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Synthesis of DNA Containing Modified Bases by Postsynthetic Substitution. Synthesis of Oligomers Containing 4-Substituted Thymine: O^4-Alkylthymine, 5-Methylcytosine, N^4-(Dimethylamino)-5-methylcytosine, and 4-Thiothymine

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A strategy is described for synthesis of oligomers modified in the 4-position of thymine by postsynthetic substitution. 4-Triazolothymine phosphoramidite monomer has been prepared in one step from thymine amidite monomer and incorporated into a 12 mer AGCGAAXTCGCT using a DNA-synthesizer. The fully protected oligomer containing 4-triazolothymine, while still bound to CPG-support, was treated at 25 °C with either alcohol/DBU, dilute aqueous NaOH, concentrated aqueous ammonia, 1,1-dimethylhydrazine, or thiolacetic acid, to produce essentially pure oligodeoxynucleotides containing O^4-alkylthymine, thymine, 5-methylcytosine, N^4-(dimethylamino)-5-methylcytosine [i.e., 4-(2,2-dimethylhydrazino)-5-methylpyrimid-2-one (T^{DH})], or 4-thiothymine respectively. This first and efficient synthesis of T^{DH} oligomers indicates that this may be a general route to the synthesis of oligomers containing thymine with a reactive group at the 4-position. The melting temperature (Tm) of a DNA duplex containing T^{DH}:G or T^{DH}:A pairs was similar to that of a duplex with A:C mismatch.

Introduction

Because of the importance of modified DNA bases in carcinogenesis, mutation, and the action of some cancer chemotherapeutic agents, there has recently been great interest in synthesis of oligodeoxyribonucleotides containing modified bases.[1,2] In general these oligomers were made by a route in which the modified nucleoside was prepared, converted to the phosphoramidite or phosphotriester monomer, and then incorporated during the synthesis of the oligomer. Each new modified base required the synthesis of a modified monomer suitable for incorporation into DNA, and often a desired modified monomer is not stable under the conditions used in DNA synthesis.

An attractive alternative strategy for synthesis of oligomers containing modified bases is to incorporate a versatile base which combines the properties of stability to the normal procedures of DNA synthesis with sufficient chemical reactivity to allow one to convert it into a number of desirable products after synthesis of the oligomer. The strategy has the following potential advantages: (1) it offers the possibility of making DNA containing a labile or chemically reactive base; (2) a single synthesis of an oligomer containing the versatile base could provide a source of oligomers each containing a different modified base; and (3) special atoms, e.g., NMR sensitive ^{17}O, ^{15}N, ^{13}C, or radioactive ^{35}S, could be introduced by simple treatment with appropriate reagents at the last step.

Because of our particular interest in O^4-alkylthymine (T^{OR}), which plays a prominent role in nitrosamine carcinogenesis[3], we started with 4-substituted thymines. We now describe the synthesis, using phosphoramidite chemistry, of the dodecadeoxyribonucleotide AGCGAAXTCGCT containing 4-triazolothymine (T^{Tri}) and the easy conversion, in high yield, of this into the parent oligomer containing thymine (T), or into five oligomers each containing a different modified base: O^4-methylthymine (T^{OMe}), O^4-ethylthymine (T^{OEt}), 5-methylcytosine (T^{NH2}), N^4-(dimethylamino)-5-methylcytosine [i.e., 4-(2,2-dimethylhydrazino)-5-methylpyrimid-2-one] (T^{DH}), or 4-thiothymine (T^{S}). So far as we know this is the first chemical synthesis of an oligonucleotide containing a base with the chemically active hydrazino side chain, and its synthesis suggests that this strategy may allow the synthesis of oligomers containing other labile bases. Preliminary results of this work have been reported.[4] While this manuscript was in preparation, MacMillan and Verdine[5] suggested a similar strategy. They incorporated 4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridine into oligomers, deprotected the oligomers, and then substituted them with various amines.

4-Triazolothymidine was introduced by Reese and Skone[6] as a precursor for the synthesis of 4-substituted thymidines. It is easily and quantitatively produced and has previously been used for synthesis of O^4-alkylthymine

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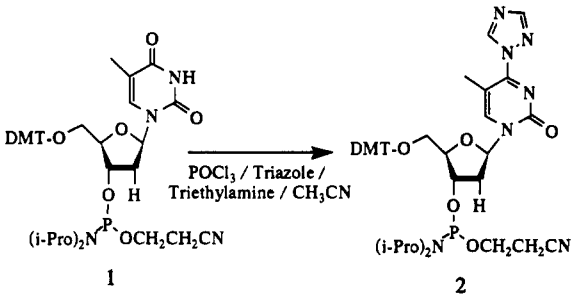
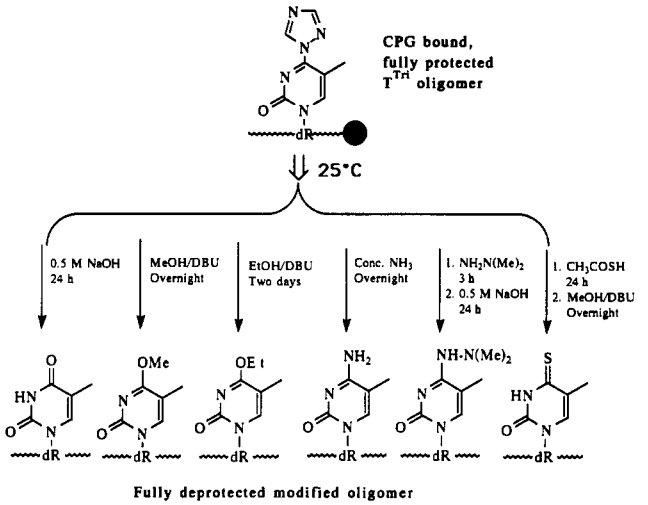
Scheme I



