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Intercalating nucleic acids: the inversion of the stereocentre in 1-*O*-(pyren-1-ylmethyl)glycerol from *R* to *S*. Thermal stability towards ssDNA, ssRNA and its own type of oligodeoxynucleotides

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Abstract—The synthesis and insertions of (*S*)-1-*O*-(pyren-1-ylmethyl)glycerol into intercalating nucleic acids is described. Insertions of this *S*-isomer as a bulge lead to reduced binding affinity towards complementary ssDNA compared to intercalating nucleic acids possessing (*R*)-1-*O*-(pyren-1-ylmethyl)glycerol in the same positions. Insertions of both (*R*) or (*S*) 1-*O*-(pyren-1-ylmethyl)glycerols as bulges into two complementary strands decreased the stability of the complex compared to dsDNA possessing the pyrene pseudo-nucleotide in one of the strands.

Synthetic oligonucleotides possessing fluorescence intercalator moieties have found wide applications in molecular biology and medicine.[1] Recently our group has reported the synthesis, hybridization and fluorescence properties of nucleic acids called INA™, which contained bulge- and end-insertions of (*R*)-1-*O*-(pyren-1-ylmethyl)glycerol (**1**, Fig. 1).[2] Intercalating nucleic acids showed greatly increased affinity for complementary single-stranded DNA (ssDNA), but reduced affinity for an identical sequence of single-stranded RNA (ssRNA).[2]

An excimer band at 480 nm generated by two pyrene pseudo-nucleotides inserted as next-nearest neighbors as bulges was quenched upon hybridization to a fully complementary strand. However, no or very little quenching was found when there were mismatch nucleobases presented between, or next to the intercalators. This allows one to detect single nucleotide polymorphisms (SNPs) without depending on differences in the thermal melting temperatures of duplexes.[3] These unprecedented properties of INA™ could be explored for diagnostic purposes identifying 'profiles' or 'signatures' of the human genome.[4] In depth structural studies of INA™ and analogues is needed in order to understand the nature of the phenomena and to discover new applications.

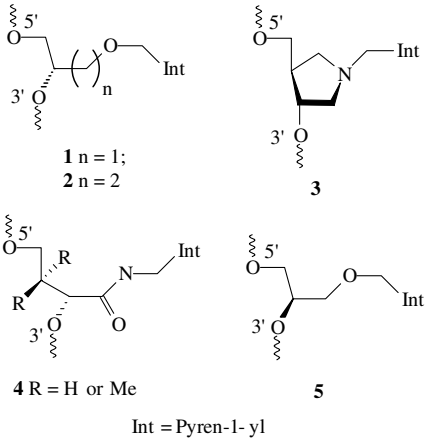

Figure 1.

From NMR and molecular modeling studies of INA™/DNA duplexes it was concluded that pyrene intercalated

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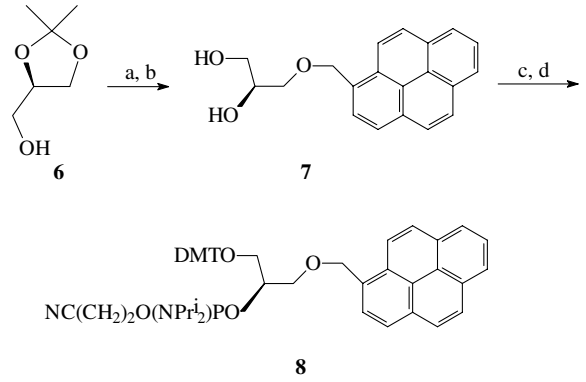
as expected but was not restrained to one site, thus inducing high mobility and a very flexible core.[5,6] Several different pyrene pseudo-nucleotides inserted as bulges have been published and their hybridization and fluorescence properties have been studied. The insertions via three-carbon moieties in the phosphate backbone (structures **3**[7] and **4**,[8] Fig. 1) or using a longer linker between the backbone and the pyrene (structure **2**,[5] Fig. 1) decreased the thermal duplex stability due to loss in entropy compared to the less flexible (*R*)-1-*O*-methyleneglycerol. It was also shown that the aromatic moiety could not reach the same position in the duplex if a shorter linker was used thus destabilizing the duplex.[5]

Using cheap, racemic 2,2-dimethyl-1,3-dioxalane-4-methanol as a starting compound for the synthesis of intercalating nucleic acids could be a competitive advantage of INA™. To evaluate the influence of the other stereoisomer of INA™ on the hybridization affinity towards ssDNA and ssRNA we report here the synthesis and insertion of (*S*)-1-*O*-(pyren-1-ylmethyl)glycerol into oligodeoxynucleotides as a bulge.

A range of synthetic oligonucleotides such as Peptide Nucleic Acid (PNA)[9] and Locked Nucleic acid (LNA)[10] having increased affinity for DNA and RNA bind even stronger to a complementary sequence of their own type. Such a high self-affinity strongly limits the number of sequences of DNA and RNA that can be targeted with the synthetic oligonucleotides, due to the formation of stable intra- and inter-molecular secondary structures. Therefore we would like to determine the binding affinity of intercalating nucleic acids to sequences with the same type of modifications possessing either *R* or *S* stereoisomers of 1-*O*-(pyren-1-ylmethyl)glycerol.

