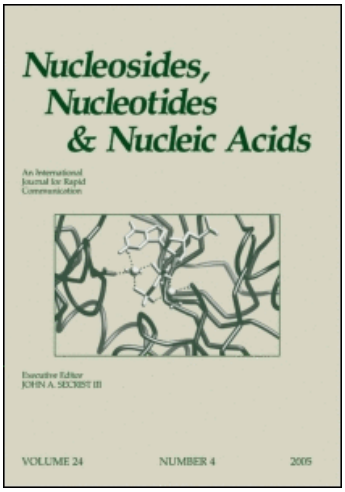

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Synthesis of Oligonucleotides Containing 5,6-Dihydro-5-Azacytosine and 5-Azacytosine at Specific CpG Sites

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SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING 5,6-DIHYDRO-5-AZACYTOSINE AND 5-AZACYTOSINE AT SPECIFIC CpG SITES

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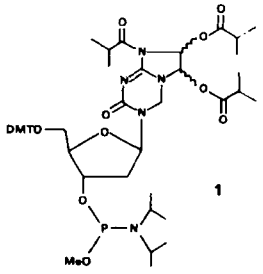
ABSTRACT: The quantitative conversion of dihydro-5-azacytosine (5-DHAC) to 5-azacytosine (5-AC) has been accomplished in a dihydro-5-azacytidine/thymidine dimer (5-DHAC$_p$T). This newly developed procedure allows similar possibilities with longer, 5-DHAC-modified oligodeoxynucleotides.

The hydrolytic instability of the triazine ring in 5-azacytosine (5-AC)-containing nucleosides is very well documented[1,2] and hence use of a conventional phosphoramidite of 2'-deoxy-5-azacytidine in DNA synthesis would be impractical. We have recently reported the synthesis of a hydrolytically stable 2'-deoxy-5,6-dihydro-5-azacytidine phosphoramidite reagent (1) that gives excellent coupling yields in the construction of dihydro-5-azacytosine (5-DHAC)-containing oligodeoxynucleotides.[3] However, the final objective of our investigation is the conversion of the incorporated 5-DHAC moiety into the corresponding 5-AC base. It is anticipated that these oligodeoxynucleotide fragments containing the modified cytosine base (5-AC), at specific CpG sites, will serve as biological tools capable of defining the mechanism of inhibition of DNA methyltransferase and the role that such inhibition plays in the regulation of gene expression.[4]



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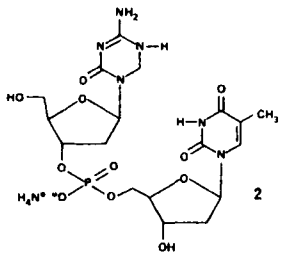

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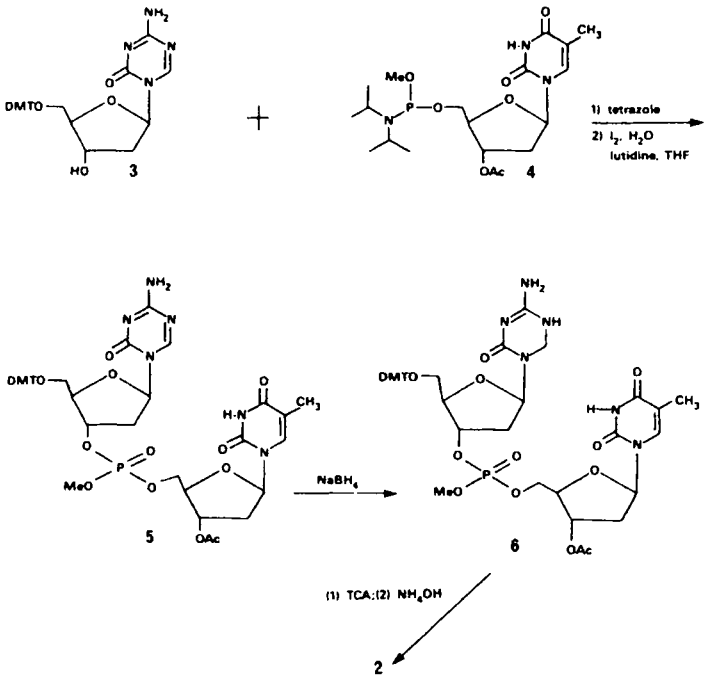

Scheme 1

Preliminary experiments towards the conversion of 5-DHAC to 5-AC have been conducted with dimer 2, which was prepared through the coupling of 1 to 3'-O-acetyl-thymidine.[3] With this dimer it is very simple to monitor the extent of oxidation of the 5-DHAC base by observing the changes in the ratio of the m/z 531 to 533 peaks in the negative ion FAB/MS.

