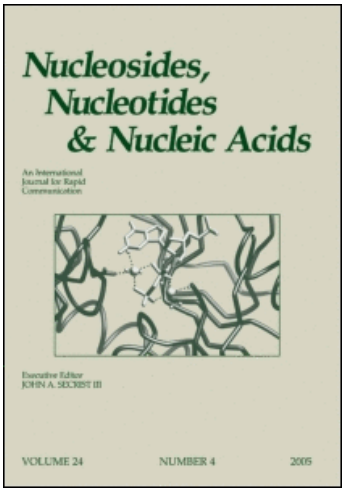

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PREPARATION OF OLIGONUCLEOTIDES CONTAINING NON-NATURAL BASE ANALOGS.

Ramon Eritja*, Viviane Adam, Anna Aviñó, Antonio R. Díaz, Carme Fàbrega, Elisenda Ferrer, Morten Grøtli, Ramón Güimil García, Mechtild Hofmann, Víctor E. Márquez# and Marten Wiersma.

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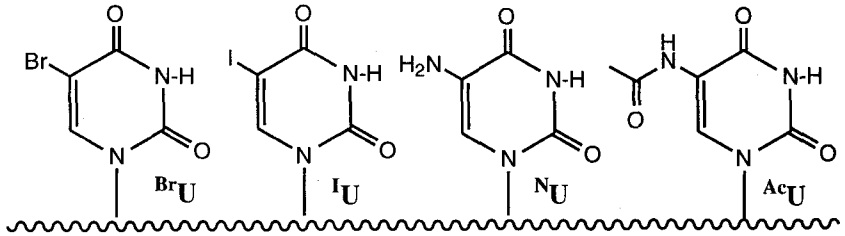
Abstract: The preparation of oligonucleotides containing 5-amino-2'-deoxyuridine, 5-N-acetamido-2'-deoxyuridine, 5-aza-2'-deoxycytidine and N^2-substituted guanosine derivatives is described. In each case selection of the appropriate protective group, synthesis and deprotection conditions is discussed.

The incorporation of modified bases into synthetic oligonucleotides offers the possibility of modifying some of the properties of the oligonucleotides including duplex and triple helix stabilization, nuclease stability and other biological properties. For example, some 5-substituted pyrimidines are known to stabilize duplex and triple helix, and some of the antiviral, antibacterial and antiproliferative properties of the 5-substituted pyrimidine nucleosides could be due to their incorporation into DNA and RNA.



In this communication we will describe the preparation of oligonucleotides carrying 5-aminouracil (^{N}U) and 5-acetamidouracil (^{Ac}U). 5-Aminouracil is obtained as side product of 5-bromouracil (^{Br}U) and 5-iodouracil (^{I}U) containing oligonucleotides during the ammonia treatment.[1] The formation of this side product could be prevented if ammonia

deprotection is performed at room temperature.[1] Oligonucleotides containing 5-bromouracil have been shown to form more stable triple helix than thymine containing oligonucleotides,[2] but the possible contribution of the side product (5-aminouracil) to the stabilization has not been evaluated. For this reason oligonucleotides containing 5-aminouracil (^{N}U) were prepared. Previously to this work the preparation of oligonucleotides containing ^{N}U was described[3] and some special triple helix properties have been found when ^{N}U was at the Watson-Crick part of the triple helix.[4] In this work we describe a modification of the ^{N}U residue during the synthesis that has not been detected previously and the use of alternative protective groups and capping conditions to avoid this side product.

In order to synthesize oligonucleotides containing ^{N}U, a protected derivative should be prepared. The reactivity of the amino group of ^{N}U is similar to aminoalkyl groups and different from the amino groups of the natural bases. Previous work has shown that benzoyl and acetyl groups are not suitable for the protection of the amino group because they are too stable to ammonia deprotection and the more labile trifluoroacetyl (TFA) group was recommended.[3] According to this work, the phosphoramidite of N-TFA-protected 5-amino-2'-deoxyuridine was prepared and some small oligonucleotide sequences carrying this base were synthesized using standard protocols.[3] During the characterization of these oligonucleotides by enzyme digestion and mass spectrometry a side product was observed. The side product was identified as 5-N-acetamido-2'-deoxyuridine (^{Ac}U). The acetylation of the desired ^{N}U residue was produced during the capping step and the acetyl derivative is stable to ammonia deprotection conditions. The acetylation of the ^{N}U residue could be produced a) by premature hydrolysis of the TFA group during synthesis conditions and subsequent acetylation during the capping step or b) direct acetylation of the trifluoacetylamide group. The N-trifluoroacetyl, N-acetyl intermediate will loose the TFA group during ammonia treatment but not the acetyl group.

