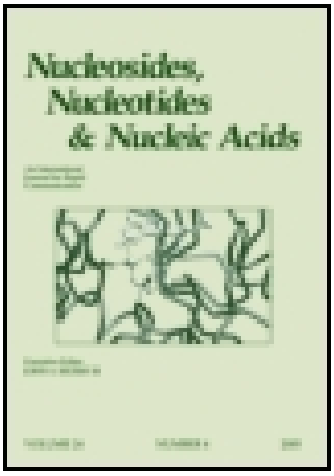

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Synthesis and Properties of Oligonucleotides Containing 5-Aza-2′-deoxycytidine

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SYNTHESIS AND PROPERTIES OF OLIGONUCLEOTIDES CONTAINING 5-AZA-2'-DEOXYCYTIDINE.

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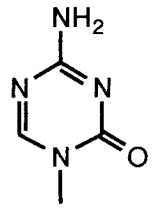
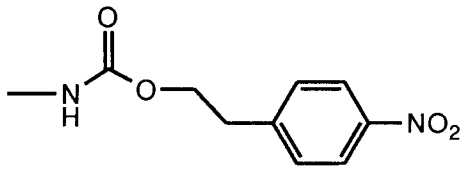
Abstract: The preparation of a protected derivative of 5-aza-2'-deoxycytidine carrying the 2-(*p*-nitrophenyl)ethyl group is described. The new derivative is useful for the preparation of oligonucleotides containing 5-aza-2'-deoxycytidine using a special methodology that avoids the use of ammonia.

5-Azacytosine (**Z**) is a cytosine analogue wherein the carbon at position 5 of the pyrimidine is replaced by a nitrogen atom. The ribo and deoxyribo nucleosides are potent cytotoxic agents and they have been used for the treatment of leukaemia. The gene regulatory effects have been ascribed to inhibition of DNA methyltransferase enzymes.[1] In order to elucidate the molecular basis of the powerful biological effects found in this nucleoside, we have undertaken the preparation of oligodeoxynucleotides containing 5-aza-2'-deoxycytidine. The hydrolytic instability of the triazine ring is well documented[2] and precludes its utilisation with the standard phosphoramidite protocol particularly during the treatment with ammonia.



Z: 5-azacytosine

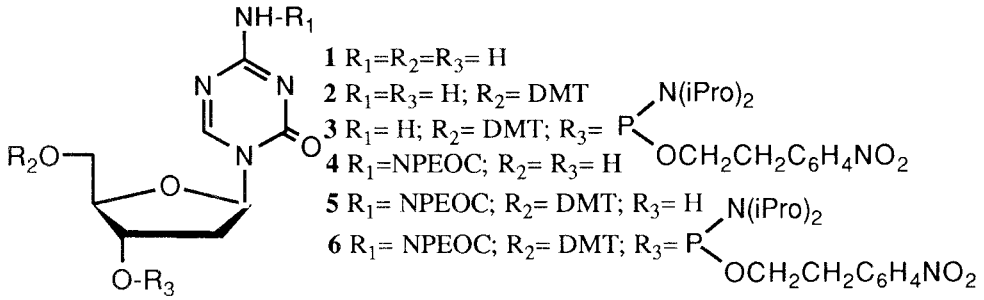


2-(4-nitrophenyl)ethoxycarbonyl, NPEOC

The preparation of oligonucleotides containing the reduced base 5,6-dehydro-5-azacytosine has been described,[3] but conversion of the reduced base to the desired 5-azacytosine was not efficient enough to yield the desired oligonucleotides in good yields.[3] Previous studies[4] using the unprotected H-phosphonate derivative of 5-aza-2'-

deoxycytidine showed that this base was stable to non-nucleophillic bases (such as 1,8-diazabiciclo[5.4.0]undec-7-ene, DBU) in anhydrous pyridine, conditions in where the *p*-nitrophenylethyl (NPEOC) groups described by Pfleiderer[5] are removed. In this communication, we will describe the use of the phosphoramidite methodology for the preparation of oligonucleotides containing 5-aza-2'-deoxycytidine (**Z**).

Due to the low reactivity of the amino group of 5-azacytosine, we first tried to use the phosphoramidite derivative of 5'-dimethoxytrityl (DMT) -5-aza-2'-deoxycytidine (without protection on the base). Reaction of the DMT-5-aza-2'-deoxycytidine (**2**) with the corresponding N,N-diisopropylamino-O-(4-nitrophenyl)ethoxychlorophosphine yielded the desired phosphoramidite (**3**) but in low yield (30%). A second product was obtained (60%) resulting from the reaction of the chlorophosphine with the unprotected amino group. Phosphoramidite (**3**) was used for the preparation of a small oligonucleotide but, after the addition of this base, coupling efficiencies were higher than expected by the DMT analysis. Moreover, HPLC analysis of the oligonucleotide after deprotection showed multiple peaks which had a molecular mass higher than expected (data not shown). All these results indicated that the amino group of 5-azacytosine ring should be protected for the synthesis of oligonucleotides using the phosphoramidite approach.