The target amidite **8** needed for oligodeoxynucleotide (ODN) synthesis was obtained applying the same sequence of reactions as described for INA™, but using the opposite stereoisomer of protected glycerol. We started from (*R*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (**6**) and 1-(chloromethyl)pyrene (Scheme 1). The latter compound was found to provoke an allergic reaction upon contact with the skin and therefore should be kept in the closed vessel or used immediately after its preparation from pyren-1-ylmethanol and thionyl chloride. The alkylation of the dioxolane **6** in the presence of KOH under Dean–Stark conditions followed by deprotection in 80% aq acetic acid gave (*S*)-1-*O*-(pyren-1-ylmethyl)glycerol (**7**) in 75% yield. Compound **7** was treated with 4,4′-dimethoxytrityl chloride (DMTCl) in dry pyridine affording (*R*)-1-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*O*-(pyren-1-ylmethyl)glycerol in 85% yield after purification on a silica gel column. The phosphoramidite **8** was obtained in 95% yield after treatment of the latter compound with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in the presence of diisopropylammonium tetrazolide in dry CH_2Cl_2 overnight followed by silica gel purification. Before ODN synthesis the phosphoramidite **8**[11] was co-evaporated twice with CH_3CN and a 0.075 M solution of **8** in 1:1 mixture of dry CH_3CN and CH_2Cl_2 was prepared for the oligo synthesis.



Scheme 1. Reagents and conditions: (a) 1-(chloromethyl)pyrene, KOH, toluene; (b) 80% aq CH_3COOH, rt, overnight, 75% over two steps; (c) DMTCl, pyridine, rt, overnight, 85%; (d) $NC(CH_2)_2OP(NPr^i_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, 0 °C to rt, overnight, 95%.

ODNs derived from compound **8** were synthesized by the solid phase phosphoramidite method on an automated Expedite™ Nucleic Acid Synthesis System Model 8909 (Applied Biosystems) in 0.2 μmol-scale using standard nucleotide coupling conditions (2 min coupling), but increased deprotection time (100 s) compared to the standard (50 s) was applied to achieve better release of the DMT-group after coupling of INA™. The coupling efficiency using 4,5-dicyanoimidazole as an activator was estimated to be over 99%. ODNs were worked up as described before[5] and confirmed by MALDI-TOF analysis.[12] The results of thermal denaturation studies of intercalating nucleic acids possessing either *R* or newly synthesized *S* isomers of 1-*O*-methyleneglycerol in the same sequences and performed under the same conditions are listed in Tables 1 and 2.

The comparison of hybridization affinities showed that **5** with inverted stereochemistry at the secondary OH-group of the 1-*O*-methyleneglycerol was less potent in increasing affinity for complementary ssDNA than **1**. The difference in T_m of these two isomers towards ssDNA varied from 3.0 °C for a single insertion (Table 1, entry 2) and up to 3.5 °C for double insertions separated by four nucleobases (Table 1, entry 4). Nevertheless the melting temperature of intercalating nucleic acids containing insertions of **1**or **5** with complementary ssRNA were nearly the same, but the discrimination of ssDNA over ssRNA was reduced in the case of **5** (Table 1).

The decreased hybridization affinity towards ssDNA when using **5** instead of **1** could be explained by the lack of flexibility of the (*S*)-1-*O*-methylglycerol linker, which cannot compensate for the inversion of the stereocentre thus decreasing the efficiency of the pyrene intercalation.

Due to differences in affinity for the two enantiomers, it is obvious that using racemic 2,2-dimethyl-1,3-dioxalane-4-methanol as a starting compound for the synthesis of intercalating nucleic acids could lead to poorly defined melting temperatures.

Table 1. Melting temperatures[a] of intercalating nucleic acids derived from **1** and **5** in duplexes with DNA and RNA

Entry	Oligo	Target DNA 5′-AGCTTGCTTGAG				Target RNA 5′-AGCUUGCUUGAG				Discrimination $T_{m(DNA)} - T_{m(RNA)}$	
1	3′-TCGAACGAACTC	48.0 °C				40.5 °C				7.5 °C	
	X =	**1**		**5**		**1**		**5**		**1**	**5**
		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)	ΔT_m (°C)
2	3′-TCGAAC**X**GA-ACTC	51.5	+3.5	48.5	+0.5	38.0	−2.5	37.0	−3.5	13.5	11.5
3	3′-TCGAAC**X**G-**X**AACTC	52.5	+4.5	49.3	+1.3	34.5	−6.0	33.5	−7.0	18.0	15.8
4	3′-TCGA**X**ACGA**X**-ACTC	61.0	+13.0	57.5	+9.5	34.8	−5.7	36.5	−4.0	26.2	21.0

[a] T_m (°C) was determined as the first derivative of melting curves by measuring the absorbance at 260 nm against increasing temperature (1.0 °C/min) on equimolar mixtures (1.0 μM of each strand) in 140 mM NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0.

