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Intercalating nucleic acids containing insertions of 1-*O*-(1-pyrenylmethyl)glycerol: stabilisation of dsDNA and discrimination of DNA over RNA

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ABSTRACT

We have studied hybridisation affinities and fluorescence behaviour of intercalator-modified oligonucleotides. The phosphoramidite of (*S*)-1-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*O*-(1-pyrenylmethyl)-glycerol, an intercalating pseudo-nucleotide (IPN), was synthesised and by standard methods inserted into 7mer and 13mer oligodeoxyribonucleotides (ODNs) to generate intercalating nucleic acids (INAs). INAs showed greatly increased affinity for complementary single-stranded DNA (ssDNA), as determined by a thermal stabilisation of the formed DNA/INA duplex of up to 10.9°C per modification when the IPN was added as a dangling end and up to 6.7°C per modification when the IPN was inserted as a bulge. There was a positive stabilisation effect of the formed DNA/INA duplex on introducing a second IPN in the INA strand, when the two IPNs were separated by at least 1 bp. The effect is more pronounced the larger the separation of the two IPNs. Contrary to the enhanced affinity for ssDNA, the IPNs lower the affinity for complementary single-stranded RNA (ssRNA), giving rise to a difference in melting temperature of up to 25.8°C for two IPN insertions in an RNA/INA duplex when compared with the corresponding DNA/INA duplex. In this way INA is able to discriminate ssDNA over ssRNA with identical sequences. Fluorescence measurements show a stronger interaction of the pyrene moiety with DNA than with RNA, indicating intercalation as the stabilising factor in DNA/INA duplexes.

INTRODUCTION

Although it has been known for some time that there are relatively large differences in the three-dimensional structure of DNA/DNA duplexes (1) and DNA/RNA duplexes (2), and that some enzymes like RNase H are able to recognise one from the other (3), there have been only a few reported modified oligonucleotides able to differentiate between ssRNA and ssDNA. Some modifications in the sugar ring, like D-hexitol nucleic acid (HNA) and locked nucleic acid (LNA), have led to oligonucleotide analogues with increased affinity for ssDNA and ssRNA in general. These modifications preferentially stabilise hybridisation to ssRNA [melting temperature (ΔT_m) +3 to +5°C and +4 to +8°C for HNA and LNA, respectively] over ssDNA (ΔT_m +1 to +3°C and +3 to +5°C for HNA and LNA, respectively) (4–6). Some modifications are reported to be totally RNA selective, meaning that these oligonucleotide analogues will hybridise only with RNA and not with DNA. Unfortunately, most of the modifications that introduce selectivity for RNA do that by lowering the affinity for both RNA and DNA, the latter being most pronounced (7–10). On the other hand, there are few data showing that modified oligonucleotides can be DNA selective. However, these data are embedded in publications where discrimination between ssDNA and ssRNA is of little interest (11,12). In only one paper has the focus been on DNA/RNA discrimination using cholic acid and its deoxy derivatives linked to the 5′-termini of short oligonucleotides (13).

Nucleoside analogues with fluorescent labels have attracted interest for the last couple of decades for the development of new methods of distinguishing and detecting specific nucleic acid sequences (14–18). Among many different fluorescent probes pyrene is commonly used (11,19–24) and among these there are several acyclic pseudo-nucleotide analogues (19,22,23).

Here, we describe the insertion into a DNA strand of a new intercalating pseudo-nucleotide (IPN), namely the phosphoramidite of (*S*)-1-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*O*-(1-pyrenylmethyl)glycerol (5). Insertion of IPNs into oligodeoxynucleotides produces oligodeoxyribonucleotide (ODN) analogues which we call intercalating nucleic acids (INAs). In the above-mentioned references there are examples of ODNs that can be considered as INAs. The INAs presented here have a higher affinity for complementary ssDNA and are able to differentiate between ssDNA and ssRNA, preferring to hybridise with ssDNA. How the affinity for and discriminating efficiency between DNA and RNA of INA depend on the number of insertions of IPNs and their positions in the INA have also been investigated.

