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Synthesis and Properties of Oligonucleotides Containing the Mutagenic Base O^4-Benzylthymidine

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Abstract—The preparation of synthetic oligodeoxynucleotides containing O^4-benzylthymidine (T^{bn}) is described. The use of standard and *t*-butylphenoxyacetyl amino protecting groups is compared. The thermal stabilities of duplexes containing T^{bn} paired with adenine and guanine have been measured.

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 in DNA in particular O^6-alkylguanine, O^4-alkylthymine and O^2-alkylthymine.[1–4] Although major attention in chemical carcinogenesis is focused on O^6-alkylguanines, the mutagenic potential of O^2- and O^4-alkylthymines has been recognized[5] and it seems to be related to an inefficient repair of *O*-alkylthymine residues in eukaryotic cells.[6] The repair of O^6-alkyl guanine and to some extent *O*-alkylthymine lesions is performed by a protein named O^6-alkylguanine-DNA alkyltransferase.[7] This protein transfers the alkyl group to a cysteine acceptor residue within its amino acid sequence, that is not regenerated. The alkyltransferase protein removes a large variety of alkyl groups, although the rate of reaction decreases as the size of the alkyl group increases. An important exception to this rule is O^6-benzylguanine, which is an excellent substrate for mammalian alkyltransferase and for that reason is an effective inactivator.[7] On the other hand, O^4-benzylthymidine (T^{bn}) does not inactivate alkyltransferases.[8] So, most probably these lesions are not repaired efficiently *in vivo*.

In order to study the influence of the alkyl group on the mutagenic properties and the repair of *O*-alkylthymidine lesions, it is important to develop efficient methods to incorporate these analogues. O^4-Alkylthymidines cannot be introduced in synthetic oligonucleotides using standard protocols because they react with ammonia during deprotection giving 5-methylcytidine derivatives.[9] This problem has been solved for O^4-methyl thymidine by using 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) solutions in methanol to remove the benzoyl (Bzl) and isobutyryl (ibu) standard amino protecting groups.[9,10] Methoxide ions present in these solutions remove the protecting groups without modification of O^4-methylthymine. The preparation of oligonucleotides with O^4-ethylthymine residues has been described using non-standard amino protecting groups because less nucleophilic ethoxide ions are not efficient to deprotected standard amino protecting groups at room temperature.[11–13] Recently, it has been described that oligonucleotides containing O^4-methyl, O^4-ethyl and O^4-propylthymine residues can be prepared using standard base protecting groups if DBU solutions in the appropiate alcohol are heated at 50 °C.[14]

In this communication, we focused on the preparation of oligonucleotides containing O^4-benzylthymine residues. For this purpose we compared standard amino protecting groups and commercially available *t*-butylphenoxyacetyl (*t*-BPA, Expedite™) amino protecting groups.[15] Similarly to the phenoxyacetyl group (Pac), the electron-withdrawing properties of the *t*-butylphenoxy group enhances the electrophilicity of the amide and *t*-BPA groups are removed in milder conditions than standard amino protecting group[15,16].The better solubility properties of the G derivative in acetonitrile makes *t*-BPA phosphoramidites more attractive for automated synthesizers.

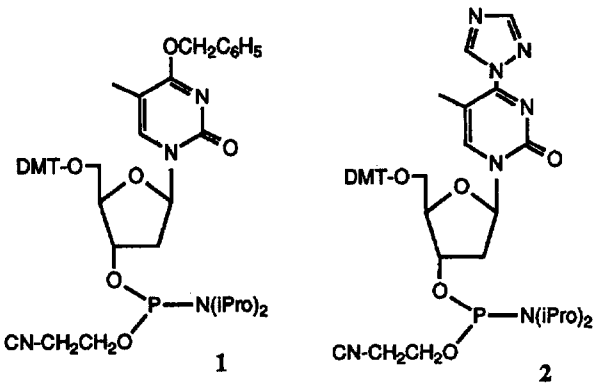
Results and Discussion

Synthesis of the phosphoramidite derivatives

The preparation of oligonucleotides containing O^4-

benzylthymidine requires the synthesis of appropriate derivative. 5'-*O*-DMT-O^4-benzylthymidine 3'-*N*,*N*-diisopropyl-*O*-2-cyanoethyl phosphoramidite was prepared following the method described for the preparation of O^4-ethylthymidine derivative.[12] Starting with DMT-T, the 3'-OH of the nucleoside was protected with trimethylsilyl group by reacting with trimethylsilyl-1,2,4-triazole. The protected derivative was reacted without isolation with phosphoryl tris-1,2,4-triazolide giving the triazolyl derivative that is displaced with sodium benzyloxide. The expected DMT-T^{bn} was isolated in good yields (70%). The phosphoramidite derivative 1 and solid support (CPG) loaded with the DMT-T^{bn} through a 3'-*O*-succinyl bond were prepared following standard protocols.[17]

