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SYNTHESIS OF A PHOSPHORAMIDITE OF 2'-DEOXY-5,6-DIHYDRO-5-AZACYTIDINE. ITS POTENTIAL APPLICATION IN THE SYNTHESIS OF DNA CONTAINING DIHYDRO-5-AZA- AND 5-AZACYTOSINE BASES.

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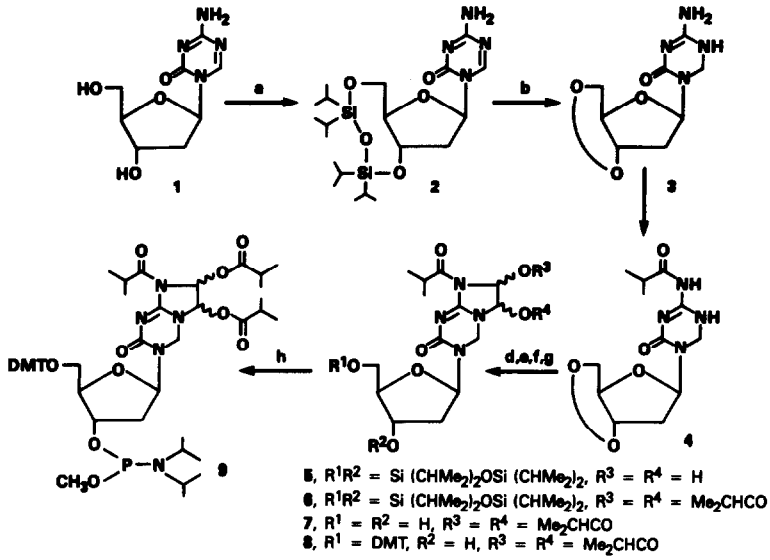
Abstract: A novel phosphoramidite (9) has been synthesized and shown to be an efficient coupling reagent for the synthesis of oligonucleotide fragments containing the modified base 5,6-dihydro-5-azacytosine. With a model dihydro-5-azacytosine/thymine dimer (12), oxidation of the dihydrotriazine ring to the aromatic base was partially successful.

In view of the well established relationship that exists between the DNA incorporation of 5-azacytosine residues and gene activation,[1] we became interested in developing a suitable methodology for the synthesis of oligonucleotide fragments containing this unnatural base. These modified oligonucleotides, which would contain 5-azacytosine residues at specific sites, could serve as tools for elucidating the mechanism of selective gene activation and the relationship that exists between the presence of the triazine base and inhibition of DNA methylation.[1]

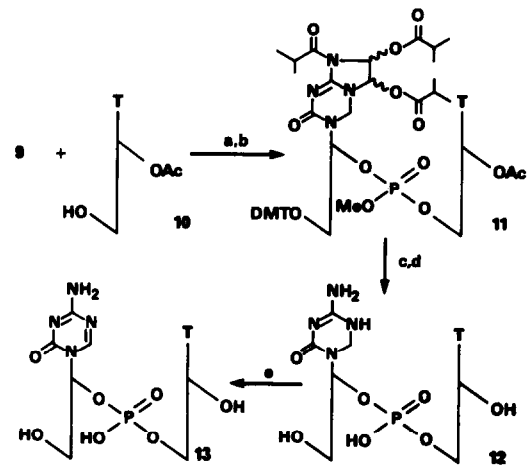
Since the hydrolytic instability of the triazine ring in 5-azacytosine nucleosides is very well documented,[2,3] use of a conventional phosphoramidite derivative of 5-azacytosine-deoxyribose (5-aza-dCR, 1) appeared impractical as it would have resulted in the base-catalyzed cleavage of the triazine ring during the last deprotection step of the synthesis.[4] In order to circumvent this problem, we decided to use a stable phosphoramidite precursor of 1 that would allow regeneration of the desired 5-azacytosine base after the conclusion of the synthesis of the oligonucleotide. The selected candidate was the protected 5,6-dihydro-5-azacytidine phosphoramidite (9) which by analogy with its parent nucleoside was expected to have a very stable triazine ring.[5] Scheme 1 outlines the synthesis of 9 starting with 5-aza-dCR (1). Protection with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane,[6] followed by borohydride reduction of 2, gave the desired dihydro analogue 3 after purification by silica gel flash chromatography (5% MeOH in ethyl acetate). The ^{1}H-NMR spectrum of 3 showed the newly generated C-6 methylene protons as an AB quartet centered at δ 4.40 (J_{gem} = 9.14 Hz).[7] The exocyclic amino group of 3 was then protected as the isobutyrylamide 4 and purified by silica gel flash chromatography (50% ethyl acetate in hexane). Complete protection of the triazine was accomplished with the introduction of the bis(isobutyryloxy)ethylene (bibe) group,[8] performed in the same manner as for 2'-deoxyguanosine.[9] Thus, the intermediate

