol:dioxane 1/1			
	1hr	2hr	4hr	1hr	2hr	4hr	10hr	24hr	32hr
C^{NPEOC}	15	50	90	60	total	20	50	80	total
A^{NPEOC}	25	50	90	60	total	20	30	70	90
$G^{NPEOC,NPE}$	50	65	total	90	total	30	50	80	total

Table 3: 4-O-Ethylthymidine containing sequences prepared with NPE-protected phosphoramidites.

Sequence	scale	amidite*	deprotection conditions
A 5'TTCT^{Et}CTT 3'	0.8 μmol	*1*	a) 0.5M DBU in pyridine, 1–5 days b) 0.5M DBU in EtOH/THF 1:1, 24 hrs
A 5'TTCT^{Et}CTT 3'	0.8 μmol	2	0.5M DBU in EtOH/THF 1:1, 24 hrs
B 5'T^{Et}T^{Et}CGACTAGT 3'	0.3 μmol	*1*	a) 0.5M DBU in EtOH/pyr 1:1, 24 hrs, b) i) 0.5M DBU in pyr 1:1, 6hr followed by ii) 0.5M DBU in EtOH/pyr 1:1, 12 hrs
C 5'GGGTT^{Et}TTCCG 3'	10 μmol	*1*	i) 0.5M DBU in pyr, 6hrs followed by ii) 0.5M DBU in EtOH/pyr 1:1, 5 treatments of 24 hrs.

* Phosphoramidite used for the incorporation of 4-O-ethylthymidine

phosphoramidite (10) were prepared as previously described. 5'-O-DMT-C^{NPEOC} was prepared following the methodology described by Pfleiderer with the only modification that *p*-nitrophenylethoxycarbonyl-N-methylimidazolium chloride (11) was used instead of *p*-nitrophenylethoxycarbonyl benzotriazole (11) as acylating reagent. Standard cyanoethyl phosphoramidites were purchased from Applied Biosystems. Oligonucleotide syntheses were done either in a home-made manual DNA synthesizer or in an automatic Applied-Biosystems DNA synthesizer.

5'-O-(4,4'-dimethoxytrityl)-N,O-(NPEOC, NPE)protected-2'-deoxyribonucleoside-3'-O-(N,N-diisopropyl)-O-2-cyanoethyl phosphoramidites.

0.6 mmols of 5'-O-(4,4'-dimethoxytrityl)-N,O-(NPEOC, NPE) protected-2'-deoxynucleoside were dried by coevaporation with anhydrous acetonitrile (2×5 mL). The residue was dissolved in anhydrous acetonitrile (1.2 mL) and a 0.4 M solution of tetrazole in anhydrous acetonitrile (1.5 mL, 0.6 mmol) was added together with 0.15 mL of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphordiamidite (0.5 mmol) under an argon atmosphere. A white precipitate of N,N-diisopropylammonium tetrazolate soon appeared. After 1 hr, a solution of 5% triethylamine in chloroform was added and the mixture was washed with a 10% sodium bicarbonate solution (2x) and a saturated sodium chloride solution. The organic phase was dried with anhydrous sodium sulphate and evaporated to dryness. The oily product was dissolved in the minimum amount of dichloromethane and added to a cold solution of hexane with stirring. The white precipitate formed was separated by centrifugation and dried *in vacuo*.

Phosphoramidite	Yield	TLC Rf*	^{31}P-NMR (Cl_3CD, 81 MHz)
A^{NPEOC}	70%	0.40	146.5, 146.4 ppm
C^{NPEOC}	70%	0.42	146.9, 146.4 ppm
$G^{NPEOC,\ NPE}$	50%	0.86	146.4, 146.2 ppm

*AcOEt/CH_2Cl_2/Et_3N (45:45:10)

5'-O-(4,4'-dimethoxytrityl)-4-O-ethylthymidine.

5'-O-(4,4'-dimethoxytrityl)thymidine (1.5 g, 2.6 mmol) was dried by coevaporation with anhydrous acetonitrile (2×10 mL). The residue was dissolved with 60 mL of anhydrous acetonitrile and 1-trimethylsilyl-1,2,4-triazole (0.78 g, 5.5 mmol) was added under argon atmosphere. The mixture was kept at 50°C during 1 hr and allowed to cool down to room temperature. The resulting solution was added to a mixture previously prepared in the following way : A suspension of 1,2,4-triazole (1.9 g, 27.6 mmol) in 48 mL of anhydrous acetonitrile was cooled in an ice-bath and 0.54 mL of phosphorous oxychloride (6.3 mmol) were added with stirring, together with 4.15 mL of triethylamine (30 mmol). The mixture was stirred for 30 minutes at 0°C before 3'-O-TMS-5'-O-DMT-thymidine was added.