We have also prepared 5'-*O*-DMT-T^{tri} 3'-*N*,*N*-diisopropyl-*O*-2-cyanoethyl phosphoramidite (**2**) using previously described protocols.[14,18] This phosphoramidite has been shown to be incorporated into synthetic DNA and, at the end of the synthesis, O^4-alkylthymidines can be generated by displacement of the triazole group with DBU solution in the appropriate alcohol.[13-14]



Preparation of oligonucleotides

Hexamer 5' GCT^{tri}AGC 3' (A) was prepared on an automatic DNA synthesizer using 2-cyanoethyl phosphoramidites of T^{tri} and the natural bases carrying standard Bzl and ibu amino protecting groups. Treatment of the solid support with a solution of DBU/dioxane/benzyl alcohol[14] at 50 °C for 3 days yielded the crude hexamer GCT^{bn}AGC (Fig. 1a). As seen in Figure 1, a large amount of side products were obtained but the special hydrophobicity of the benzyl group helped the purification by retarding the elution of the desired product. The last product in the chromatogram was purified to homogeneity and was characterized by enzymatic digestion (Fig. 1b). The more polar impurities were assigned to hydrolysis and/or incomplete transformation of T^{tri} to T^{bn} during the DBU/benzyl alcohol deprotection–modification treatment. A synthesis of the same hexamer using *t*-BPA phosphoramidites and T^{bn} phosphoramidite gave only one peak with the same retention time and nucleoside composition as the product obtained with standard phosphoramidites (data not shown). The more polar impurities were not present on the chromatogram. These results were encouraging because deprotection of amino groups was judged to be completed in the hexamer even though the less reactive benzyloxide ions were used.

Pentadecamer 5' GCAATGGAT^{tri}CCTCTA 3' (B) was prepared using standard and more labile *t*-BPA phosphoramidites. Acetic anhydride and *t*-butylphenoxyacetyl anhydride were used as capping reagents for the synthesis using *t*-BPA phosphoramidites. The supports were treated with a solution of DBU/benzyl alcohol/dioxane at 50 °C for 3 days. Results are shown in Figure 2. No defined peaks were observed in the HPLC profile obtained when standard amino protecting

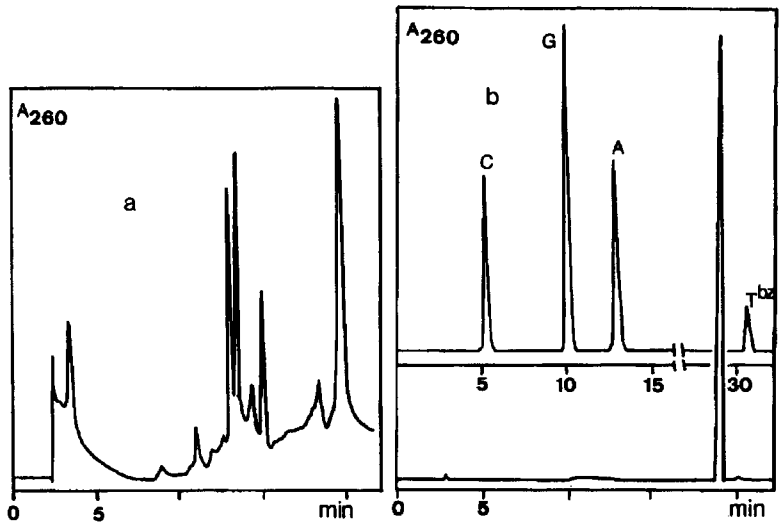


Figure 1. Purification and characterization of hexamer A prepared using standard and T^{tri} phosphoramidites. (a) HPLC profile of the product obtained after treatment with DBU/benzyl alcohol/dioxane at 50 °C for 3 days and desalting with Sephadex G-10. The last product of the chromatogram was characterized as the desired T^{bn} hexamer; (b) lower part: analytical HPLC profile of purified hexamer; upper part: HPLC analysis of the enzymatic digestion of this product.

groups were used. A huge mass of different products having a similar retention time was obtained. When *t*-BPA protecting groups and acetic anhydride were used, three or four defined peaks started to show but still a large mass of non-defined peaks were found surrounding these peaks. Finally, the use of *t*-BPA phosphoramidites and *t*-butylphenoxyacetyl anhydride yielded a defined peak with small amounts of side products mostly on the back part (less polar) of the main peak. The difference between the results obtained with *t*-BPA phosphoramidites when acetic anhydride is used as capping reagent could be explained as a result of the conversion of the *t*-butylphenoxyacetamide groups to acetamide during the capping reaction. The resulting acetamide groups are less labile than the *t*-BPA groups and they are probably not completely removed in the DBU/benzyl alcohol deprotection.