Scheme 1



a) 2.2 eq. 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine, rt, 1 h, 97%. b) 8 eq. $NaBH_4$, THF, rt, 1 h, 78%. c) i. 6 eq. isobutyryl chloride, pyridine/chloroform, 0°, ii. MeOH, rt, 16 h, 84%. d) i. 40 eq. glyoxal, pyridine, rt, thrice reduced to dryness, ii. chloroform/water extraction. e) 2 eq. isobutyryl chloride, pyridine, rt, 2 h, 61%. f) 1.2 eq. tetrabutylammonium fluoride, THF, rt, 1/2 h, 60%. g) 1.2 eq. 4,4'-dimethoxytrityl chloride, pyridine, rt, 2 h, 50%. h) 2.2 eq. chloro-N,N-diisopropylamino methoxyphosphine, 4.2 eq. tetrazole, chloroform, rt, 15 min, 71%.

Scheme 2



a) 1.5 eq. phosphoramidite 9, 5 eq tetrazole, MeCN, rt, 15 min. b) Iodine, 3%; water, 2%; 2,6-lutidine, 2%; THF, 93%; rt, 10 min. c) trichloroacetic acid, 2% in dichloromethane, rt, 10 min. d) conc. NH_4OH, 50°, 15 h. e) i. 250 eq bis(trimethylsilyl)-trifluoroacetamide, MeCN, reflux, 1 h, ii. 50% methanol in water, rt, 1 h.

diol 5, isolated from the reaction of 4 with glyoxal, was reacted with isobutyryl chloride to give fully protected 6 (mixture of two isomers)[10] after purification by silica gel flash chromatography (15% ethyl acetate in hexane). Removal of the tetraisopropyldisiloxane group in 6 with tetrabutylammonium fluoride gave compound 7 following a simple extraction in methylene chloride/water. Protection of the 5'-hydroxyl group was accomplished by the standard procedure[11] employing 4,4'-dimethoxytrityl chloride to afford compound 8 as a crystalline solid (mp 89-91°C, hexane). Finally, phosphytilation of compound 8 with chloro-N,N'-diisopropylamino methoxyphosphine[11] gave the desired phosphoramidite 9 as a white solid (mp 67-69°C) after purification by silica gel flash chromatography (25% ethyl acetate in hexane); ^{31}P-NMR δ 148.48, 148.56, 148.97 and 149.07. Since formation of the phosphoramidite introduces a new chiral center in each of the two isomers generated after the synthesis of 6, the ^{31}P-NMR spectrum displayed four peaks instead of the two signals commonly observed with nucleoside phosphoramidites.

The reactivity of the new phosphoramidite (9) was initially tested under the standard conditions employed for DNA synthesis in a typical tetrazole-catalyzed condensation reaction with 3'-O-acetyl-thymidine (10, Scheme 2).[12] After 15 min, the reaction was complete and immediately oxidized in situ to give a quantitative yield of the fully protected dimer 11. Removal of the dimethoxytrityl group with trichloroacetic acid and further treatment of the residue with concentrated ammonium hydroxide afforded the fully deblocked dimer 12. An analytical sample of 12 was obtained after reversed phase chromatography (J.T. Baker C-18 silica gel, 40 μm, 5% MeOH in water) and as shown in Figure 1, the FAB/MS was consistent with the expected structure.