Since we anticipated using significant amounts of this dimer in optimizing the conditions for the 5-DHAC to 5-AC conversion, and we wanted to reserve the phosphoramidite 1 for the synthesis of larger oligodeoxynucleotides, an alternative synthesis for 2 was developed (Scheme 1). Thus, starting with thymidine, the 5'-phosphoramidite intermediate 4 was prepared and coupled to the dimethoxytrityl protected 5-aza-deoxycytidine riboside 3 to give dimer 5. This 5-AC-containing dimer was readily reduced with sodium borohydride to the 5-DHAC dimer 6, which, after removal of the protecting groups, produced 2 which was identical to the material obtained when 1 was used as a reagent.

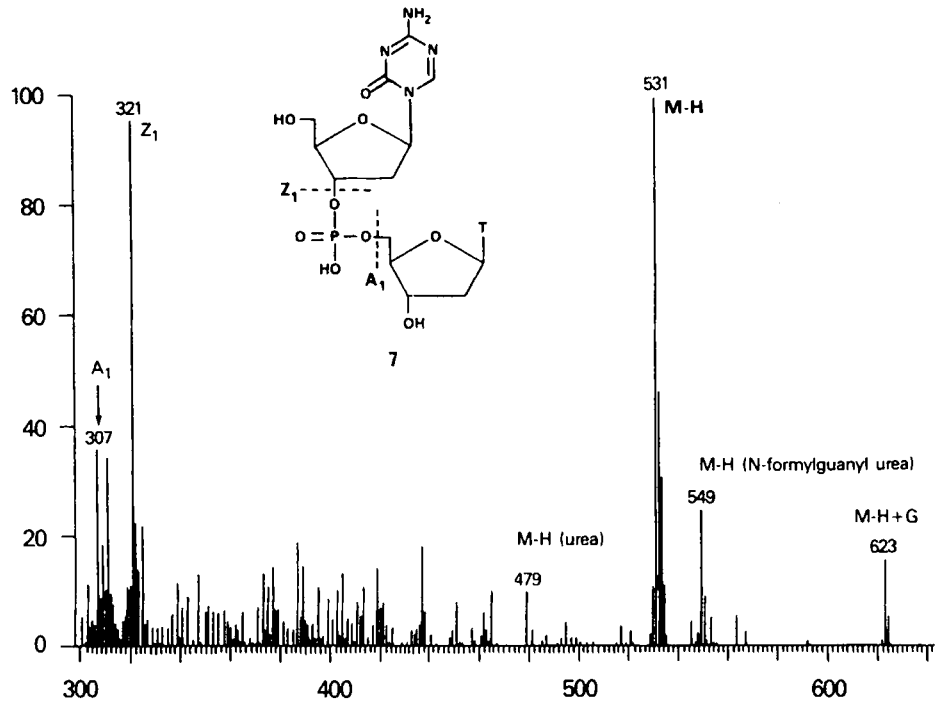

Fig. 1. Fragmentation pattern for dimer 7 obtained by negative ion FAB mass spectroscopy

In our initial attempts to oxidize the 5-DHAC moiety in dimer 2, the silylation-mediated oxidation procedure for the aromatization of dihydrotriazine nucleosides[5,6] produced only 34% yields of the desired dimer 7.[3] Since this oxygen-dependent, radical oxidation requires the complete silylation of the substrate,[5] it was possible that the reagent used, bis(trimethylsilyl)trifluoroacetamide (BSTFA), failed to form the requisite trimethylsilyl ester with the unsubstituted phosphate hydroxyl group 2 (ammonium salt). The use of trimethylsilyl chloride, in combination with BSTFA, overcame this difficulty by readily silylating the $P\text{-}O^- NH_4^+$ linkage. This allowed the reaction to proceed quantitatively as indicated by the FAB/MS of the desired dimer 7 (Fig. 1). In addition to the expected m/z 531 (100%) peak, it is interesting to observe the m/z 549 peak which corresponds to either the hydrated form of 5-AC or its open tautomer, N-formylguanyl urea. Similar hydrated forms have been detected to exist in equilibrium in aqueous solution of triazine nucleosides.[1,2]

A key intermediate postulated in this oxygen-dependent oxidation reaction is trimethylsilyl peroxide.[5] Since dissolved oxygen could be a limiting reagent, we synthesized this putative intermediate[7] and used it as an additional reagent in our reaction mixture. Although the conversion of 2 to 7 appeared to have been completed in a shorter time, a detailed kinetic analysis of this reaction deserves further investigation.

Use of 1 in the preparation of 5-DHAC modified 26mers at the indicated CpG sites (bold face), was also accomplished. The selection of the parent unmodified oligomer (5'-CCGGCCATTACGGATCCGTCCTGGGC-3') was based on its reported excellent substrate properties for DNA methyltransferase.[8] Conversion of the 5-DHAC moiety into the 5-AC base in these oligomers is currently under investigation.

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