The N,N-dimethylformamidine (Dmf)[5] and the 2-(4-nitrophenyl)ethoxycarbonyl (NPEOC)[6] were tested for the protection of the amino group of ^{N}U. The model dimer $5'A^{N}U3'$ was prepared because allows the easy quantification of the acetylation product by HPLC. When TFA was used for protection of 5-amino group a 60 % of acetylated product was observed. When the Dmf group was used a 40% of acetylation was observed. Finally, when the NPEOC group was used a 10% of side product was observed (FIG. 1). In order to avoid the side reaction, alternative capping reagents were investigated. The use of trifluoroacetic anhydride together with the TFA-protected ^{N}U phosphoramidite did not give good results. Although the dimer was prepared free of acetylated side product, when longer oligonucleotides were prepared, a complex mixture of oligonucleotides was obtained. On the other hand, the use of a 2% solution of NPEOC chloroformate replacing

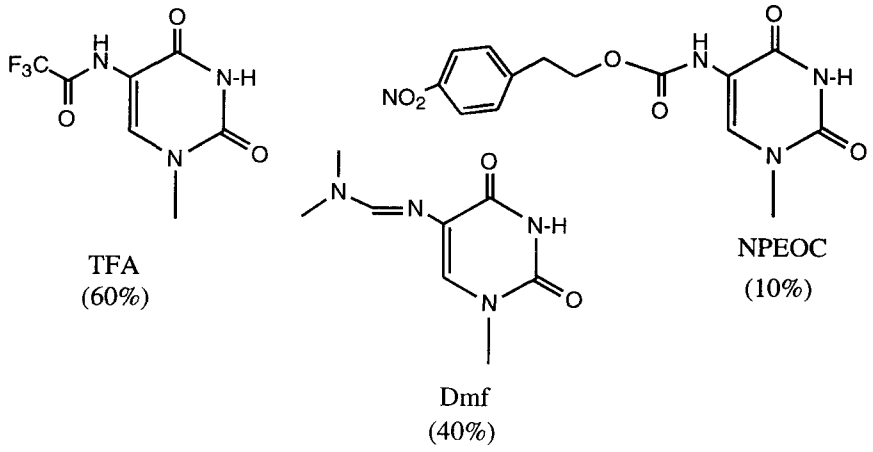


FIGURE 1: Groups used for the protection of 5-aminouracil residue. The number in brackets indicates the amount of acetylated side product found during the synthesis of the model dinucleotide $5'A^{N}U3'$.

the acetic anhydride solution together with the NPEOC-protected phosphoramidite gave the desired oligonucleotides in pure form.

Using the NPEOC-protected ^{N}U phosphoramidite and NPEOC-Cl as capping agent, the following sequences were prepared: 15b-^{N}U: $5'$GCA ATG GA^{N}U CCT CTA$3'$, and S_{11}-C^{N}U: $5'$C^{N}U^{N}U CC^{N}U CC^{N}U CT$3'$. Deprotection was performed in two-steps, first removal of NPEOC groups with a DBU solution followed by the standard ammonia deprotection. Purification was performed using the DMT on protocol and reversed-phase HPLC columns. In all cases a major peak was obtained that was collected and repurified after removal of the DMT group. Analysis of purified products by enzyme digestion and mass spectrometry gave the correct nucleoside composition and the expected mass. Also, the phosphoramidite derivative of 5-N-acetamido-dU (^{Ac}U) was prepared and incorporated into similar oligonucleotide sequences to study base-pairing properties of this derivative.

The selection of appropriate protecting groups is also the critical point for the synthesis of oligonucleotides containing 5-azacytidine (**Z**) residues. Due to the sensitivity of this base to ammonia, the use of non-standard deprotection conditions is needed. When NPE, NPEOC-protected phosphoramidites[6,7] are used together with the NPE[8] or oxalyl linkage,[9] oligonucleotides can be prepared using DBU (1,8-diazabiciclo[5.4.0]undec-7-ene) solutions in aprotic solvents for the deprotection avoiding the use of any nucleophile.[7,8] Previous studies using the unprotected H-phosphonate derivative of 5-aza-2'-deoxycytidine showed that this base was stable to DBU solutions and small

oligonucleotides carrying this base were prepared.[10] We have also tried to use the phosphoramidite derivative of unprotected 5-aza-2'-deoxycytidine. Although the phosphoramidite could be obtained, the yield was low. Also, overcoupling was observed after the addition of this base, indicating that protection of the amino function of 5-aza-2'-deoxycytidine was necessary when phosphoramidite chemistry was used.

