MULTIPLE LID STABILIZATION OF THE DNA THREE-WAY JUNCTION. INSERTION OF N^3-(1-PYRENYLMETHYL)THYMIDINE

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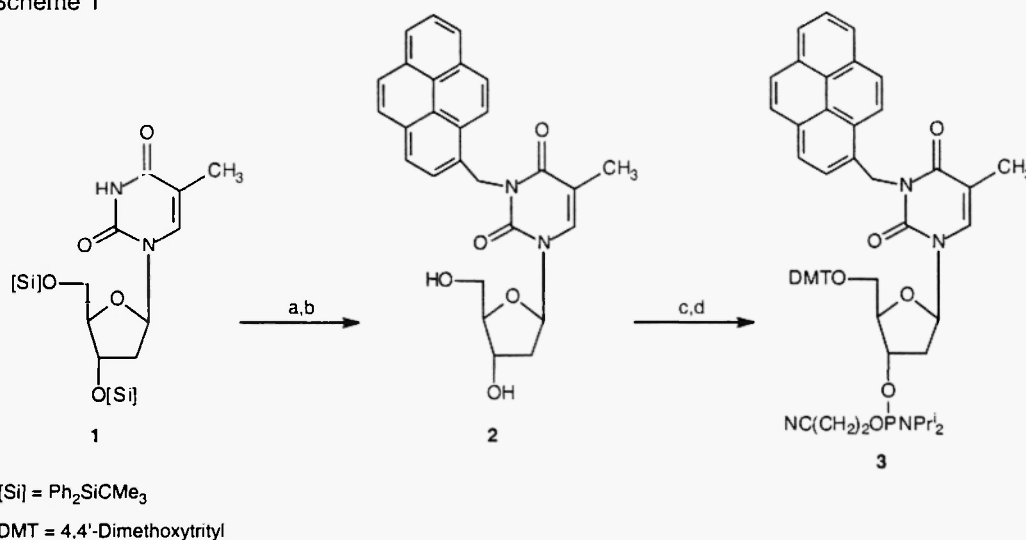
Abstract
The DNA three-way junction is stabilized when stacking moieties cover the inclined arm at the branch point and at the free ends of the duplex arms. N^3-(1-pyrenylmethyl)thymidine, used as the stacking moiety, is easily synthesized by N^3-alkylation of adequately protected thymidine.

Oligonucleotides have generated great interest as antisense agents because of their potential to inhibit gene expression. Helène and co-workers (1) developed an alternative way to recognise secondary structures in nucleic acid through oligonucleotides that are complementary to two non-adjacent sequences which are brought in close proximity through hairpin formation. They investigated the hairpin structure by melting curves, osmium tetroxide reactions and copper phenanthroline cleavage. The attachment of a polycyclic aromatic hydrocarbon as an intercalating group to an oligodeoxynucleotide (ODN) usually increases its binding affinity for the target nucleic acid, as well as improving cellular uptake due to increased lipophilicity and retaining the high specificity of the antisense oligonucleotide. In a previous study, (2) we investigated the insertion of 5-methyl-N^4-(1-pyrenylmethyl)cytidine into DNA, and studied the duplex, triplex and three-way junction stabilities when the intercalating moiety is inserted. We proved that the best stability of the three-way junction is found when the insertion is into the junction region. Here, we report the synthesis of N^3-(1-pyrenylmethyl)thymidine, its incorporation into oligonucleotides and its stabilization effect on DNA duplexes and DNA three-way junctions.

N^3-(1-Pyrenylmethyl)thymidine was prepared by the reaction of 1-pyrenylmethyl chloride and 3',5'-di-*O*-(*tert*-butyl-diphenylsilyl)thymidine in the presence of potassium carbonate in DMF at 40 °C to give the alkylated protected nucleoside in 81% yield which was deprotected using tetrabutylammonium fluoride (TBAF) in THF to give the compound 2 in 91% yield (3).

Scheme 1



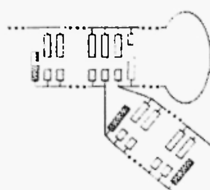
Scheme 1. a) Py-CH_2Cl, K_2CO_3, DMF, 40 °C, 12h, 81%; b) TBAF, THF, r.t., 2h, 91%; c) DMTCl, pyridine, r.t., 6h, 89%; d) $(Pr^i_2N)(O(CH_2)_2CN)PCl$, Pr^i_2NEt, CH_2Cl_2, r.t., 1h, 85%.

The phosphoramidite 3 was prepared in successive standard 4,4'-dimethoxytritylation and phosphitylation reactions in 89% and 85% yield, respectively. The purity of the obtained phosphoramidite 3 was 91% according to ^{31}P-NMR; 9% impurity was non-phoshoramidite phosphor. Using the standard phosphoramidite method (4) the coupling efficiency (20 min coupling) for the modified amidite 3 was approximately 85% compared to the 99% (2 min coupling) for the commercial ones. The insertion of 3 at the 3'-end was performed using the commercially available Biogenex Universal CPG support and deblocking with 2% LiCl in 25% NH_4OH for four days. All ODNs were desalted on a Pharmacia NAP-10 columns. DNA duplexes and DNA three-way junctions were formed from equimolar amounts in each strand at pH 7.0 in a 1 mM EDTA, 10 mM Na_2HPO_4 and 140 mM NaCl buffer.

Making use of the 36-mer DNA hairpin proven by Francois et al. (1) to form a three-way junction (TWJ) we want to study the effect of inserting 2 into the junction region of the TWJ and at the 3' and 5'-ends of the targeting ODN to see whether insertion in each position are giving additive effects on stabilizing the TWJ. The hybridization data are summarized in Table 1. Also we found it of interest to study the duplex stabilities when inserting 2.