After 2 hrs of magnetic stirring at room temperature, the reaction mixture was diluted with dichloromethane and washed with a 10% sodium bicarbonate solution. The organic phase was dried with anhydrous sodium sulphate and evaporated to dryness. The product was dissolved in 20 mL of absolute ethanol and 20 mL of a 0.08 M solution sodium ethoxide were added. The

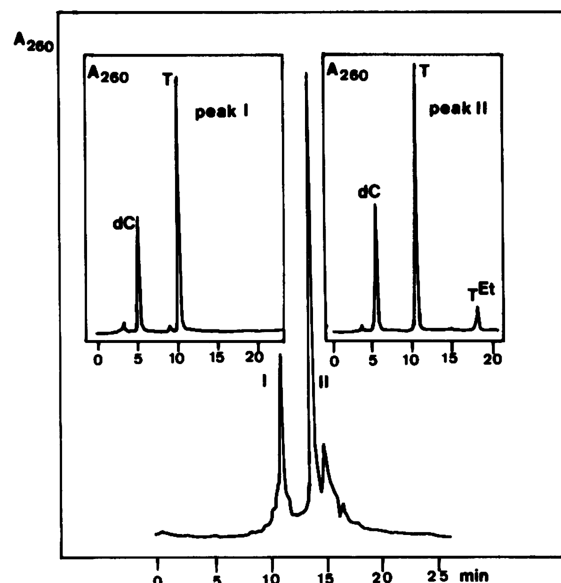

Figure 3: HPLC purification of heptanucleotide A (TTCT^{Et}CTT) prepared with NPEOC-protected and 4-O-ethylthymidine phosphoramidites after the treatment with 0.5 M DBU in EtOH/THF 1:1 being I : TTCTCTT and II : TTCT^{Et}CTT. Inserts show HPLC analysis of the enzymatic digestions of peak I and II.

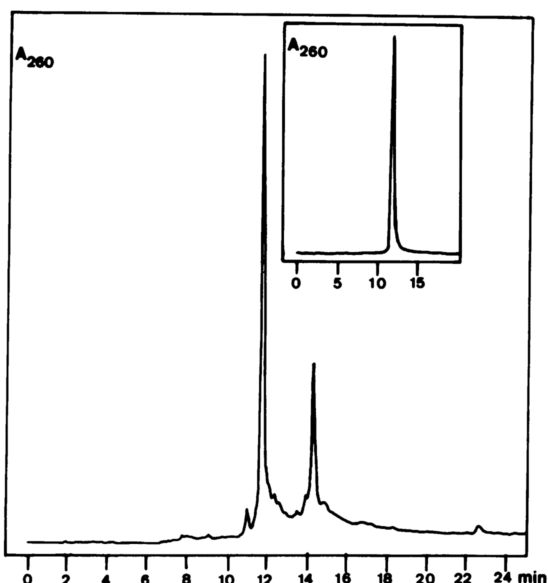

Figure 4: HPLC purification of decanucleotide C (GGGTT^{Et}TTCCG). In the insert, analytical HPLC of the purified product.

solution was stirred for 30 min, neutralized with 0.2 mL of glaciar acetic acid and evaporated to dryness. The product was purified on a silica gel column eluted with a solution from 0% to 5% of methanol in dichloromethane.

Fractions containing the desired product were pooled and evaporated to dryness. The oily product was dissolved with the minimal amount of dichloromethane and precipitated from a mixture of ethyl ether / hexane (1:1), obtaining 0.42 g of a white solid (67% yield), m.p. 86–92°C. Homogenous by TLC (CH_2Cl_2/MeOH 90:10) Rf= 0.48. UV (MeOH) max. 275.1 nm (log ϵ 3.60). ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) = 7.9 (1H, s, H-5); 7.3 (9H, m, Ar); 6.9 (4H, m, Ar); 6.2 (1H, t, H-1'); 5.4 (1H, d, 3'-OH); 4.3 (3H, m, -OCH_2-CH_3, H-3'); 3.9 (1H, m, H-4'); 3.4 (2H, m, H-5'); 2.87 (3H, s, -OCH_3); 2.85 (3H, s, -OCH_3); 2.2 (2H, m, H-2'); 1.5 (3H, s, -CH_3); 1.15 (3H, t, -O-CH_2-CH_3).