HPLC analysis of the enzymatic digestion of the product from the main peak showed deprotection of natural bases was completed but T^{bn} was not present (data not shown). The same solid support (prepared with *t*-BPA phosphoramidites and T^{tri}) when treated with a solution of DBU in ethanol/dioxane at room temperature for 3 days gave the expected O^4-ethylthymine oligonucleotide. So, we conclude that T^{tri} was not converted to T^{bn} in appreciable yields if the oligonucleotide was 15 bases long. The lack of conversion of T^{tri} to T^{bn} and the failure on the deprotection of the standard amino protecting groups in the pentadecamer are due to the lower nucleophilicity of benzyloxide ions compared with ethoxide and methoxide ions. So, the methodology described for the preparation of oligonucleotides containing O^4-ethyl and O^4-methylthymines[13,14] could not be directly applied to the preparation of O^4-benzylthymine oligonucleotides. The use of T^{bn} phosphoramidite and the more labile *t*-BPA phosphoramidites is required together with the use of the corresponding *t*-butylphenoxyacetic anhydride during capping reaction.

Following this rationale, pentadecamers 5' GCAATGGAT^{bn}CCTCTA 3' (C) and 5' TCCCAGTCACGACGT^{bn} 3' (D) were prepared using T^{bn} phosphoramidite and solid support loaded with DMT-T^{bn}, *t*-BPA phosphoramidites and *t*-butylphenoxyacetic anhydride. Deprotection was performed with a DBU solution in benzyl alcohol/dioxane at 50 °C for 3 days, yielding products with a correct analytical HPLC (Fig. 3). The main product of the chromatogram was isolated and analyzed by enzymatic digestion presenting the correct nucleoside composition.

Circular dichroism

The CD spectra for pentadecamer duplexes containing O^4-benzylthymidine and O^4-ethylthymidine paired with guanine and adenine are shown in Figure 4. Only small differences were observed in the spectra of the different duplexes. All spectra showed a positive (270 nm) to negative (245 nm) splitting and the CD magnitudes of both bands are similar. These observations indicate that all duplexes have a similar structure and it has the characteristics found in B-type double helical DNA.

Base-pairing properties of O^4*-benzylthymine*

Figure 5 shows the melting curves for duplexes containing O^4-benzylthymine, O^4-ethylthymine paired with guanine and adenine. Melting temperatures of duplexes containing O^4-benzyl and O^4-ethylthymine are shown in Table 1. O^4-ethyl and benzyl thymine:G

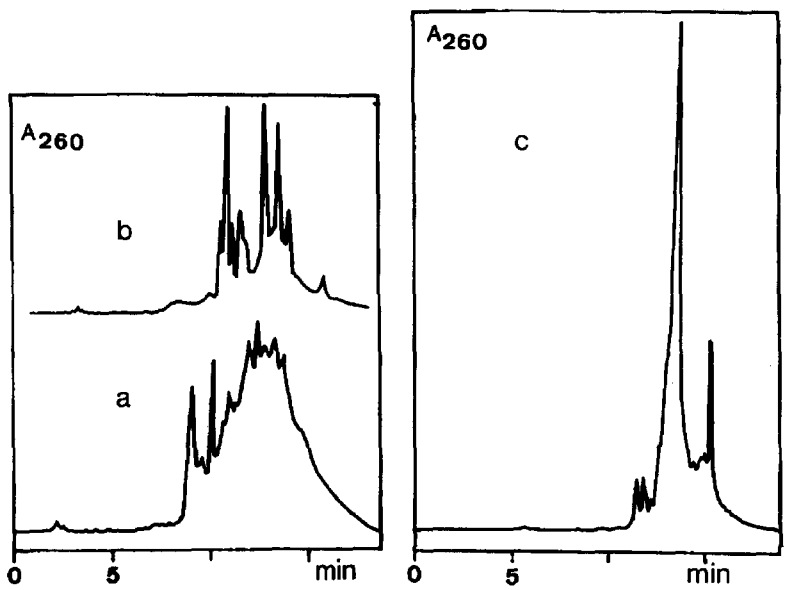


Figure 2. Analysis of the products obtained after synthesis of pentadecamer B. Deprotection and work-up as described in Figure 1. (a) Product from the synthesis using standard and T^{tri} phosphoramidites; (b) product from the synthesis using *t*-BPA and T^{tri} phosphoramidites and acetic anhydride as capping reagent; (c) product from the synthesis using *t*-BPA and T^{tri} phosphoramidites and *t*-butylphenoxyacetic acid as capping eagent.