Scheme II



phosphoramidite monomers to be used in oligonucleotide synthesis,[7] and very recently we have found that it can be easily converted into 4-thiothymidine at room temperature with thiolacetic acid.[8] Two previous papers have also reported the preparation of oligomers containing 4-triazolothymine with the subsequent transformation of these to oligomers with a new 4-modified base.[9,10] Webb and Matteucci[9] used it to synthesize oligomers containing 5-methyl-N^4,N^4-ethanocytosine. The acid-labile ethano group was successfully introduced after removal of the 4,4'-dimethoxytriphenylmethyl (DMT) group protecting the 5'-OH, but there was then great difficulty in removing the protecting groups from the bases. The 9-fluorenylmethoxycarbonyl (Fmoc) group was successfully removed from adenine and cytosine, but the Fmoc group was found unsuitable for guanine and it was impossible to make oligomers containing both guanine and the ethanocytosine. Similarly Fernandez-Forner et al.[10] have used it to make oligomers containing O^4-ethylthymine, but again had considerable difficulty deprotecting the final product. The experience of these workers indicates that for the strategy to be successful one has to match the deprotection procedure to the nature of the base which has been introduced, and furthermore one has to have an analytical and preparative technique which will allow the separation of the desired product from the impurities. In this regard we used the labile protecting groups,[11] i.e., the phenoxyacetyl group on adenine and guanine and the isobutyryl group on cytosine, together with ion-exchange chromatography[12] at pH 12 for separation and analysis of the desired products.

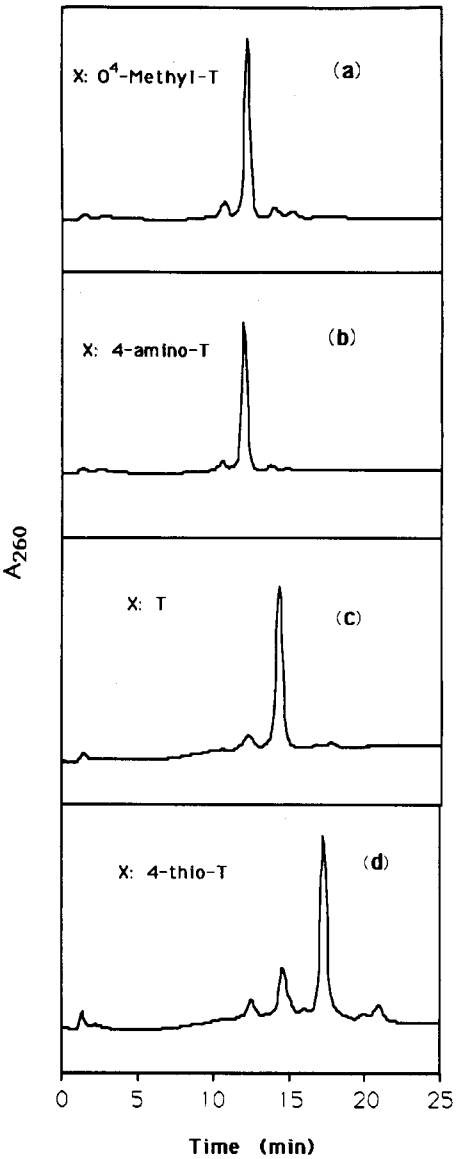


Figure 1. FPLC profiles of crude modified oligomers AGCGAAXTCGCT (X: T^{OMe}, T^{NH2}, T, and T^{S}). The oligomers were chromatographed on a Mono Q column using 0.4 M NaCl, 0.01 M NaOH for 2 min, and then 1.2 M NaCl, 0.01 M NaOH increasing to 15% over 3 min, and then to 40% over the following 20 min at a flow rate of 1 mL/min.

Results and Discussion

4-Triazolothymine phosphoramidite monomer (compound 2) was prepared from commercially available compound 1 (Scheme I) in high yield (97%) as described earlier.[9] The purity assessed by HPTLC and ^{31}P NMR was >95%. The modified monomer was incorporated into the DNA-oligomer with a coupling yield >98%.

As a model, a self-complementary dodecamer AGCGAAXTCGCT was assembled (X standing for 4-triazolothymine), and then the protected oligomer still attached to the controlled-pore-glass (CPG) support was treated at 25 °C with different reagents to substitute 4-triazolothymine in the oligomer to cleave the oligomer from the CPG support and to remove the protecting groups from

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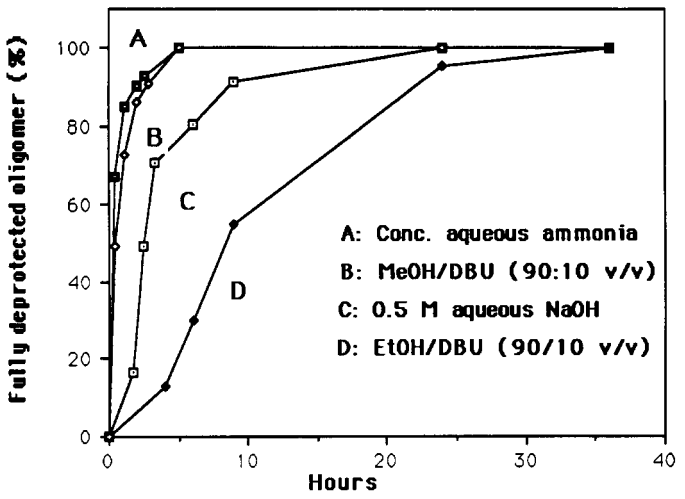

Figure 2. Time courses of deprotection of an oligomer AGCGAAXTCGCT (X: T^{Tri}) protected with PAC groups. The CPG support bearing the protected T^{Tri} 12 mer, with the 5'-DMT removed, was treated at 25 °C with concd ammonia, MeOH/DBU, EtOH/DBU, or 0.5 M aqueous NaOH, respectively. The course of the deprotection was followed by FPLC on a mono Q column.

the oligomer, thus to produce modified oligomers (Scheme II). Although we were not able to check the stability of compound **2** during oligonucleotide synthesis, comparison of the chromatographic profile of the 12 mer containing O^4-methylthymine (Figure 1a) or 5-methylcytosine (Figure 1b) with that of the oligomer containing thymine (Figure 1c) (all of these oligomers were produced by postsynthetic substitution) shows that the 4-triazolothymine residue is sufficiently stable during the synthesis of the oligomer.