5'-O-(4,4'-Dimethoxytrityl)-4-O-ethylthymidine-3'-O-(N,N-diisopropylamino) 2-cyanoethyl phosphoramidite

0.3 g of 5'-O-(4,4'-dimethoxytrityl)-4-O-ethylthymidine (0.52 mmol) were reacted in acetonitrile with 35.2 mg of tetrazole (0.50 mmol) and 1.143 mL of 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphordiamidite (0.5 mmol) under a dry argon atmosphere as described above. The resulting product was dessicated and stored under argon at −20 °C. It weighed 0.40 g (85% yield) and was used without further purification. TLC (AcOEt/CH_2Cl_2/Et_3N 45:45:10) Rf 0.6. ^{31}P-NMR ($CDCl_3$, 81 MHz); δ (ppm): 148.8, 148.5.

Oligonucleotide syntheses

All the oligonucleotides were synthesized using standard solid-phase phosphite-triester procedures. Oligonucleotide C was prepared on a 10 μmol scale and using an automatic DNA synthesizer. The rest of the oligonucleotides were synthesized on a 0.3–1 μmol scale using a manual synthesizer. The support used in all the syntheses was long-chain amino alkyl controlled-pore glass that was functionalized following the methodology described in ref. 18 with the only modification that N-methylimidazole was used as catalyst in the preparation of the DMT-nucleoside succinates instead of N,N-dimethyl-aminopyridine.

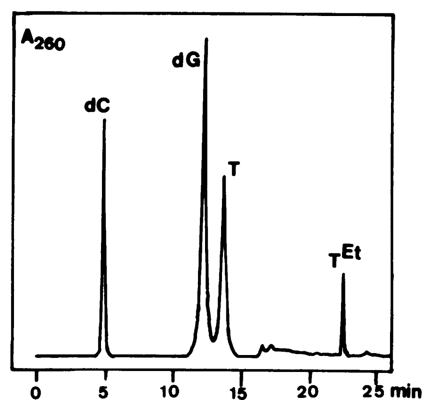

Figure 5: HPLC analysis of the enzymatic digestion of oligonucleotide C.

Deprotection and purification

In a typical experiment 10–20 mg of oligonucleotidyl-resin were treated with aprox. 0.5 mL of the appropiate DBU solution. After a period of time ranging from 12 to 72 hrs, the resin was filtered and washed with the same solvent used for the deprotection. The filtrates were pooled, neutralized with acetic acid (1.5 equiv. with respect to the amount of DBU) and diluted with aprox. 10 mL of water. The aqueous phase was washed with ether, CH_2Cl_2, and ether and concentrated to dryness. The residue was dissolved in water and chromatographed on a Sephadex G-10 column (30×1 cm) eluted with 50 mM triethylammonium bicarbonate. The fractions corresponding to the first UV-absorbing peak were pooled and lyophilized. The resulting residue was purified by reversed-phase HPLC using a C-18 Nucleosil (5μm) column (250×4 mm) with a 5–30% gradient of acetonitrile in 10 mM triethylammonium acetate over 20 min.

In the 10 μmol preparation of 5'-d(GGGTT^{Et}TTCCG), 400 mg of the resin were treated twice with 10 mL of a 0.5 M DBU solution in acetonitrile at room temperature for 3 hrs. Afterwards, the resin was washed with acetonitrile and treated with 10 mL of a 0.5 M DBU solution in EtOH/pyridine (1:1). After 24 hrs, the solution was filtered from the resin, and the resin was washed with pyridine. The collected filtrates were pooled, neutralized with 0.15 mL of acetic acid and diluted with 3 mL of a 50 mM aqueous solution of triethylammonium bicarbonate. The treatment of the resin with 0.5M DBU in EtOH/pyridine was repeated until no more oligonucleotide was cleaved from the resin (a total of 5 treatments were needed). The filtrates were pooled and evaporated to dryness. The residue was chromatographed on a Sephadex G-10 column obtaining 550 OD units at 260 nm of a crude material that was purified by reversed-phase HPLC using a μ-Bondapak C-18 (5μm) column (150×19 mm). Flow rate: 5 mL/min. Solution A: 50 mM triethylammonium acetate; solution B: acetonitrile/water (1:1). A 20 min gradient from 5 to 30%B was used. Yield : 240 OD units at 260 nm (33% yield). The product was characterized by its nucleoside content.

Synthesis of 5'-d(AAGT^{Et}CTG) 3' by alkoxy exchange at the oligonucleotide level

DMT-AAGT^{tri}CTG-resin was prepared with standard phosphoramidites as described above. 10 mg of the resin were

treated with a solution of DBU/MeOH/THF (1:3:3 v/v/v) at room temperature for 60 hrs. After the work-up described above, the resulting DMT-oligonucleotide was purified by reversed-phase HPLC, treated with 80% acetic acid and rechromatographed. The product 5'-d(AAGT^{Me}CTG) was characterized by its nucleoside content analysed by enzymatic hydrolysis followed by HPLC analysis of the digestion.