Table 2. Melting temperatures[a] of INA™/DNA and INA™/INA™ duplexes

Entry	Oligo		Target INA	
			X = **1**	**5**
			5′-AGCTTG**X**CTTGAG	
1	3′-TCGAACGAACTC		50.3	47.5
2	3′-TCGAAC**X**GAACTC	**1**	43.6	41.8
3		**5**	41.0	41.0
4	3′-TCGA**X**ACGA**X**ACTC	**1**	58.0	
5		**5**		54.0
			5′-AGCTTG**X**C**X**TTGAG	
6	3′-TCGAACGAACTC		49.7	45.0
7	3′-TCGAAC**X**G**X**AACTC	**1**	45.0	39.7
8		**5**	39.0	37.5
			5′-AGCT**X**TGCT**X**TGAG	
9	3′-TCGAACGAACTC		50.0	47.1
10	3′-TCGA**X**ACGA**X**ACTC	**1**	32.0	30.0
11		**5**	30.0	29.5

[a] T_m (°C) was determined as in Table 1.

In other studies of INA™ we found that ΔT_m can vary in the range of +1.0 to +11.0 °C per modification (unpublished data). The sequence dependence is clearly illustrated by the fact that the double insertion of **1** separated by four base pairs in one strand gave only a ΔT_m of +2.0 °C (Table 2, entry 9) in comparison with +13.0 °C (Table 1, entry 4) when **1** was placed in the other strand.

It could be expected that the incorporation of **1** or **5** in two complementary strands, opposite to each other could increase the hybridization affinity, as it allows the large pyrene moieties to co-axially stack with each other. However in all cases a decrease in melting temperatures was observed (Table 2, entries 2, 3, 7, 8, 10, 11) when compared to the wild-type duplex (Table 1, entry 1). This means that coaxial stacking of two pyrene moieties is not large enough to compensate for a distortion of the double helix by two ethylene glycol linkers situated in opposite DNA strands. Decreased melting temperatures were also detected when intercalating nucleic acids having a double insertion of either **1** or **5**, separated by four nucleobases, hybridized to complementary DNA possessing a pyrene pseudo-nucleotide in the middle of the strand (Table 2, entries 4 and 5) when compared to hybridization with unmodified ssDNA (Table 1, entry 4).

When two insertions of **1** or **5** were separated by four nucleobase pairs and placed opposite to each other in both strands (Table 2, entries 10 and 11), the change of hypochromicity in melting transitions was considerably lower than in all other cases. This could be due to partly interrupted base-stacking in the duplex and/or because of the formation of different secondary structures having small changes in hypochromicity at their transition states.

From this study it can be concluded that intercalating nucleic acids with bulge insertions of (*R*)-1-*O*-(pyren-1-ylmethyl)glycerol (**1**) have higher affinity for complementary ssDNAs compared to insertions of (*S*)-1-*O*-(pyren-1-ylmethyl)glycerol (**5**). When intercalating pseudo-nucleotides **1** or **5** are positioned opposite each other, the corresponding duplexes have lower thermal stability than wild-type complexes. We believe that INA™ is the first group of synthetic DNAs having a high affinity for the natural DNA and not for its own type of oligodeoxynucleotides.

Insertions of **5** into intercalating nucleic acids can be used to level a melting temperature if required, while still maintaining its reduced affinity for ssRNA. We believe that intercalating nucleic acids possessing **1** or **5** as bulges can become a powerful tool for demanding hybridizations and can substitute normal DNA oligos when they form secondary structures or have high self-affinity.

Acknowledgement

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11. Compound **8**: ^{1}H NMR ($CDCl_3$) δ 1.20 (m, 12H, 4×Me), 2.42 (m, 2H, CH_2CN), 3.22 (m, 2H, O*CH*$_2$CH), 3.50–3.90 (m, 12H, CH_2ODMT, O*CH*$_2$CH$_2$CN, 2×CH [Pr^i], 2×OCH$_3$), 4.18 (m, 1H, CH_2*CH*CH_2), 5.25 (m, 2H, CH_2–pyrene), 6.60–6.70, 7.10–7.45 (m, 13H, DMT), 7.80–8.40 (m, 9H, pyrene); ^{31}P NMR ($CDCl_3$) δ 150.20, 150.24 in ratio 3:2, respectively; HRMS, found m/z 831.357, calcd for $C_{50}H_{53}N_2O_6P$ $[M+Na]^+$ 831.353.
12. MALDI-TOF analysis of intercalating nucleic acids:

Oligo	X =	m/z, Calcd. (Da)	m/z, Found (Da)
3′-TCGAAC**X**GAACTC	**5**	3981	3977
3′-TCGAAC**X**G**X**AACTC	**5**	4348	4349
3′-TCGA**X**ACGA**X**ACTC	**5**	4348	4350
5′-AGCTTG**X**CTTGAG	**1**	4042	4043
	5	4042	4041
5′-AGCTTG**X**C**X**TTGAG	**1**	4409	4407
	5	4409	4409
5′-AGCT**X**TGCT**X**TGAG	**1**	4409	4410
	5	4409	4408