The conversion of the 4-triazolothymine (T^{Tri}) oligomer into the modified base oligomer involves two major reactions: substitution and deprotection. Experiments were done to follow the progress of these reactions and to discover the optimum conditions for the subsequent synthesis of oligomers.

a. O^4-Methylthymine. The fully protected T^{Tri} 12 mer was exposed to MeOH/DBU at 25 °C. The cleavage from the CPG support and the deprotection are complete within 5 h (Figure 2B), and the substitution was finished within 12 h. Therefore, overnight MeOH/DBU treatment was generally employed in the production of the methylated oligomer. This gave essentially pure T^{OMe} oligomer (Figure 1a). Base analysis of the main product confirmed the correct composition.

b. O^4-Ethylthymine. Because ethoxide is less nucleophilic than methoxide, a more prolonged exposure to EtOH/DBU at 25 °C is needed. A lipophilic counterion, cetyltrimethylammonium, was added to the reaction mixture to keep the partially deprotected oligomer in solution.[7] Less than 10% of the T^{Tri} residue was left unsubstituted after 12 h, and the substitution was finished within 24 h. All protecting groups were cleaved within 36 h (Figure 2D), so 2 days of EtOH/DBU treatment was generally employed. An essentially pure oligomer was easily obtained, and the correct composition was confirmed by base analysis. It is worth mentioning that most commercial anhydrous ethanol contains quite a high percentage of methanol. This must be avoided otherwise methylated oligomers will be produced to contaminate ethylated products.

We have previously synthesized oligomers containing T^{OMe} or T^{OEt} by incorporation of either T^{OMe} or T^{OEt} monomer[7] but the present method can provide T^{OMe} and T^{OEt} oligomers in a single synthesis without the need to prepare T^{OMe} and T^{OEt} monomers. Comparison of our previous results,[7] and the results reported now, with those of Fernandez-Forner et al.[10] shows the advantage of the labile base-protecting groups used here. Fernandez-Forner et al. showed that the conventional base protecting groups (benzoyl on adenine and cytosine and isobutyryl on guanine) are unsuitable for the synthesis of oligomers containing O^4-ethylthymine, and although they employed a new protecting group (*p*-nitrophenylethyl) for this purpose it also was very difficult to remove.

Table I. Yield and Purity of Synthetic Oligomers[a]

oligomer	scale	yield (OD)	purity (%)
AGCGAATTCGCT	$^1/_5 \times 1$ μmol	12.1	>90
AGCGAATTCGCT	0.2 μmol	14.0	>90
---AT^{OMe}---	$^1/_5 \times 1$ μmol	12.4	>90
---AT^{OEt}---	$^1/_5 \times 1$ μmol	10.6	>90
---AT^{NH2}---	$^1/_5 \times 1$ μmol	12.4	>90
---AT^{NH2}---	0.2 μmol	7.0	>90
---AT^{DH}---	$^1/_5 \times 1$ μmol	8.0	60
---AT^{S}---	$^1/_5 \times 1$ μmol	9.3	70

[a] The full sequence is shown for the parent dodecamer. Only the central dimer containing the modified thymine is shown for the modified oligomers. Yield refers to the amount of oligomer (A_{260} units) recovered from the Nensorb cartridge. Purity is the purity, estimated from the FPLC profile, of the oligomers recovered from the Nensorb cartridge. The $^1/_5 \times 1$ μmol scale syntheses were carried out by substitution of $^1/_5$th of the contents of a 1 μmol cartridge containing a synthetic 4-triazolothymine oligomer. The 0.2 μmol scale syntheses were done for comparison, and in these only commercially available monomers were used.

c. Thymine. Treatment of the T^{Tri} 12 mer with 0.5 M aqueous NaOH at 25 °C for 24 h was sufficient for cleavage of the oligomer from the CPG support, for substitution of the triazolo group, and for deprotection of the other bases (Figure 2C). Base analysis of the main product (Figure 1c) gave only four unmodified bases (dA, dG, dC, and T), and a more prolonged exposure (3 days) to 0.5 M NaOH caused no obvious destruction of the oligomer. Only deprotected thymine-containing oligomer and no deprotected 4-triazolothymine-containing oligomer was formed during the course of the reaction. Although oligomers containing thymine can be easily prepared from thymine monomer, this substitution allows a reference sample containing thymine to be obtained during the same synthesis as the modified oligomer and would also allow ^{17}O to be introduced from ^{17}O-water for NMR studies.

d. 5-Methylcytosine. The T^{Tri} 12 mer was treated with concd aqueous ammonia (overnight, 25 °C), which cleaved the oligomer from the CPG support, substituted the 4-triazolo group of the base, removed the protecting groups, and gave pure 5-methylcytosine-containing oligomer (Figure 1b). The correct composition of the oligomer was confirmed by base analysis. Deprotection is complete within 5 h (Figure 2A), and the longer treatment (overnight) was adopted simply because it is routinely used for deprotecting unmodified oligomers. Although 5-methylcytosine monomer is commercially available, the present procedure can provide 5-methylcytosine-containing oligomer as well as the parent containing thymine in a single synthesis. Furthermore, it is possible to introduce NMR-sensitive ^{15}N from easily available ^{15}N-ammonia. Comparison of the oligomer containing 5-methylcytosine made from the T^{Tri} oligomer with the same sequence made from commercial 5-methylcytosine monomer shows that the purity was similar, but the yield from the T^{Tri} oligomer was slightly better (Table I).

e. N^4-(Dimethylamino)-5-methylcytosine [4-(2,2-Dimethylhydrazino)-5-methylpyrimid-2-one]. This was chosen as an example of the application of this strategy to the synthesis of oligomers containing labile amino de-