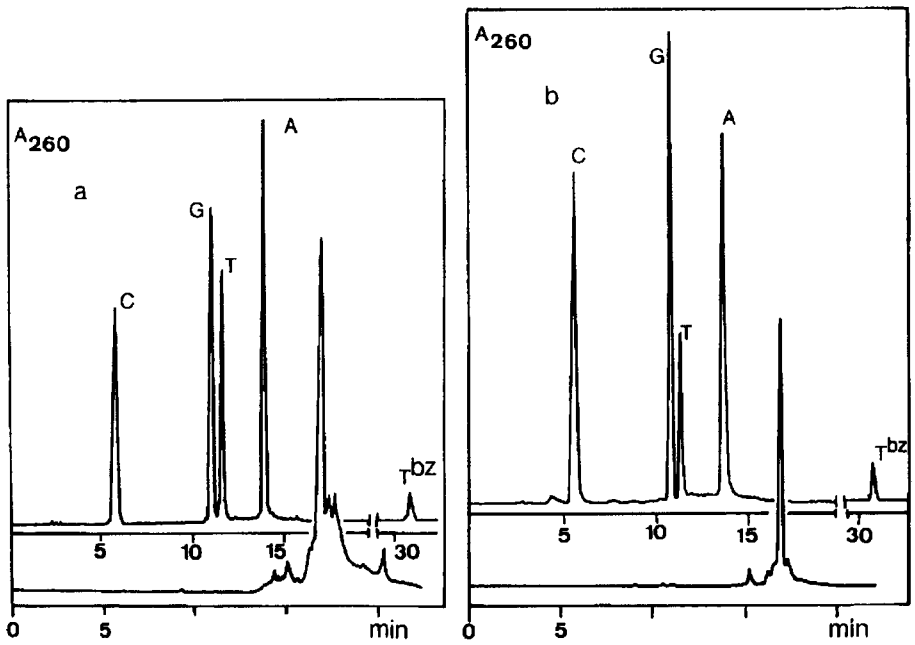


Figure 3. Purification and characterization of pentadecamers C and D. Lower part: HPLC profile of the product obtained after deprotection and work-up as described in Figure 1. Upper part: HPLC analysis of the enzymatic digestion of the main peak. (a) Pentadecamer C; (b) pentadecamer D.

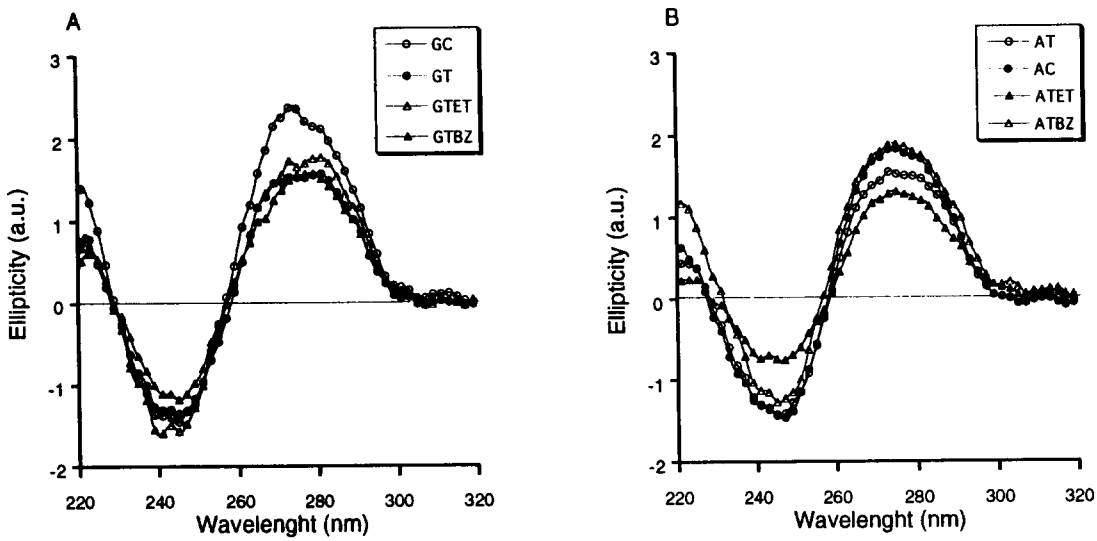


Figure 4. CD spectra of pentadecamer duplexes. (A) Duplexes containing G:C, G:T, G:T^{et} and G:T^{bn} base pairs; (B) duplexes containing A:T, A:C, A:T^{et} A:T^{bn}. Ellipticity is expressed in arbitrary units.

basepair are more stable than the corresponding O^4-alkylthymine:A basepair. A similar trend was observed for O^4-methylthymine[19] basepairs. The melting curves of duplexes containing T^{et} and T^{bn} extends over a wider temperature range compared with the perfectly matched duplexes. Melting profiles of duplexes containing T^{bn} paired with adenine and guanine are biphasic while duplexes containing T^{et} and duplexes containing T^{bn} paired with cytosine and thymine are wide but they do not show the biphasic character so clearly. Mispairs A:C and G:T look similar to T^{et} duplexes. First derivative of the melting profiles obtained with T^{bn}:A and T^{bn}:G duplexes gave two maxima from which two apparent Tm could be measured. The hyperchromicity associated to each of the two transitions has the same magnitude in both T^{bn}:A and T^{bn}:G duplexes. We do not know why the melting curves were biphasic but it has been observed previously in three self-complementary dodecamers containing T^{me}:G, T^{me}:A and G^{me}:T base pairs.[9] As suggested by these authors, one explanation could be a preferential melting around the alkylated base. But also, owing to the possible *syn–anti* orientation of the alkyl group at position 4 with respect to N-3 of alkylated thymine, it could be feasible to have two distinct types of T^{bn} duplexes. One duplex with the benzyl group in a *syn* orientation, less stable