Purified AAGT^{Me}CTG (0.5 OD units at 260 nm) was treated with the appropriate ethoxide solution (see table 1). At different times, acetic acid (1 equivalent with respect to the amount of ethoxide) was added and the mixture was concentrated to dryness. The residue was purified by gel filtration chromatography on a Sephadex G-10 column (30×1 cm) eluted with 50 mM triethylammonium bicarbonate followed by reversed-phase HPLC using a C-18 Nucleosil (5 μm) column (250×4 mm) with a 0 −20% gradient of acetonitrile in 50 mM triethylammonium acetate over 15 min, and 5 min isocratic at 20% acetonitrile. The elution times were as follows : AAGTCTG, 14.5 min; AAGT^{Me}CTG, 15.0 min; and AAGT^{Et}CTG, 15.5 min. All the products were characterized by its nucleoside content.

Deprotection of NPEOC, NPE-protected nucleosides

Approx. 1−2 mg of NPEOC,NPE-protected nucleosides were dissolved in 0.2 mL of 0.5M DBU solutions in dioxane, pyridine and ethanol/dioxane (1:1) with stirring, At different times, aliquots were taken, and were analyzed immediately by TLC (dichloromethane/methanol (9:1)). Results are shown in table 2.

Enzymatic digestions of oligonucleotides

The polymers (0.5 OD at 260 nm) were incubated in 50 mM tris.HCl pH 8.0 and 10 mM magnesium chloride with snake venom phosphodiesterase (0.4 μg) and bacterial phosphatase (0.4 μg) in a total volume of 20 μL at 37°C overnight. The resulting mixture was diluted and analyzed by HPLC. Column: C-18 Nucleosil (5 μm). Flow rate: 1 mL/min. Solvent A: 10 mM triethylammonium acetate, solvent B: acetonitrile/water (1:1). A 20 min linear gradient from 0%B to 50%B. The retention times observed under these conditions were dC 6.7 min, dG 9.8 min, T 10.2 min, dA 12.4 min, T^{Me} 15.5 min, T^{Et} 19.8 min.

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REFERENCES

1. Singer, B. and Kusmierek, J.T. (1986) Ann. Rev. Biochem., *52*, 655−693.
2. Saffhill, R., Margison, G.P. and O'Connor, P.J. (1985) Biochim. Biophys. Acta, *823*, 111−145.
3. Pegg, A.E. (1977) Adv. Cancer Res., *25*, 195−269.
4. Singer, B. (1986) Cancer Res., *46*, 4879−4885.
5. Dolan, M.E., Oplinger, M. and Pegg, A.E. (1988) Mutation Res., *193*, 131−137.
6. Singer, B., Kroger, M. and Carrano, M. (1978) Biochemistry, *17*, 1246−1250.
7. Li, B.F.L., Reese, C.B. and Swann, P.F. (1987) Biochemistry, *26*, 1086−1093.
8. Borowy-Borowski, H., Chambers, R.W. (1989) Biochemistry, *28*, 1471−1477.
9. Nielsen, J., Taagaard, M., Marugg, J.E., van Boom, J.H. and Dahl, O. (1986) Nucleic Acids Res., *14*, 7391−7403.
10. Webb, T.R. and Matteuci, M.D. (1986) Nucleic Acids Res., *14*, 7661−7674.
11. Himmelsbach, F., Schulz, B.S., Trichtinger, T., Charubala, R. and Pfleiderer, W. (1984) Tetrahedron *40*, 59−72.
12. Herdewijn, P., Charubala, R. and Pfleiderer, W. (1989) Helv. Chim. Acta, *72*, 1729−1738.
13. Uhlmann, E. and Pfleiderer, W. (1980) Tetrahedron Lett., *21*, 1181−1184.
14. Pfleiderer, W., Schwarz, M. and Schirmeister, H. (1986) Chemica Scripta, *26*, 147−154.
15. During the preparation of the manuscript a communication on the synthesis of oligonucleotides using NPE and NPEOC-protected phosphoramidites has appeared: Stengele, K.P. and Pfleiderer, W. (1990) Tetrahedron Lett. *31*, 2549−2552.
16. Borowy-Borowski, H. and Chambers, R.W. (1987) Biochemistry *26*, 2465−2471.
17. Connolly, B.A. and Newman, P.C. (1989) Nucleic Acids Res. *17*, 4957−4974.
18. Gait, M.J. in 'Oligonucleotide synthesis a practical approach'. IRL Press. Oxford 1984, pp 47.