Reaction of 5-aza-2'-deoxycytidine with hexamethyldisilazane yielded the silylated nucleoside that was reacted with NPEOC chloroformate. Removal of the trimethylsilyl groups was performed by keeping the silylated nucleoside in dimethylformamide for 4 days at room temperature yielding the desired NPEOC-protected nucleoside in good yields (65% after purification). Other conditions were harmful for the 5-azacytosine ring. Reaction of NPEOC-protected nucleoside with dimethoxytrityl (DMT) chloride gave the 5'-DMT-protected derivative that was reacted with the appropriate chlorophosphine to yield the desired phosphoramidite.

The following oligonucleotide sequences were prepared on 1 μmol scale: A (5' TAG **Z**TG A 3'); B (5' TA**Z** G**Z**T GA 3'); C (5' GCA ATG GA**Z** CCT CTA 3'); using phosphoramidite and NPE phosphoramidites of the natural bases protected with the NPE and NPEOC groups.[6,7] Controlled-pore glass supports having the NPE,NPEOC-protected nucleosides attached through an oxalyl linkage were used.[8,9] 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 and the resulting solutions were purified as previously described.[7] Mass spectrometry analysis of purified products using electrospray in neutral conditions gave the expected molecular weights, together with 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.[14] The M-290 product corresponds to an oligonucleotide that lacks 5-aza-2'-deoxycytidine. Ion-exchange HPLC on neutral conditions was successfully used to removed these impurities.

Finally, the preparation of oligonucleotides containing guanosine derivatives carrying spermine, spermidine and 3-aminopropylimidazole groups at position 2 is described. These groups have been found to increase the stability of G.C base pairs.[11,12]

The preparation of oligonucleotides carrying these groups at position 2 of guanine could be achieved by using 2-fluoro-2'-deoxyinosine as precursor (FIG. 2). Treatment of oligonucleotides carrying 2-fluorohypoxanthine residues with the appropriate amine produces the oligonucleotide carrying the N^2-substituted guanine residue. The displacement reaction could be done before, during or after removal of the base protecting

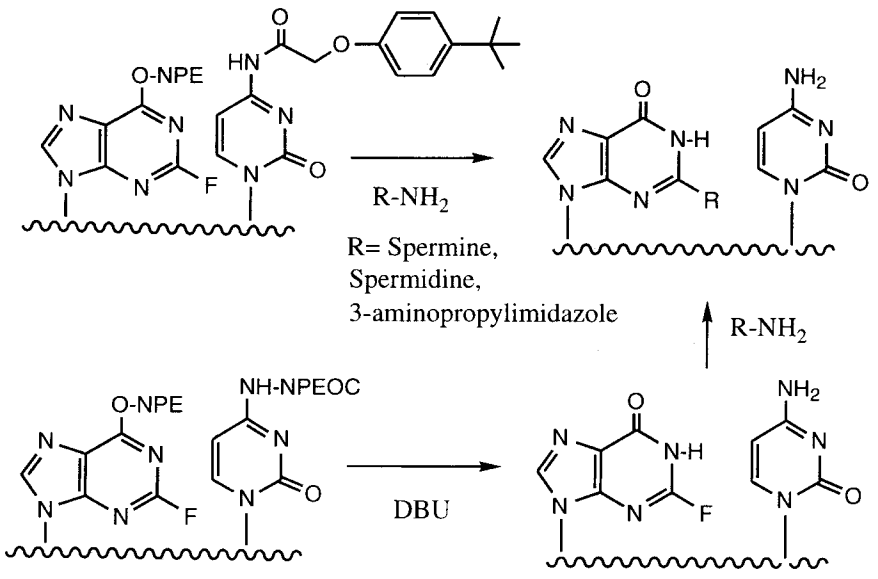


FIGURE 2: Preparation of oligonucleotides carrying N^2-substituted guanine derivatives.

groups, depending of the protecting groups used for the natural bases. Usually, the displacement reaction is performed at the same time than the removal of the protecting groups but modification of cytidine residues could happen if standard (benzoyl, isobutyryl) protecting groups are used.[13] The use of more labile *tert*-butylphenoxyacetyl protecting groups reduces the formation of modified cytosine residues. Also, the use of β-elimination protecting groups such as NPEOC groups[6] eliminates the modification of C residues and allows the isolation and characterization of oligonucleotides containing 2-fluorohypoxanthine residues.

In summary, the preparation of oligonucleotides carrying different non-natural base analogues has been described. In every case, the search for the appropriate protective groups has been a crucial step to avoid decomposition or side-reactions. Studies on the structural and biological properties of these oligonucleotides are in progress.

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