because of the presence of the bulky benzyl group near the base-pairing groups and another duplex with the benzyl group in an *anti* orientation pointing out the major groove and more stable because it allows the base pairing with adenine or guanine (Fig. 6). NMR studies have shown that the methyl group O^4-methylthymine adopts the *syn* orientation with respect to the N-3 of the base in both T^{me}:A and T^{me}:G mispairs.[20] But, on the other hand, primer-extension experiments have shown the presence of two distinct conformations of O^6-methylguanine with a large difference on their extension kinetics by DNA polymerases.[21] In our melting experiments, the fact that a biphasic transition is found when T^{bn} is paired with adenine and guanine but not when T^{bn} is opposed to C and T could indicate that the *syn–anti* orientation of the benzyl group together with the possibility of stabilizing the *anti* conformer by H-bonding are the main causes of the biphasic melting profile, but more detailed experiments are needed to confirm this hypothesis.

In conclusion, we have shown that oligonucleotides carrying O^4-benzylthymine residues can be prepared using a modification of the solid-phase phosphite-triester synthesis protocols. Although the modification of the deprotection conditions has been described for oligonucleotides containing O^4-methyl, O^4-ethyl and O^4-propylthymine, the low reactivity of DBU solution in

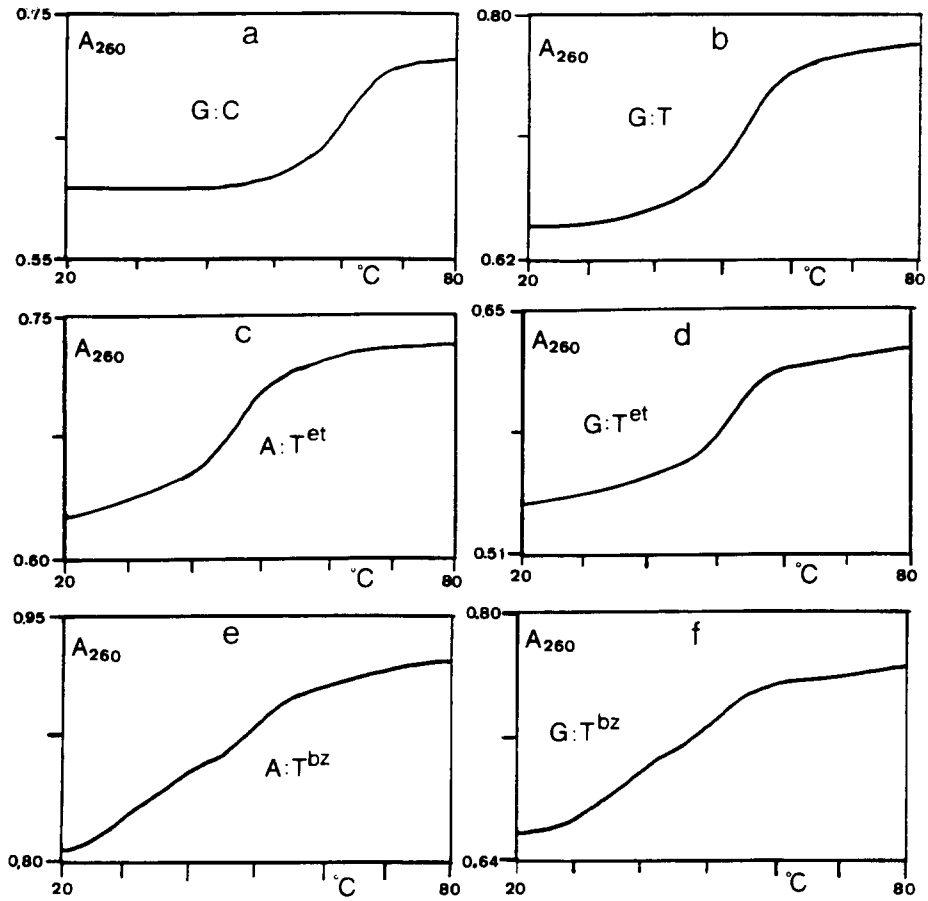


Figure 5. Thermal melting curves of pentadecamer duplexes containing the following basepairs at the central position: (a) G:C, (b) G:T, (c) A:T^{et}, (d) G:T^{et} (e) A:T^{bn}, and (f) G:T^{bn}.

