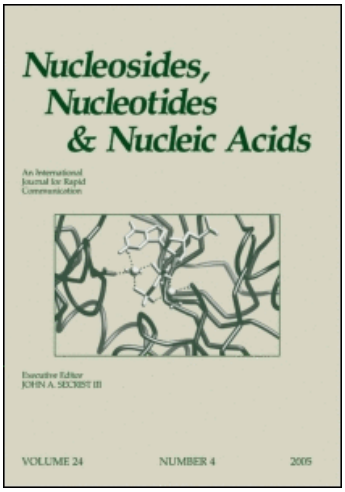

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Solid Phase Synthesis of Oligodeoxynucleotides Containing O^6-Alkylguanine and O^4-Alkylthymine

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SOLID PHASE SYNTHESIS OF OLIGODEOXYNUCLEOTIDES CONTAINING O6-ALKYLGUANINE AND O4-ALKYLTHYMINE

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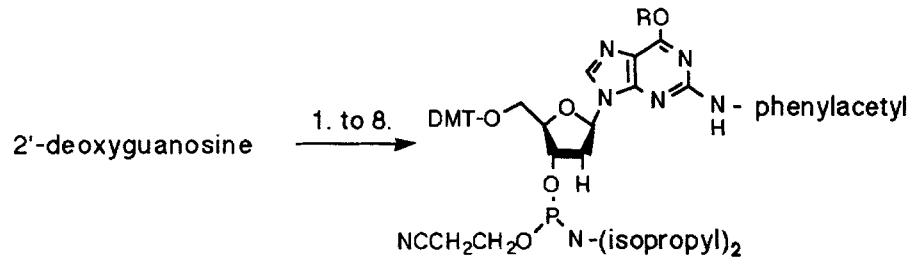
ABSTRACT: Solid phase synthesis of pure oligodeoxynucleotides containing O6-alkylguanine and O4-alkylthymine are described. 4-triazolothymine has been incorporated into oligomers and by choice of the appropriate alkoxide or of ammonia these oligomers can be deprotected and the triazolide converted into the O4-alkylthymine or 5-methylcytosine in a single step.

O6-alkylguanine and O4-alkylthymine are produced in DNA by the carcinogenic N-nitroso compounds and are believed to play a crucial role in their carcinogenicity[1,2]. The chemical synthesis of oligodeoxynucleotides containing these alkylated bases presents two major problems. First the monomer has to be made, then any difficulties consequent upon the susceptibility of the modification to the reagents of synthesis or deprotection have to be overcome. Here we wish to report simple methods to synthesize oligodeoxynucleotides containing these alkylated bases.

O6-alkyldeoxyguanosine could be made by direct alkylation of the nucleoside, but yield is so low as to be impractical. Gaffney et al[3] prepared O6-methyldeoxyguanosine with sulphonation of O6 of deoxyguanosine, followed by successive displacement with an amine, then with alkoxide ion. We have used a similar route to the phosphoramidite monomer (scheme 1).

For the synthesis of oligodeoxynucleotides the N2- of guanine is usually protected with isobutyryl but as the alkyl group on O6 of guanine greatly stablizes the protecting group on N2 severe conditions are required to remove the isobutyryl group (eg. exposure to ammonia at 65°C for 72 hr)[4]. This prolonged exposure can convert part of the alkylguanine to 2,6-diaminopurine. To avoid this problem we have used the phenylacetyl group for protection of the N2 position of O6-alkylguanine because its half life in ammonia is only 45 min.

We have used the N2-phenylacetyl protected monomer for the mechanised solid phase synthesis of oligomers by the phosphoramidite method[5]. Both methyl and cyanoethyl groups have been used for protection of the internucleotide phosphorus, but methyl is unsuitable for oligomers containing O6-alkylguanine[4] because the thiophenol used to remove it may remove the 6-O-alkyl group. Therefore we used the cyanoethyl group.



1. acetic anhydride 2. phenylacetic anhydride 3. mesitylene sulphonyl chloride
4. N-methylpyrrolidine 5. ROH/1,8 diazabicyclo[5,4,0]undec -7-ene (DBU)
6. NaOH 7. 4,4'-dimethoxytritylchloride 8. $NCCH_2CH_2OP(Cl)N(isopropyl)_2$

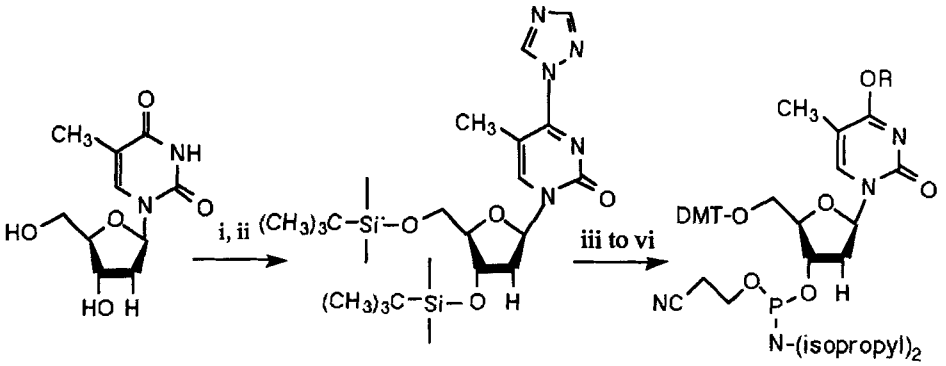
SCHEME I

If the phenylacetyl group is used to protect N^2 of the alkylguanine then the length of exposure to ammonia needed for the deblocking depends on the rate at which the natural bases can be deblocked. We have previously exploited the fact[6] that deprotection is faster if it is done with a mixture of ammonia, *E*-2 nitrobenzaldoxime and tetramethylguanidine than with ammonia alone, but removal of oximate and tetramethylguanidine adds an extra step to the purification which might reduce the recovery of the synthetic oligomer. To avoid this we have recently used monomers of the natural bases in which the N^6 of adenine and N^2 of guanine are protected with the phenoxyacetyl group and the N^4 of cytosine is protected with the isobutyryl group (Pharmacia PAC amidites) when making oligomers containing O^6-alkylguanine. These protecting groups are so labile that all the protecting groups can be removed by treatment with ammonia at room temperature for two days.