Afterwards, the protection of the amino group of 5-aza-2'-deoxycytidine with the *p*-nitrophenylethyl (NPEOC)[5] group was studied. Reaction of 5-aza-2'-deoxycytidine (**1**) with hexamethyldisilazane yielded the silylated nucleoside that was treated with 2-(4-nitrophenyl)ethoxycarbonyl chloroformate to produce the N^4-NPEOC-derivative of 3',5'-O-bis(trimehtylsilyl)-5-aza-2'-deoxycytidine. The removal of the trimethylsilyl groups without damaging the base was troublesome but it could be achieved by keeping the silylated nucleoside in dimethylformamide (DMF) for 4 days at room temperature. Other conditions such as different fluoride solutions, sodium ethoxide, *p*-toluensulfonic acid caused the removal of the NPEOC group and/ or the breakdown of the base. Dissolving the silylated nucleoside in DMF the trimethylsilyl groups were removed slowly without detectable side reactions and the NPEOC-protected nucleoside (**4**) was obtained in a 65% yield after silica gel purification. Reaction of **4** with DMT-Cl followed by reaction with the appropriate chlorophosphine gave the desired phosphoramidite (**6**) in good yields.

The following oligonucleotide sequences were prepared on 1 μmol scale: A (5' TAG ZTG A 3'); B (5' TAZ GZT GA 3'); C (5' GCA ATG GAZ CCT CTA 3'); D (5' ATT GCG CAT TCC GGA TCZ GCG ATC 3'); and E (5' ATT GCG CAT TCC GGA TCC GZG ATC 3') using phosphoramidite **6** and the 2-(4-nitrophenyl)ethyl phosphoramidites of the natural bases protected with the NPE and NPEOC groups.[5,6] Some phosphoramidites were not very soluble in acetonitrile and for this reason were dissolved in dry dichloromethane to obtain 0.1 M solutions. Controlled-pore glass supports having the NPE,NPEOC-protected nucleosides attached through an oxalyl linkage were used.[7] The oxalyl bond is efficiently cleaved by DBU solutions with higher efficiency than the previously described NPE linkage.[8] Standard cycles were used with slight modifications. Coupling times were increased from 30 sec to 2 min. In all cases the last DMT group was left on the oligonucleotide to facilitate purification. Coupling efficiencies were >96% as measured by DMT analysis.

Deprotection was carried out by treatment of oligonucleotide-supports with a 0.5 M DBU solution in pyridine containing 5 mg of thymine for 15 hours at room temperature.[6] The resulting solutions were neutralized with acetic acid and concentrated to dryness. Purification of products was performed by HPLC (after desalting with a Sephadex G-10 column) using the standard DMT-on and DMT-off protocols. During the characterization of oligonucleotides by enzyme digestion, it was not possible to observe the presence of 5-azacytidine probably due to degradation of the nucleoside during the enzyme digestion (pH 8.5).[2] But, on the contrary, mass spectrometry using electrospray in neutral conditions gave the expected molecular weights. The analysis by mass spectrometry of purified oligonucleotides showed also the presence of low amounts of two other compounds: one having 10 units less than the expected mass (M-10) and a second with 290 units less (M-290). The M-10 product was assigned to the oligonucleotide resulting from the opening of the 5-azacytosine ring and subsequent loss of a formyl group. This product has been described to be formed spontaneously from 5-azacytosine derivatives.[2] The M-290 product corresponds to an oligonucleotide that lacks 5-aza-2'-deoxycytidine. The presence of this product could only be explained if 5-azacytosine was not completely stable to oxidation or detritylation steps because coupling yields of 5-aza-2'-deoxycytidine phosphoramidite were high and the capping step will prevent the elongation of unreacted sequences. We confirmed this hypothesis by analysing the oligonucleotides by ion-exchange perfusion chromatography using neutral conditions. Small amounts of a product that could be a N-1 sequence were observed. We tried to replace the classical iodine solution used during the oxidation step (because of the presence of water and base that are known to be harmful to 5-azacytosine ring) with a 1M solution of *t*-butylperoxide in dry dichloromethane, but the iodine solution gave always better results.

In conclusion, we have described the preparation of the phosphoramidite derivative of 5-aza-2'-deoxycytidine protected with the NPEOC group. This phosphoramidite allows the preparation of oligonucleotides containing this base if non-hydrolytic conditions are used for the deprotection. Studies on the biological properties of these oligonucleotides are in progress.

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