Table 1. Melting temperatures (Tm, °C) of DNA duplexes containing O^4-benzyl and O^4-ethylthymine in 0.15 N NaCl, 0.05 N Tris–HCl pH 7.8, 1 mM EDTA. The differences in Tm values in two different melting experiments are less than 0.2 °C

5' GCAATGGAXCCTCTA 3'
3' CGTTACCTYGGAGAT 5'

X:Y	Tm (°C)	Hyperchromicity	X:Y	Tm (°C)	Hyperchromicity
T:A	58.5	22 %	T^{bn}: A	32.7, 47.5	10%
C:G	62.0	20 %	T^{bn}: G	37.6, 50.3	14%
T:G	53.8	20 %	T^{bn}: C	45.1	13%
C:A	48.5	25 %	T^{bn}: T	43.5	12%
T^{et}:A	44.5	16%			
T^{et}:G	48.6	12%			

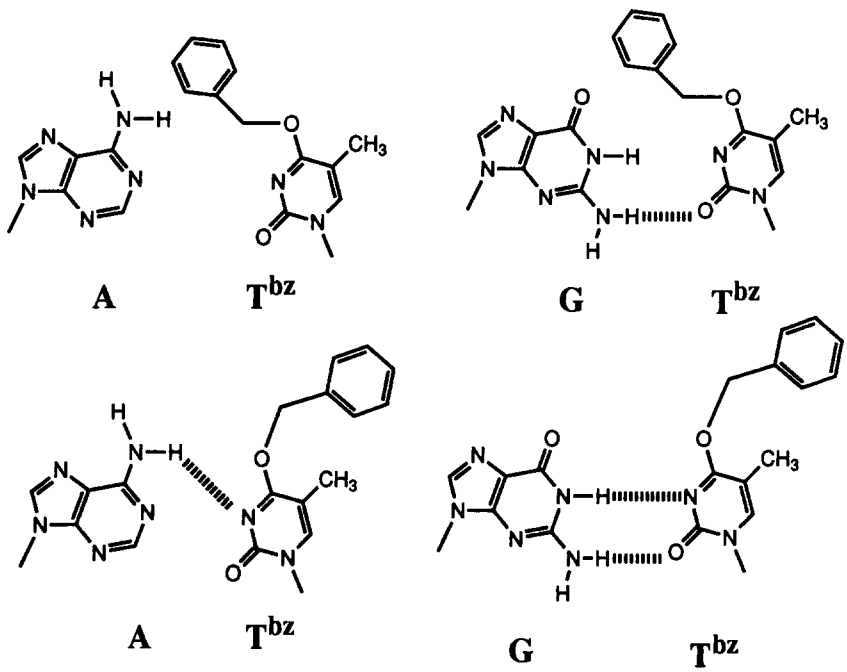


Figure 6. Postulated structures for A:T^{alkyl} and G:T^{alkyl} basepairs showing both *anti* and *syn* orientation of the alkyl group with respect to N-3.

benzyl alcohol made and the direct extension of previously described protocols difficult.[9–14] The use of more labile *t*-BPA (Expedite™) phosphoramidites with the corresponding anhydride for capping was indispensable for the obtention of oligonucleotides containing the desired O^4-benzylthymine. Thermal stabilities of duplexes containing O^4-benzylthymine show a different behaviour from other mispairs but still the T^{bn}:G basepair was slightly more stable than T^{bn}:A. Whether the greater stability of the T^{bn}:G basepair will yield a better efficiency of dGTP in front of dATP in a template with T^{bn} is still unknown, although it has been shown recently for O^4-methyl and O^4-ethylthymine.[22] This question and the repair efficiency of this lesion could be answered using the methodology described here for the preparation of oligonucleotides containing O^4-benzylthymine.

Experimental

Abbreviations: AcOEt: ethyl acetate, Bzl: benzoyl, *t*-BPA: *tert*-butylphenoxyacetyl, DBU: 1,8-diazabicyclo-[5.4.0]undec-7-ene, CPG: controlled pore glass, DCM: dichloromethane, DIEA: ethyldiisopropylamine, DMT: dimethoxytrityl , Et_3N: triethylamine, ibu: isobutyryl, MeOH: methanol, Npe: 2-(4-nitrophenyl)ethyl, Pac: phenoxyacetyl, pyr: pyridine, T^{bn}: O^4-benzylthymidine, T^{et}: O^4-ethylthymidine, T^{tri}: 4-(1,2,4-triazol-1-yl)-5-methyl-2-pyrimidone-2'-deoxyriboside. DMT-T^{tri} *N,N*-diisopropyl-2-cyanoethyl phosphoramidite was prepared essentially as described in Refs 14 and 18.

5'-O-(4,4'-Dimethoxytrityl) O^4-benzylthymidine. 5'-*O*-Dimethoxytrityl-thymidine (1 g, 1.8 mmol) was dried by coevaporation with dry acetonitrile. The residue was dissolved with 45 mL of dry acetonitrile and 0.53 g (3.73 mmol) of 1-trimethylsilyl-1,2,4-triazole was added under argon atmosphere. The mixture was kept at 55 °C for 70 min and allowed to cool down to room temperature. The resulting solution was added to a mixture previously prepared as follows: a suspension of 1.3 g (48.9 mmol) of 1,2,4-triazole in 45 mL of dry acetonitrile was cooled in an ice-bath and 0.39 mL (4.3 mmol) of phosphorous oxychloride was added with stirring, together with 3 mL (20.7 mmol) of Et_3N. The mixture was stirred for 30 min at 0 °C before 3'-*O*-trimethylsilyl-5'-DMT-thymidine was added.