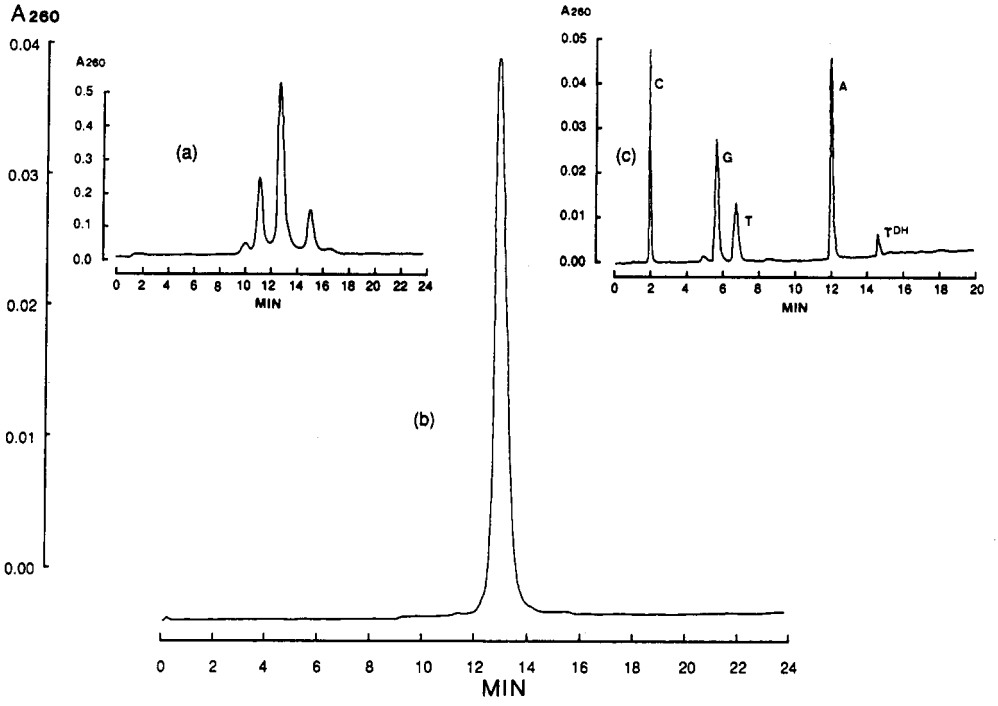

Figure 3. FPLC profiles of crude (a) and purified (b) AGCGAAT^{DH}TCGCT and HPLC base analysis (c) of the purified oligomer. FPLC was performed on a Mono Q column using 0.4 M NaCl, 0.01 M NaOH for 2 min, then 0.8 M NaCl, 0.01 M NaOH increasing to 30% over 3 min, then to 70% over the following 20 min at a flow rate of 1 mL/min. HPLC was performed on an 8MBC18 10-μm reversed-phase column using 0.05 M KH_2PO_4 (pH 4.5) and 0.05 M KH_2PO_4 (pH 4.5) containing 33% CH_3CN at a flow rate of 3 mL/min.

rivatives in the 4-position of pyrimidines. To our best knowledge, no paper has previously been published describing the chemical synthesis of an oligonucleotide containing such a labile hydrazino derivative.

The T^{Tri} 12 mer was treated with 1,1-dimethylhydrazine/CH_3CN for 3 h at 25 °C to substitute the triazolo group. Because the substituted 12 mer was still attached to the CPG support, the excess 1,1-dimethylhydrazine could be washed off with CH_3CN, and then the oligomer was cleaved from the CPG support and deblocked with 0.5 M aqueous NaOH for 24 h at 25 °C. This gave the desired oligomer (t_R = 13 min) (Figure 3a and b). The yield was slightly less than those of the other oligomers (Table I). One possible explanation is that during the substitution step the dimethylhydrazine, as a weak base, might slightly cleave the oligomer from the CPG support and this cleaved oligomer was washed off before the deprotection step. Because this is the first report of chemically synthesized oligomer containing a hydrazino derivative, the HPLC profile of the nucleoside digest is also presented (Figure 3c). As well as the four peaks corresponding to the four unmodified nucleosides (dA, dG, dC, and T) there was one peak which coeluted with authentic 1-(2′-deoxyribofuranosyl)-4-(2,2-dimethylhydrazino)-5-methylpyrimid-2-one. Aqueous NaOH was employed rather than concd NH_3 for deblocking because NaOH converts any remaining 4-triazolothymine into thymine, and the thymine-containing oligomer (t_R = 15 min) can be separated easily from the desired modified oligomer (t_R = 13 min) by FPLC (Figure 3b). From the results described above, we believe that other amino derivatives could be introduced in a similar way.

f. 4-Thiothymine. The T^{Tri} 12 mer was treated with CH_3COSH/CH_3CN at 25 °C, which replaced the triazolo group with an SH group.[8] Interestingly, this treatment does not remove the 5′-DMT group, which is particularly useful for later separation of failure sequences from desired oligomer. Thiation was finished within 12 h, but overnight treatment with CH_3COSH/CH_3CN was generally used. As the oligomer was still attached to the CPG support, the excess CH_3COSH was washed off with CH_3CN. For deprotection, either MeOH/DBU or concd aqueous NH_3 can be used. In agreement with a previous report[2] we observed that the 4-thiothymine in the dodecamer was converted to 5-methylcytosine by prolonged exposure to concd aqueous NH_3. Therefore, MeOH/DBU (16 h, 25 °C) is preferred (Figure 1d). This conversion, at the oligomer level, of thiothymine into other modified thymine derivatives by nucleophiles, like ammonia, may be useful for preparing olgomers containing other 4-modified pyrimidines, for example, those containing an aziridine group. The present method offers an easy way to produce T^S oligomers with a good yield and without the need to prepare a 4-thiothymine monomer.