Duplex: It has been established that inserting the modified nucleotide at the terminals of the antisense ODNs results in the greatest stability of the formed duplexes, (2,5) whereas the insertion of the modified nucleotide as a bulge in the middle of the an antisense ODN can cause destabilizing geometric distortions to the double helix, resulting in a much lower stability, and this is also what we found in this study.

Table 1. Thermal melting data T_m /°C of TWJ and the corresponding duplex (deletion of the stem loop region in the TWJ) on insertion of the modified nucleoside 2 and C (cytidine).



Entry	Oligonucleotide sequence	DNA hairpin T_m (°C)		Duplex T_m (°C)	
		X = 2	X = C	X = 2	X = C
1	5'-TTTTTTCT TCTCTTTCC-3'	28.0		47.6	
2	5'-TTTTTTCT **X** TCTCTTTCC-3'	39.2	28.8	42.4	38.0
3	5'-**X**TTTTTTCT **X** TCTCTTTCC-3'	40.8	30.0	44.8	39.2
4	5'-**X**TTTTTTCT TCTCTTTCC-3'	32.0	27.6	51.2	48.0
5	5'-TTTTTTCT TCTCTTTCC**X**-3'	34.8	28.4		
6	5'-TTTTTTCT **C** TCTCTTTCC**X**-3'	36.6	30.0		
7	5'-TTTTTTCT **X** TCTCTTTCCC-3'	40.0	30.0		
8	5'-TTTTTTCT **X** TCTCTTTCC**X**-3'	43.2	30.0		
9	5'-**X**TTTTTTCT TCTCTTTCC**X**-3'	36.4			
10	5'-**X**TTTTTTCT **C** TCTCTTTCC**X**-3'	38.4	30.0		
11	5'-**X**TTTTTTCT **X** TCTCTTTCC**X**-3'	46.0	30.0		

The target ODNs used in this study was a 24-mer derived from the hairpin 36-mer, but lacking the hairpin sequence. From Table 1, it can be seen that a higher stability of the duplex formed is achieved when 2 is inserted (Entry 4) at the 5'-end (ΔT_m = 3.6 °C) compared to the stability of the duplex without insertion. The steric contribution of inserting 2 in the middle of the ODN (Entry 2) is clarified by the destabilization that took place (ΔT_m = −5.2 °C). Destabilization is also observed when 2 is inserted at both the 5'-end and in the middle (ΔT_m = −2.8 °C) when compared with the 5'-end insertion.

TWJ: We found a high binding affinity to the DNA-hairpin when 2 is inserted in the junction region (Entry 2) with ΔT_m = 10.4 °C compared to the reference with a cytidine in the same position. When 2 was inserted into the junction region and at the same time at both ends (Entry 11), there is obtained a higher stabilisation with ΔT_m = 18 °C when compared to a reference without insertions and with ΔT_m = 15.4 °C when compared with inserted C instead of 2. This should be compared with ΔT_m = 8.4 °C (Entry 10) when C is inserted only at both ends. In order to confirm whether targeting of ODNs is taking place on both

sides of the hairpin, we compared the melting temperatures of the hybridized hairpin with those when the antisense DNAs were hybridized with each of the two halves of the DNA hairpin sequence obtained when the loop was cut in the middle. The 3'-side of the hairpin sequence showed T_m < 15 °C when hybridized with the same targeting ODNs. The 5'-side of the hairpin sequence showed higher T_m values (data not shown) than the 3'-side which could be attributed to the higher ratio of GC base pairs on the 5'-side. However, T_m values were always higher when the full hairpin was hybridized to targeting DNAs indicating that the targeting ODNs bind to both sides of the hairpin.

The approximately additive effects on stabilizing the TWJ stability on multiple insertions of 2 into the TWJ are ascribed to the lid effect which has been described by Kool and coworkers (6) who studied self complementary DNAs with a dangling pyrene moiety. In this work the TWJ is assumed stabilized by placing the non-polar pyrene as a stacking moiety covering the end of the inclined helical arm at the branch point. The TWJ is assumed further stabilized by the lid effect by additional placing pyrenes as stacking moieties at the 3'-end and 5'-end of the targeting oligo (see figure in Table 1).

REFERENCES AND NOTES

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(3) **2'-Deoxy-N^3-(1-pyrenylmethyl)thymidine** 2. ^{1}H NMR (DMSO-d_6): δ 8.28-8.04 (m, pyrene),7.68,(s,H6), 6.26 (m, H1'), 5.74 (s, N-CH_2-Py), 5.24 (m, OH), 5.03 (m, OH), 4.28 (m, H3'), 3.82 (m, H4'), 3.81 (m, H5'), 2.47 (m, H2'), 2.16 (m, H2'), 1.89 (s, CH_3, thymidine). ^{13}C NMR (DMSO-d_6): δ 163.08 (C4), 150.83 (C2), 135.36 (C6), 130.86, 130.50, 130.28, 129.92, 127.79, 127.57, 127.29, 127.05, 126.23, 125.27, 125.16, 125.02, 124.80, 124.09, 123.99, 122.92 (pyrene), 108.67 (C5), 87.47 (C4'), 85.01 (C1'), 70.21 (C3'), 61.14 (C5'), 41.65 (CH_2-N), 40.28 (C2'), 12.57 (CH_3, thymidine). FAB MS ($CHCl_3$ + 3-nitrobenzyl alcohol): 457 (M + H^+).

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