Reasonably pure oligomers containing O^6-alkylguanine can be recovered by simply passing the deblocked-trityl on product through a Nen-sorb prep cartridge (Du Pont). Oligomers up to 12 mers can be further purified by reverse phase HPLC and longer oligomers (up to 21 mers) can be well separated from any oligomer that has lost the alkyl group (see Fig 1) by FPLC on monoQ (Pharmacia) at pH12, because as O^6-alkylguanine and O^4-alkylguanine do not have an imino proton they have one less negative charge than guanine or thymine at this basic pH.

The synthesis of oligomers containing O^4-alkylthymine present similar, but more severe, problems to those encountered with O^6-alkylguanine because the O^4-alkyl group is more susceptible to base and to nucleophilic attack[7,8]. However we have been able to evolve a method[9] which will give essentially pure oligomers containing O^4-methyl or O^4-ethylthymine.

We first converted 3',5'-disilylthymidine into its 4-triazolo derivative, followed by substitution with alkoxide ions (the appropriate alcohol and 1,8 diazabicyclo[5,4,0]undec -7-ene (DBU)) to give O^4-alkylthymidine, which after desilylation could be converted into the 5'-trityl-3'-cyanoethylphosphoramidite O^4-alkylthymine monomer in a very good yield (scheme 2).



i. t-butyldimethylsilyl chloride (96% Yield) ii.$POCl_3$/triethylamine/triazole(99%)

iii. ROH/DBU iv. $(Bu)_4N^+F^-$ (60-70% for iii and vi)

v. 4,4'-dimethoxytrityl chloride (80%) vi. $NCCH_2CH_2OP(Cl)N(isopropyl)_2$ (70-80%)

SCHEME 2

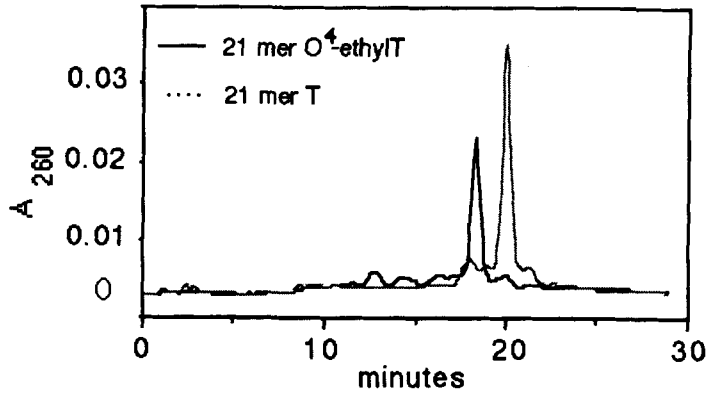


FIGURE 1

Separation of an oligomer containing 4-O-ethylthymine from the control oligomer containing thymine by chromatography on MonoQ at pH 12

This O^4-alkylthymine monomer has been used together with Pharmacia PAC monomers of the naturally occurring bases to synthesize oligomers containing O^4-alkylthymine. Deprotection of these oligomers can be easily achieved with appropriate alcohol/DBU (methanol for methylthymine and ethanol for ethylthymine) for 2 days at room temperature in the presence of a lipophilic counter ion (cetyltrimethylammonium) to increase solubility of the partially deprotected oligomer in alcohol. Oligomers with sufficient purity can usually be obtained by passing the product just through a Dowex anion exchange column, to remove most of the DBU and counter ion, and a Nen-sorb column (Du Pont) . Using this procedure we have made oligomers containing O^4-methyl or O^4-ethylthymine up to 48 bases long.

The observation that 4-triazolothymidine can be easily and quantitatively converted into O^4-alkylthymine by the appropriate alcohol/DBU, and that the same treatment can be used for deprotection of the oligomer suggested the possibility that the conversion of the triazolide and the deprotection might be carried out simultaneously after the synthesis of the oligomer is complete. To test this possibility we have prepared 4-triazolothymine phosphoramidite monomer[10]. It was found that this could be incorporated by a DNA synthesizer into oligomers and a single treatment with ROH/DBU at room temperature for 2 days or with ammonia at room temperature overnight gave essentially pure oligomers containing O^4-methyl, O^4-ethylthymine or 5-methylcytosine (i.e.4-aminothymine). Fig.1 shows a FPLC profile on a Mono Q column (pH 12) of an unpurified 21 mer containing O^4-ethylthymine (GGA CCT CCC TC$^{4\text{-et}}$T TGA CCT GCT) which had been converted from an unpurified 21 mer containing 4-triazolothymine by treatment with 10% DBU in ethanol for 2 days at room temperature. The analogous unpurified oligomer containing thymine is shown as control. This direct conversion at the oligomer level may be of great value in the preparation of oligomers containing thymine with different groups on the 4-position, particularly for those monomers which are difficult to prepare or which are unstable during the synthesis procedure. Currently we are attempting to directly convert 4-triazolothymine into 4-thiothymine at the oligomer level.

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