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Table 1. Calculated and found masses of modified ODNs

Sequences	Calculated *m/z*	Found *m/z*
5′-XCG CGC G	2160	2161
5′-XTC GCG CGA	2777	2778
5′-CTC AAG XCA ACC T	3981	3979
5′-CTC AAG XXC AAC CT	4348	4347
5′-CTC AAX GXC AAC CT	4348	4348
5′-CTC AXA GXC AAC CT	4348	4353
5′-CTC AXA GCA XAC CT	4348	4349
5′-CTC AAG YCA ACC T	3736	3742
5′-CTC AAG ZCA ACC T	3750	3751

X represents the inserted compound **5**, Y represents the flexible, abasic ethylene glycol backbone linker and Z the 1,3-propane-diol backbone linker.

MATERIALS AND METHODS

ODN and INA synthesis, purification and measurement of melting temperatures

The ODN and INA synthesis was carried out on a Pharmacia LKB Gene Assembler Special using Gene Assembler Special software v.1.53. The pyrene amidite **5** was dissolved in a 1:1 mixture of dry acetonitrile and dry dichloromethane, as a 0.1 M solution, and inserted into the growing oligonucleotides chain using the same conditions as for normal nucleotide coupling (2 min coupling). The coupling efficiency of the modified nucleotides was >99%. The ODNs were synthesised with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-1 column. Buffer A, 950 ml of 0.1 M NH_4HCO_3 and 50 ml of MeCN, pH 9.0; buffer B, 250 ml of 0.1 M NH_4HCO_3 and 750 ml MeCN, pH 9.0. Gradients, 5 min 100% A, linear gradient to 100% B in 40 min (product peak at ~37 min). The ODNs were DMT deprotected in 925 µl of H_2O and 75 µl of CH_3COOH and purified by HPLC, again using the same column, buffer system and gradients (product peak at ~26 min). To get rid of the salts, the ODNs were redissolved in 1 ml of water and concentrated *in vacuo* three times. The yields after purification for the synthesis of INAs were for the self-complementary sequences from 2.6 to 6.6 OD, for the mono inserted IPN in the 13mer INA 17.0 OD and for the double IPN inserted INAs from 7.4 to 15.2 OD.

All modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems (Table 1). The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and PECSS software package v.4.3. Melting temperature measurements of the self-complementary sequences were made in 1 M NaCl, 10 mM sodium phosphate, pH 7.0, 1.5 µM each DNA strand. All other ODNs were measured in 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, 1.5 µM each strand. All melting temperatures are with an uncertainty ±1.0°C as determined by repetitive experiments.

Molecular calculations

The molecular calculations used the Amber force field (25) done in MacroModel 6.0 and 7.0 with water as solvent and minimisation was by the conjugant gradient method. Lam and Au-Yeung solved the structure of the self-complementary sequence by NMR and their data were used in this work (26). Their structure was extended with the pyrene nucleoside at the 5′ end of each strand and was used for the structural calculations. The 13mer sequence is a highly conserved HIV-1 long terminal repeat region (27). Using the coordinates from the corresponding duplex from the Brookhaven Protein Databank, G7 was replaced by the pyrene nucleotide and calculations for the duplex were made with and without an opposite C nucleotide. The pyrene was placed in the interior of the duplex from the beginning. All bonds were free to move and to rotate.

Fluorescence measurements

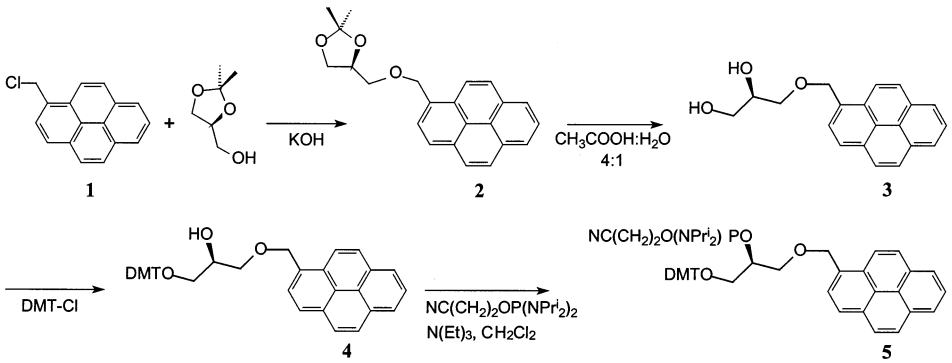
Fluorescence measurements were carried out in a Perkin-Elmer MPF-3 fluorescence instrument in 2 ml plastic cuvettes with excitation at 340 nm and detection at 360–600 nm. All measurements were conducted in 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, with a concentration of 1.5 µM each strand. The plastic cuvettes did not interfere with the measurements.