Comparison of these results with the two previous reports[2,13] shows the advantages of the postsynthetic substitution strategy. Originally, T^S oligomers were prepared by incorporation of a 4-thiothymine phosphoramidite monomer in which the sulfur atom was protected with a methylsulfenyl (-SCH_3) group.[2] Unfortunately, this monomer was not very stable toward some of the reagents used in oligonucleotide synthesis and there was only a moderate yield (10–15%) of T^S oligomer. For this reason the same authors very recently used the *p*-nitrophenyl group to protect the sulfur.[13]

For purification, the resulting T^{NH2}, T^{DH}, or T oligomers with DMT groups still remaining on their 5′ ends were passed through a Nensorb Prep cartridge to remove the failure sequences which have no DMT groups on their 5′ ends, and the resulting T^{OR} or T^S oligomers with DMT groups still remaining on their 5′ ends were passed through Dowex ion-exchange column to remove DBU, followed by Nensorb Prep cartridge to remove the failure sequences. These steps result in reasonably pure products (cf. Figures 1 and 3) with high yields (Table I). More highly purified

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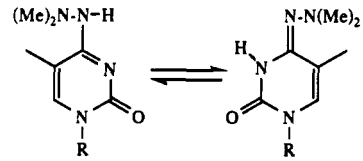
Scheme III



Amino form Imino form

oligomers could be obtained by FPLC. Under basic conditions, the oligomers containing O^4-alkylthymine (Figure 1a), 5-methylcytosine (Figure 1b), or N^4-substituted 5-methylcytosine (Figure 3a) were eluted earlier than their parent oligomer containing thymine (Figure 1c) because they have one less negative charge due to lack of an imino proton at the 3-position.[12] Because 4-thiothymine has a lower pK_a than thymine,[14] the T^S oligomer (Figure 1d) was eluted later and easily separated from the parent containing thymine. The nucleoside O^4-methylthymidine is very slowly converted to thymidine in alkaline solution,[7] but the O^4-alkylthymine residue in the oligomer is more resistant to destruction by alkali, perhaps because of steric hindrance by the neighboring nucleotides. This allows one to use FPLC under basic conditions for the purifications. After purification followed by immediate neutralization, the purified oligomer was checked again by FPLC and still gave a single peak.

By combination of the direct base substitution at the oligomer level and separation by FPLC under basic conditions, we are able to provide a general and easy method for synthesis and purification of oligodeoxyribonucleotides containing different modified thymines avoiding the tedious steps of preparation of modified monomers. The method also provides potential for preparation of oligomers containing modified bases which are not stable during the assembly steps and for easily introducing atoms such as radioactive ^{35}S from ^{35}S-thiolacetic acid or NMR sensitive atoms such as ^{13}C from ^{13}C-methanol, ^{15}N from ^{15}N-ammonia, and ^{17}O from ^{17}O-water.

Base-Pairing Properties of Oligomers Containing N^4-(Dimethylamino)-5-methylcytosine [4-(2,2-Dimethylhydrazino)-5-methylpyrimidin-2-one] (T^{DH}). T^{DH} was made because it was expected to exist in about equimolar proportions of the amino and imino form (Scheme III) and thus might base-pair equally with adenine or guanine. This expectation was based on the observation that in the series cytosine, N^4-aminocytosine, and N^4-hydroxy(or N^4-methoxy)cytosine the ratio of amino to imino form is 10^4, 10–0.1 (depending on solvent), and 0.1–0.03, respectively, presumably reflecting increasing electronegativity in the series H, NH_2, and OH (or OCH_3).[15-17] Little is known in this respect about DNA containing N^4-aminocytosine[18] or N^4-(alkylamino)cytosine because the necessary oligomers have not been available. For this reason we developed the method for preparation of T^{DH} oligomers. However, melting temperature studies on a self-complementary 12-mer AGCGAAT^{DH}TCGCT in 0.1 M Hepes pH 7.5, 0.2 M NaCl and 0.02 M $MgCl_2$ solution, did not show a distinct transition temperature indicating that the self-complementary oligomer cannot form double-stranded DNA, or even a hairpin structure, under these experimental conditions. Non-self-complementary duplexes containing T^{DH} paired to guanine or to adenine had sharp transition temperatures like the controls containing G:C or A:T pairs, but in both cases the Tm was 17–19 °C lower than the control (Table II). The depression of Tm was nearly equal to the effect of mismatch base-pairing (Table II). This indicates that T^{DH} is not a good substitute for either thymine or cytosine. Possibly this is a consequence of steric hindrance by the dimethylamino group.

Table II. Tm Values of DNA Duplexes Containing the Modified Base

5' CAG GAA TXC GC 3'
3' GTC CTT AYG CG 5'

X	Y	Tm
T	A	58.7
T^{DH}	A	42
T^{DH}	G	40.6
C	G	59.2
C	A	42

In order to further evaluate the base-pairing properties of T^{DH} a synthetic 20 mer CGCTCTTA-CAT^{DH}GTATCGGAT, was used as template for DNA synthesis by the Klenow fragment of E. coli DNA polymerase I. The ^{32}P labeled complementary strand ATCCGATAC, as primer, was annealed to the template, and Klenow fragment with dATP or dGTP added. Preliminary experiments show that T^{DH} does not block DNA synthesis but the elongation of the primer with both G and A appears to proceed at a rate similar to that previously reported[19] for the mismatch incorporation of G opposite T in the template strand. These results are consistent with the Tm measurement and confirm that T^{DH} does not replace either C or T.