After 90 min of magnetic stirring at room temperature, the reaction mixture was diluted with DCM and washed with a saturated aqueous $NaCO_3H$ solution. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to dryness. The product was dissolved in 10 mL of dry acetonitrile and 5 mL of a 0.72 M solution of sodium benzyloxide was added. The solution was stirred overnight, neutralized with 0.21 mL glacial acetic acid and concentrated. The remaining solution was then poured slowly into 100 mL of hexane with stirring. The precipitate was collected and purified on a silica gel column eluted with 0 to 5% MeOH in DCM. Fractions containing the desired product were pooled and concentrated to dryness, obtaining 0.8 g (70% yield). TLC (DCM:MeOH 95:5) R_f 0.4. ^{1}H NMR ($CHCl_3$, 200 MHz): δ 1.56 (3H, *s*, CH_3 Thy), 2.27 (1H, *m*, H-2'), 2.68 (1H, *m*, H-2'), 3.44 (2H, *m*, H-5'), 4.16 (1H, *m*, H-4'), 4.59 (1H, *m*, H-3'), 5.41 (2H, *s*, CH_2Bz), 6.45 (1H, *t*, H-1'), 6.84 (4H, *m*, Ar), 7.33 (9H, *m*, Ar), 7.9 (1H, *s*, H-6).

5'-O-(4,4'-Dimethoxytrityl) O^4-benzylthymidine 3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite. 5'-*O*-Dimethoxytrityl-O^4-benzylthymidine (0.23 mmol) (150 mg) was dried by coevaporation with dry acetonitrile.

The residue was dissolved in dry DCM, 0.16 mL (0.97 mmol) of DIEA was added and the mixture was kept under argon atmosphere. The solution was cooled with an ice-bath and 82 mg (0.34 mmol) of chloro-2-cyanoethoxy-*N*,*N*-diisopropylamino phosphine added with a syringe. After magnetic stirring for 1 h at room temperature, MeOH (1 mL) was added and the solution was concentrated to dryness. The residue was dissolved in 10% Et_3N in AcOEt and the solution washed with 10% aqueous $NaCO_3H$ and saturated aqueous NaCl. The organic layer was dried with anhydrous Na_2SO_4 and concentrated to dryness. The residue was purified by column chromatography (silica gel) eluted with $CHCl_3$:AcOEt:Et_3N (45:45:10), giving 160 mg (83% yield) of the desired product. R_f ($CHCl_3$:AcOEt:Et_3N 45:45:10): 0.8. ^1H NMR ($CHCl_3$, 300 MHz) two diastereoisomers, selected signals: 1.58 (3H, *s*, CH_3 Thy), 3.78 (6H, *s*, CH_3, DMT), 4.16 (2H, *m*, H-4'), 4.62 (2H, *m*, H-3'), 5.4 (2H, *s*, CH_2 Bz), 6.83 (2H, two *t*, H-1'), 7.89 and 7.95 (1H, two *s*, H-6). ^{31}P NMR ($CHCl_3$, 300 MHz) 2 diastereoisomers: 149.06 and 149.69 ppm.

5'-O-(4,4'-Dimethoxytrityl) O^4-benzylthymidine 3'-O-succinate and preparation of the solid support loaded with DMT-T^{bz}. To a solution of 38 mg (0.06 mmol) of DMT-T^{bn} in 5 mL of dry DCM, 7.7 mg (0.07 mmol) of succinic anhydride and 9.4 mg (0.07 mmol) of dimethylaminopyridine were added. After 16 h of magnetic stirring at room temperature, the mixture was diluted with 15 mL of DCM and the resulting solution was washed with a 0.1 M solution of monosodium phosphate pH 5 and water. The organic layer was dried with anhydrous Na_2SO_4 and concentrated to dryness. The product was dissolved in the minimum amount of DCM and precipitated with hexane yielding 30 mg (68%) of the desired product as a white solid. TLC (DCM:MeOH 95:5): R_f 0.35.

The product obtained above (0.04 mmol) was dissolved in dimethylformamide and the solution was cooled with ice. 6.6 mg (0.04 mmol) of 1-hydroxybenzotriazole and 8.5 mg (0.04 mmol) of dicyclohexylcarbodiimide were added and the solution was kept 10 min on ice. This solution was added to a flask containing 0.2 g of long-chain amino alkyl CPG (0.07 mmol g^{-1}) and 3 mg (0.02 mmol) of dimethylaminopyridine was added. The reaction was kept at room temperature for 2 days and the solution was filtered. The solid support was washed with acetonitrile, treated with a 10% acetic anhydride solution in pyridine for 15 min and washed with acetonitrile and DCM. Loading was determined by measuring the DMT absorbance released in a small aliquot of the support and was 0.033 mmol g^{-1}.