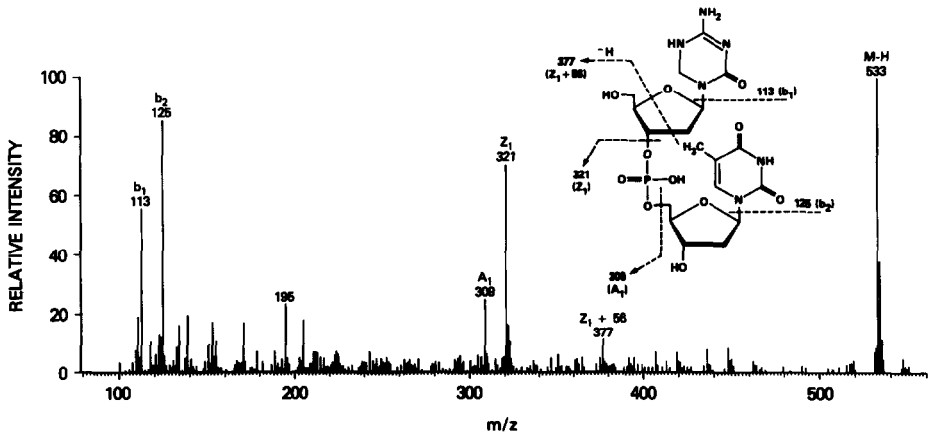


Fig. 1. Fragmentation pattern for dimer 12 obtained by negative ion FAB mass spectroscopy.

In order to test the utility of our new reagent, two decamers (Figure 2, lanes 4 and 5) in which the cytosine base at positions 3 and 6 was replaced by the 5,6-dihydro-5-azacytosine moiety, were synthesized in an Applied Biosystems model 380A automated DNA synthesizer. Based on the trityl assay data, the stepwise yield was 98.5% and 98.4%, respectively, compared to 99.09% for the unmodified decamer (Figure 2, lane 3).

The final conversion of the dihydrotriazine base to the aromatic triazine in dimer 12 was successful in moderate yield. Despite the demonstrated success of the silylation mediated oxidation procedure for the aromatization of 5,6-dihydro-5-azacytidine and 5,6-dihydro-5-aza-aracytidine,[14,15] persilylation of dimer 12 with bis(trimethylsilyl)trifluoroacetamide in refluxing oxygen-containing acetonitrile, afforded only a 34% conversion to the aromatic triazine, as judged by the ratio of the M-H peaks at m/z 533 and 531 in the negative ion FAB/MS. This yield, while low, supports our original synthetic strategy, although it is clear that more experimentation is required to improve the final conversion step. However, since recent evidence suggests that 5,6-dihydro-5-azacytidine behaves in the same manner as 5-azacytidine in inhibiting DNA methylation and inducing gene expression, it would appear that both types of modified oligonucleotides would be of biological interest.[16,17] Moreover, it is possible that the additional stability of the of the reduced triazine ring might prove to be a desirable property.

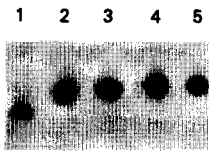


Figure 2. Autoradiography of synthetic oligonucleotides obtained after 5′-end labeling and polyacrylamide gel electrophoresis.[13]
Lane 1: $(CA)_3$, hexamer marker.
Lane 2: $(AT)_4$, octamer marker.
Lane 3: TACGTCGCAG, parent decamer.
Lane 4: TA**X**GTCGCAG, 3-modified decamer.
Lane 5: TACGT**X**GCAG, 6-modified decamer.
X = 5,6-dihydro-5-azacytosine

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