Experimental Section

Chemicals and Enzymes. The CPG-linked monomers and the chemicals used on the synthesizer were obtained from Cruachem (Glasgow, Scotland) and the monomers of (2-cyanoethyl)phosphoramidites 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; 5-methylcytosine (cyanoethyl)phosphoramidite monomer protected with a benzoyl group on the 4-amino position was from Glen Research Corporation. Anhydrous methanol (MeOH, 99+%, Gold Label), 1,1-dimethylhydrazine (98%) (NB this is a suspected carcinogen), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 96%), and thiolacetic acid (96%) were from Aldrich. Absolute ethanol (99.7+%, Analar) and concd aqueous ammonia (d = 0.880, Aristar) were from BDH. Acetonitrile (HPLC Grade, Rathburn) was dried with molecular sieves (4A) at least overnight. All other chemicals were from either Aldrich or Sigma. All chemicals and solvents, unless stated otherwise, were used directly without further purification. The water content of anhydrous solvents was checked by Karl Fischer titration. Snake venom phosphodiesterase I (*crotalus durissus*) was from Sigma and alkaline phosphatase from Boehringer Mannheim.

Chromatography and Purification. Reversed-phase HPLC for base analysis was carried out on a Gilson 320, with a 620 Datamaster for integration and Shimadzu SPD6A UV spectrophotometetric detector, using a Waters 8MBC18 10μ column. Gradients were formed from 0.05 M aqueous KH_2PO_4 (pH 4.5) (buffer A) and 0.05 M aqueous KH_2PO_4 (pH 4.5) containing 33% CH_3CN (buffer B) at a flow rate of 3 mL/min. Fast protein liquid

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chromatography (FPLC) was carried out on a Dionex BIOLC system with a Dionex variable-wavelength detector using a Pharmacia mono Q HR5/5 column. Gradients were formed from 0.4 M NaCl, 0.01 M NaOH, aqueous solution (pH 12) (buffer C) and 0.8 M NaCl, 0.01 M NaOH, aqueous solution (pH 12) (buffer D) or 1.2 M NaCl, 0.01 M NaOH, aqueous solution (pH 12) (buffer E) at a flow rate of 1 mL/min. High-performance thin-layer chromatography (HPTLC) was carried out on Merck Kieselgel 60 F_{254} aluminum-backed TLC sheets developed with 2.5% $CH_3OH/CHCl_3$. Nensorb Prep Cartridges (NEN Research Products, Du Pont Co., Boston, MA 02118, USA) were used according to the maker's instructions.

Base Analysis. The purity of the oligomers was assessed by analysis of a nucleoside digest. In general, 0.5 A_{260} unit of an oligomer was dissolved in 160 μL of H_2O and 20 μL of 600 mM Tris-HCl, 60 mM $MgCl_2$, pH 8.5. Snake venom phosphodiesterase I (10 μL, 10 μg of protein) was added and the mixture incubated (37 °C, 30 min), and then alkaline phosphatase (10 μL, 5 μg of protein) was added and incubation continued for 30 min. The deoxyribonucleosides were separated by HPLC using 96.5% buffer A and 3.5% buffer B for first 8 min and then with a linear gradient from 3.5% to 50% of buffer B over the following 15 min. The chromatography was monitored at 260 nm, but for the T^S oligomer the first 13 min of the run was monitored at 260 nm for the detection of dC, dG, T, and dA and the remainder at 335 nm for the detection of T^S. The amount of each nucleoside was measured by integration of the absorbance of each peak.[2,7,20] Retention times were as follows: dC, 1.9 min; 5-Me-dC, 3.7 min; dI (from enzymatic deamination of dA), 4.8 min; dG, 5.5 min; T, 6.5 min; dA, 12 min; T^{DH}, 14.8 min; T^{OMe}, 15.2 min; T^S, 15.6 min; T^{Tri}, 15.9 min; T^{OEt}, 19.4 min. Authentic compounds used for reference were prepared as follows: T^{OMe} and T^{OEt} as before;[7] T^S as before;[8] and T^{Tri} as literature.[9] T^{DH} [1-(2'-deoxyribofuransoyl)-4-(2,2-dimethylhydrazino)-5-methylpyrimid-2-one] was prepared as follows: to an CH_3CN (40 mL) solution of 3',5'-*O*-bis(*tert*-butyldimethylsilyl)-4-(1,2,4-triazolo)thymidine (1.02 g, 2 mmol), prepared as before,[7] was added 1,1-dimethylhydrazine (600 μL, 10 mmol) at room temperature. After the mixture was stirred overnight, TLC ($CH_3OH/CHCl_3$ (5:95)) showed that most of the starting material had disappeared. Additional 1,1-dimethylhydrazine (400 μL) was added, and stirring continued for 5 h. TLC showed that besides the main spot (R_f 0.5), there were two UV absorbing spots (R_f 0.7, small amount, and R_f 0.25, tiny amount). The reaction solution was concentrated and the residue diluted with ethyl acetate (100 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 100 mL) and then with saturated aqueous NaCl. The organic layer was dried over Na_2SO_4, evaporated into an oily residue, and purified by silica gel column. The compound with R_f 0.7 was found to be 3',5'-bis(*tert*-butyldimethylsilyl)thymidine, and the main compound (R_f 0.5) was confirmed to be 1-(2'-deoxy-3',5'-*O*-bis(*tert*-butyldimethylsilyl)ribofuranosyl)-4-(2,2-dimethylhydrazino)-5-methylpyrimid-2-one by NMR spectroscopy: ^1H NMR data (in DMSO-d_6) 0.07 (2 s, 12 H, 3'- and 5'-$Si(CH_3)_2R$), 0.86 (2 s, 18 H, 3'- and 5'-$Si(R)_2(CH_3)_3$), 2.04 (m, 2 H, 2'- and 2''-H), 2.10 (s, 3 H, 5-CH_3), 3.05 (s, 6 H, $NN(CH_3)_2$), 3.73 (m, 2 H, 5'-H), 3.79 (m, 1 H, 4'-H), 4.33 (m, 1 H, 3'-H), 6.14 (t, 1 H, 1'-H), and 7.37 (s, 1 H, 6 H). This compound was desilylated with tetrabutylammonium fluoride in tetrahydrofuran,[21] and the resulting nucleoside (T^{DH}) was purified by silica gel column and crystallized from acetone/methanol (90/10, V/V): ^1H NMR data (in DMSO-d_6) 1.95–2.07 (m, 2 H, 2'- and 2''-H), 2.11 (s, 3 H, 5-CH_3), 3.06 (s, 6 H, $NN(CH_3)_2$), 3.45–3.59 (m, 2 H, 5'-H), 3.71 (m, 1 H, 4'-H), 4.27 (m, 1 H, 3'-H), 6.09 (t, 1 H, 1'-H), and 7.75 (s, 1 H, 6 H); UV (MeOH) λ_{max} 289, $A_{260/289}$ 0.653; mp 197 °C.