Oligonucleotide synthesis

The following sequences were prepared on an Applied Biosystems automatic DNA synthesizer on a 1 μmol scale. Sequences A–B and E–H were prepared using standard 2-cyanoethyl phosphoramidites and the appropiate T^{tri} phosphoramidite. Sequences A–D were prepared using *t*-butylphenoxyacetyl (Expedite®) amino protected 2-cyanoethyl phosphoramidites and the appropriate T^{bn} phosphoramidite. During the preparation of oligonucleotide B two capping A solutions were tested: the commercially available acetic anhydride and a 5% *tert*-butylphenoxyacetyl anhydride solution in tetrahydrofuran. Oligonucleotides C and D were prepared using a *t*-butylphenoxyacetyl anhydride solution as capping A.

A) 5' GCT^{tri}AGC 3' 6 mer
B) 5' GCAATGGAT^{tri}CCTCTA 3' 15 mer
C) 5' GCAATGGAT^{bn}CCTCTA 3' 15 mer
D) 5' TCCCAGTCACGACGT^{bn} 3' 15 mer
E) 5' GCAATGGATCCTCTA 3' 15 mer
F) 5' GCAATGGACCCTCTA 3' 15 mer
G) 5' TAGAGGATCCATTGC 3' 15 mer
H) 5' TAGAGGGTCCATTGC 3' 15 mer
I) 5' TAGAGGCTCCATTGC 3' 15 mer
J) 5' TAGAGGTTCCATTGC 3' 15 mer

Oligonucleotides E–J were deprotected with concentrated aqueous ammonia using published protocols.[17] Oligonucleotides A–D were deprotected by treatment of the supports with a solution of benzyl alcohol:DBU:dioxane (45:10:45) at 50 °C for 3 days. The deprotection mixtures were cooled, neutralized with acetic acid, filtered and washed with water. The combined filtrates were concentrated to dryness until a viscous oil was obtained. The residual products were dissolved in water (4 mL) and benzyl alcohol was extracted three times with diethyl ether. The resulting aqueous layers were desalted on a Sephadex G-10 column eluted with 20 mM triethylammonium acetate aqueous solution. Finally, oligonucleotide fractions were analyzed and purified by HPLC (see Figs 1–3).

Oligonucleotide B prepared with *t*-BPA protecting groups was also treated with ethanol:DBU:dioxane (45:10:45) at room temperature for 3 days to obtain the 15 mer with O^4-ethylthymidine. Work-up and purification was performed as described above for T^{bn} oligonucleotides.

Purified oligonucleotides were obtained in the following yields: oligonucleotide A using standard phosphoramidites and T^{tri} (1 μmol): 13 O.D.; oligonucleotide A using *t*-BPA phosphoramidites and T^{bn} (0.2 μmol): 2.2 O.D.; oligonucleotide B: the desired product was not obtained; oligonucleotide C (*t*-BPA, T^{bn}, 1 μmol): 12 O.D.; oligonucleotide D (*t*-BPA, T^{bn}, 1 μmol): 13 O.D.; oligonucleotides E–H (standard, 0.2 μmol): 14, 16, 15, 18 O.D., respectively.

The homogeneity of purified polymers was checked by HPLC. The composition of polymers containing O^4-alkyl thymidines was confirmed by hydrolysis with snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis.[12]

HPLC conditions

In all cases solvent A was 20 mM triethylammonium

acetate (pH 7.8) and solvent B was a 1:1 mixture of water and acetonitrile. The following conditions were used: column: Nucleosil 120C18, 10 μm, 250 × 4 mm, flow rate: 1 mL min^{-1}. (A) 10–60% B linear gradient in 20 min for oligonucleotides with T^{bn}; (B) 5–50% B linear gradient in 20 min for oligonucleotides with T^{et} and unmodified oligonucleotides; (C) 5–95% B linear gradient in 40 min for the analysis of enzymatic digestions with T^{bn}. Retention times of nucleosides in conditions C are: dC 6 min, dG 11 min, T 11 min, A 13 min, T^{et} 21 min, T^{bn} 32 min.

Melting studies and CD measurements

Pentadecamer duplexes were made by mixing equimolar amounts of two pentadecamer strands dissolved in 50 mM Tris–HCl buffer pH 7.8, 0.15 N NaCl and 1 mM EDTA. Duplexes were annealed by slow cooling from 80 °C to 4 °C. UV absorption spectra and melting curves (absorbance vs temperature) were recorded in 1 cm path-length cells using a Shimadzu 2100 spectrophotometer having a temperature controller with programmed temperature increase of 1 deg min^{-1}. Melts were run on duplex concentrations of 3–6 μM at 260 nm.

CD spectra were obtained using a Jasco J-710 spectropolarimeter with temperature-controlled cell holder. The same samples from UV melting curves were used for CD measurements after re-annealing as described above. CD measurements were taken at 20 °C with a 1 mm pathlength cell.

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