(*S*)-2,2-Dimethyl-4-(1-pyrenylmethoxymethyl)-1,3-dioxalane (2)

Pulverised KOH (25 g) and 1-(chloromethyl)pyrene (**1**, 6.0 g, 23.9 mmol) were added to a solution of (*S*)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (2.6 g, 19.7 mmol) in dry toluene (250 ml). The mixture was refluxed under Dean–Stark conditions for 16 h, cooled to room temperature and H_2O added (150 ml). The organic phase was washed with H_2O (3 × 100 ml), dried ($MgSO_4$ and Na_2SO_4) and concentrated under reduced pressure to give a viscous oil. The residue was purified by silica gel chromatography with CH_2Cl_2 as eluent, which afforded the pure compound **2** (6.1 g, 90%) as an oil. ^{1}H NMR ($CDCl_3$): δ 1.39 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 3.57–3.75 (m, 2H, H-5′), 3.88–4.06 (m, 2H, $CHCH_2OCH_2$), 4.32 (m, 1H, H-4′), 5.27 (m, 2H, OCH_2pyrenyl), 7.99–8.39 (m, 9H, H_{arom}). ^{13}C NMR ($CDCl_3$): δ 25.25 (CH_3), 26.68 (CH_3), 66.82 (C-1′), 70.96 (C-2′), 72.06 (OCH_2pyrenyl), 74.70 (C-3′), 109.44 [$C(CH_3)_2$], 123.44, 124.46 (pyrene CH), 124.73, 124.97 (pyrene C_{quat}), 125.29 (2 × pyrene CH), 125.99, 127.14, 127.42, 127.52, 127.78 (pyrene CH), 129.46, 130.85, 131.02, 131.27, 131.43 (pyrene C_{quat}).

(*R*)-1-*O*-(1-Pyrenylmethyl)glycerol (3)

(*S*)-2,2-Dimethyl-4-(1-pyrenemethoxymethyl)-1,3-dioxalane (**2**, 6.1 g, 17.6 mmol) was added to a mixture of CH_3COOH and H_2O (100 ml, 4/1 v/v) and stirred at room temperature for 19 h. The mixture was concentrated under reduced pressure, giving an oil in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 3.56–3.62 (m, 4H, H-1′, H-3′), 3.88 (m, 1H, H-2′), 5.14 (s, 2H, CH_2), 7.89–8.26 (m, 9H, H_{arom}). ^{13}CNMR: δ ($CDCl_3$) 63.79 (C-3′), 70.05 (C-2′), 71.45 (CH_2), 72.0 (C-1′), 123.08, 124.44 (pyrene CH), 124.65, 124.89 (pyrene C_{quat}), 125.33 (2 × pyrene CH), 126.00, 127.12, 127.33, 127.56, 127.92 (pyrene CH), 129.36, 130.73, 131.20, 131.47 (pyrene C_{quat}). Maldi HRMS, found *m/z* 329.1137; calculated for $C_{20}H_{18}O_3$ ($M + Na^+$), 329.1148. Found, C 76.64%, H 5.84%; calculated for $C_{20}H_{18}O_3 \cdot 0.35H_2O$, C 76.61%, H 6.05%.



Scheme 1. Synthesis of the phosphoramidite **5**.

(*S*)-1-*O*-(4,4′-Dimethoxytriphenylmethyl)-3-*O*-(1-pyrenylmethyl)glycerol (4)