DNA Synthesis on a Template Containing N^4-(Dimethylamino)-5-methylcytosine [4-(2,2-Dimethylhydrazino)-5-methylpyrimidin-2-one] (T^{DH}).

5'-^{32}P Labeling. A mixture (20 μL) containing 10 μM primer DNA (5'-ATCCGATAC-3'), 10 mM $MgCl_2$, 0.5 μM [γ-^{32}P]-ATP (3000 Ci/mmol), and 4 units of T_4 polynucleotide kinase in 50 mM Tris-HCl (pH 7.4) was incubated at 37 °C for 30 min.

Duplex Preparation. A solution (100 μL) containing 1 μM 5'-end-labeled primer DNA, 9 μM cold primer DNA, 10 μM cold template DNA (5'-CGCTCTTACAT^{DH}GTATCGGAT-3'), 5 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.4) was hybridized by heating at 100 °C for 2 min and then cooling to room temperature over 2 h.

Elongation and Electrophoresis. A reaction mixture (5 μL) containing 1 mM duplex, 150 μM dNTP (dATP or dGTP), 5 mM $MgCl_2$, and 0.2 μM E. coli DNA polymerase I (Klenow fragment) in 50 mM Tris-HCl (pH 7.4) was incubated at room temperature for 30 s and quenched directly with 5 μL of denaturing gel loading buffer (80% formamide and 0.1% each of bromophenol blue and xylene cyanol FF in electrophoresis buffer). Aliquots (2 μL) were then loaded on a 20% polyacrylamide gel (32 × 18 × 0.06 cm) containing 7 M urea. Electrophoresis was carried out at 2000 V, 30 W, for 2 h. The gel was autoradiographed and the amount elongated determined by cutting the gel into sections followed by scintillation counting in 3 mL of scintillation fluid.

Melting Curve Measurement. Non-self-complementary oligomers (Table II) were annealed with an equimolar quantity of each complementary strand in 0.1 M Hepes, pH 7.5, 0.2 M NaCl, and 0.02 M $MgCl_2$. The temperature-dependent change in absorbance at 260 nm was followed using a CARY3 spectrophotometer connected to a Cary temperature controller (Varian Techtron Pty Ltd, Australia). The temperature was increased by 1 °C/min. The Tm values were determined as the maximum values of the first derivative graph of the absorbance vs temperature graph.

Preparation of T^{Tri} Monomer: 5'-*O*-[(4,4'-Dimethoxytriphenyl)methyl]-4-triazolothymidine 3'-*O*-(2-Cyanoethyl (*N*,*N*-diisopropylamino)phosphoramidite) (2). Compound 2 was prepared by modification of a previous procedure.[9] 1,2,4-Triazole (1.38 g, 20 mmol) was suspended in dry CH_3CN (25 mL) at 0 °C (ice bath), and 0.4 mL of $POCl_3$ was slowly added with rapid stirring. Triethylamine (3 mL) was added dropwise and the suspension left stirring for 30 min. 5'-*O*-[(4,4'-Dimethoxytriphenyl)methyl]thymidine 3'-*O*-(2-cyanoethyl (*N*,*N*-diisopropylamino)phosphoramidite) (250 mg, 0.335 mmol, compound 1) in dry CH_3CN (5 mL) was added over 20 min and stirring continued for 1 h. The reaction was stopped with saturated aqueous $NaHCO_3$ (30 mL) and then extracted with CH_2Cl_2 (50 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (30 mL) and saturated aqueous NaCl (30 mL) and then dried (Na_2SO_4), evaporated under reduced pressure into a small volume, coevaporated with toluene twice, and then precipitated from toluene into cold *n*-pentane (salt–ice bath). The resulting white precipitate, washed twice with fresh *n*-pentane, was dissolved in anhydrous benzene and lyophilized to give a white powder (260 mg, 97%). Its purity was checked by ^{31}P NMR (149.45 and 150.13 ppm in $CDCl_3$) and by TLC in 2.5% $CH_3OH/CHCl_3$ on HPTLC aluminum sheets (Silica gel 60 F_{254}, from E. Merck) which showed only two bright spots (phosphoramidite steric isomers) with R_f's (0.25 and 0.37) differing from the starting material (compound 1, usual UV absorbing spots) with R_f's (0.35 and 0.42).

Synthesis of Oligodeoxyribonucleotides. Oligonucleotides were synthesized on a Cruachem PS200 automatic DNA synthesizer (Cruachem Ltd., Glasgow, Scotland) using PAC amidities of the normal bases (see above). The portion of the oligonucleotide 3' to the 4-triazolothymine was synthesized on the machine, and then the T^{Tri} monomer was added manually. In both 1.0- and 0.2-μm scale 10 mg of compound 2 in a 2-mL conical glass vial with septum top (Wheaton reactivial) was dissolved in 0.1 mL of anhydrous CH_3CN 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 the 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, and 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. For comparison, a 5-methylcytosine-containing oligomer was also synthesized manually from the commercial monomer (Glen Research Corporation) using the same procedure as above. The 5-methylcytosine oligomer was deprotected (concd aqueous NH_3,

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55 °C, overnight) and purified with Nensorb Prep cartridge.

Preparation of Oligomers Containing O^4-Methylthymine (T^{OMe}) and O^4-Ethylthymine (T^{OEt}).

a. Optimization of Substitution. The CPG support (5 mg) bearing the fully protected T^{Tri} 12 mer (AGCGAAT^{Tri}TCGCT) was put into Eppendorf tubes and either MeOH/DBU (1 mL, 9:1, v/v) alone or EtOH/DBU (1 mL, 9:1 v/v) with 10 mg of cetyltrimethylammonium bromide were added to each tube and left at 25 °C, for 12, 24, 48, and 68 h, respectively. The solution was neutralized (90 μL of 50% aqueous acetic acid, 1.5 equiv to DBU) and immediately passed through a Dowex 50 × 8, Na^+ form, 400 mesh ion-exchange column (10 mL wet volume), eluted with water, and collected in 1-mL fractions. The oligomers were usually found in fractions 4–6 by measuring A_{260} nm. The oligonucleotides were separated from failure sequences and the DMT group finally removed using a Nensorb Prep cartridge. The resulting oligomers were hydrolyzed enzymically and the base composition measured (cf. base analysis). The extent of substitution by MeOH or EtOH was assessed by comparison of percentage of 4-triazolothymidine (t_R 15.9 min) with that of O^4-methylthymine (t_R 15.2 min) or with that of O^4-ethylthymine (t_R 19.4 min) using reversed-phase HPLC.

b. Synthesis of O^4-Methylthymine and O^4-Ethylthymine Oligomers. The fully protected T^{Tri} 12 mer described above while still attached to the CPG support was treated with either methanol/DBU or ethanol/DBU at 25 °C overnight or for 2 days, respectively. The solution was neutralized and immediately passed through Dowex 50 as described above. The oligonucleotides were purified with a Nensorb Prep Cartridge (yield in Table I).

Preparation of Oligomers Containing Thymine from the T^{Tri} Oligomer. The fully protected T^{Tri} 12 mer described above while still attached to the CPG support was treated with 0.5 M aqueous NaOH (24 h at 25 °C). This cleaved the oligomer from the CPG support, substituted the 4-triazolothymine, and removed all protecting groups except the DMT group at the 5′ end of the oligomer. The resulting oligomer was purified with a Nensorb Prep cartridge as above (yield in Table I).

Preparation of Oligomers Containing 5-Methylcytosine. The fully protected T^{Tri} 12 mer described above while still attached to the CPG support was treated with concd aqueous ammonia (d = 0.880, overnight, 25 °C). This cleaved the oligomer from the CPG support, substituted the 4-triazolothymine, and removed all protecting groups except 5′-DMT. The resulting oligomer was purified with a Nensorb Prep cartridge (yield in Table I).

Preparation of Oligomers Containing N^4-(Dimethylamino)-5-methylcytosine (T^{DH}). The fully protected T^{Tri} 12 mer described above while still attached to the CPG-support was treated with $NH_2N(Me)_2/CH_3CN$ (10/90, v/v, 3 h, 25 °C) to substitute the 4-triazolothymine. As the oligomer was still attached to the CPG support, it can be washed with CH_3CN (5 × 1 mL) to completely remove 1,1-dimethylhydrazine. The oligomer was then cleaved from the CPG support, and all protecting groups except 5′-DMT were removed with aqueous 0.5 M NaOH (1 mL, 24 h, 25 °C). The resulting oligomer was purified with a Nensorb Prep cartridge (yield in Table I). The FPLC profile of the product and the HPLC of the nucleoside digest is shown in Figure 3.

Preparation of Oligomers Containing 4-Thiothymine.

a. Optimization of Thiation. To determine the optimum condition for thiation, 0.5 mL of CH_3COSH/CH_3CN (10/90, v/v) was added to each of four Eppendorf tubes containing 3 mg of the CPG support bearing the protected T^{Tri} 12 mer, with the 5′-DMT removed, and left at 25 °C for 4, 8, 12, and 24 h, respectively. Then each sample was washed with CH_3CN (5 × 1 mL) and treated with MeOH/DBU (0.5 mL, 90/10, v/v) overnight to cleave the oligomer from the CPG support and to remove the protecting groups. The products were separated by FPLC and the extent of thiation was calculated by comparing the integrated absorbance (at 260 and 335 nm) of the T^S oligomer with that of T^{OMe} oligomer which had been formed from substitution of T^S and of any remaining T^{Tri} during the deprotection.

b. Synthesis of Oligomers Containing 4-Thiothymine. The fully protected T^{Tri} 12 mer described above while still attached to the CPG support was treated with CH_3COSH/CH_3CN (1 mL, 10 %, v/v) for 24 h to convert 4-triazolothymine in the oligomer into 4-thiothymine. As the oligomer was still attached to the CPG support, it can be washed with CH_3CN (5 × 1 mL) to completely remove CH_3COSH. MeOH/DBU (1 mL, 90/10, v/v) was added and left overnight for deprotection. The resulting product was then purified as described above for the T^{OMe} oligomer (yield in Table I).

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Registry No. 1, 98796-51-1; 2, 101712-10-1; AGCGAAT^{Tri}CGCT, 141344-58-3; CGCTCTTACAT^{DH}GTATCGGAT, 141507-63-3; ATCCGATAC ^{32}P labeled, 141344-59-4; AGCGAATTCGCT, 130583-08-3; AGCGAAT^{OMe}TCGCT, 130583-09-4; AGCGAAT^{OEt}TCGCT, 130583-10-7; AGCGAAT^{NH_2}TCGCT, 141344-60-7; AGCGAAT^{DH}TCGCT, 141344-61-8; AGCGAAT^STCGCT, 141096-11-9; 5′CAGGAATTCGC3′·3′GTCCTTAAGCG5′, 141076-26-8; 5′CAGGAATT^{DH}CGC3′·3′GTCCTTAAGCG5′, 141374-93-8; 5′CAGGAATT^{DH}CGC3′·3′GTCCTTAGGCG5′, 141344-64-1; 5′CAGGAATCCGC3′·3′GTCCTTAGGCG5′, 141344-66-3; 5′CAGGAATCCGC3′·3′GTCCTAAGCG5′, 141344-67-4; DNA polymerase, 9012-90-2; triazole, 288-88-0.