(*R*)-1-*O*-(1-Pyrenylmethyl)glycerol (760 mg, 2.48 mmol) was dissolved in dry pyridine (20 ml) and dimethoxytrityl chloride (DMT-Cl) added (920 mg, 2.72 mmol). The reaction mixture was stirred for 24 h and concentrated under reduced pressure. The residue was purified by silica gel chromatography with EtOAc/cyclohexane/triethylamine (49/49/2 v/v/v) as eluent, affording compound **4** (1.20 g, 80%) as a white foam. ^{1}H NMR($CDCl_3$): δ 2.8 (s, 1H, OH), 3.20 (m, 2H, H-3′), 3.68–3.72 (m, 6H, OCH_3, H-1′, H-2′), 3.80 (s, 3H, OCH_3), 5.26 (s, 2H, O*C*H_2pyrenyl), 6.72–8.33 (m, 22H, H_{arom}). ^{13}C NMR ($CDCl_3$): δ 55.06 (2 × CH_3), 64.50 (C-1′), 70.00 (C-2′), 71.89 (CH_2), 72.00 (C-3′), 86.08 (O*C*Ph_3), 113.00 (4 × trityl CH), 113.00 (pyrene CH), 123.35, 124.40 (pyrene CH), 124.67, 124.88 (pyrene C_{quat}), 125.21, 125.26, 125.94, 126.70, 127.06, 127.38 (pyrene CH), 127.42 (trityl CH), 127.75 (2 × trityl CH), 128.06 (2 × trityl CH), 129.31 (pyrene C_{quat}), 129.98 (4 × trityl CH), 130.75, 131.07, 131.19, 131.32 (pyrene C_{quat}), 135.90 (2 × trityl C_{quat}), 144.81 (trityl C_{quat}), 158.37 (2 × trityl C_{Ome}). Maldi HRMS, found *m/z* 631.2448; calculated for $C_{41}H_{36}O_5$ (M + Na^+) 631.2455. Found, C 79.36%, H 6.07%; calculated for $C_{41}H_{36}O_5 \cdot 0.7H_2O$, C 79.26%, H 6.06%.

Phosphoramidite of (*S*)-1-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*O*-(1-pyrenylmethyl)glycerol (5)

(*S*)-1-*O*-(4,4′-Dimethoxytriphenylmethyl)-3-*O*-(1-pyrenylmethyl)glycerol (**4**, 458 mg, 753 µmol), 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (453 mg, 429 µl, 1.51 mmol) and diisopropylammonium tetrazolide (193 mg, 1.13 mmol) were mixed in dry CH_2Cl_2 (7 ml) and stirred under a nitrogen atmosphere for 6 days. The product was purified by silica gel chromatography with EtOAc/cyclohexane/triethylamine (49/49/2 v/v/v) and dried under reduced pressure. Yield 438 mg (72%) as a white foam. ^{31}P NMR ($CDCl_3$): δ 150.17, 150.21. Maldi HRMS, found *m/z* 815.3810; calculated for $C_{41}H_{36}O_5$ (M + Li^+) 815.3801.

RESULTS AND DISCUSSION

Synthesis of the building block

1-Pyrenemethanol is commercially available, but it is also easily prepared from pyrene by Vilsmeier–Haack formylation (28) followed by reduction with sodium borohydride. Subsequent conversion of the alcohol with thionyl chloride affords 1-(chloromethyl)pyrene (29).

The acyclic amidite **5** was prepared from (*S*)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol and 1-(chloromethyl)-pyrene in 52% overall yield (Scheme 1). The synthesis of **5** was accomplished using KOH in the first step for the alkylation reaction (30) and then using 80% aqueous acetic acid (31) to give the diol **3**. Protection by DMT-Cl (32) and finally reaction with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite affords the target compound **5** where the yield in the latter step was 72% (21). This yield decreased from 72 to 14% if 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite was used as the phosphitylating reagent.

To increase the coupling yield in oligonucleotide synthesis, **5** was dissolved in a 1:1 mixture of acetonitrile and dichloromethane instead of in pure acetonitrile, in which **5** is poorly soluble. The phosphoramidite **5** was inserted into the growing chain of an ODN using standard nucleotide coupling conditions (2 min couplings). The coupling efficiency was over 98% as determined by detritylation measurement.

Self-complementary INAs with an intercalating pseudo-nucleotide as a dangling end

To investigate the stacking ability of **5**, it was added to the 5′ end of two different self-complementary strands (5′-XCGCGCG and 5′-XTCGCGCGA, X = **5**). The melting temperatures of modified and unmodified self-complementary oligonucleotides are shown in Table 2. Incorporation of the pyrene moiety at the 5′ end to give a dangling end stabilises the DNA duplex by 17.2 or 21.8°C (8.6 or 10.9°C per modification) depending on the underlying base pair. The stabilisations of the duplexes due to incorporation of **5** at the 5′-termini of the nucleic acid strands are similar to those found by Guckian *et al*. (33), who added a 2-deoxyribose pyrene nucleotide at the 5′ terminus of one of the very same self-complementary ODNs.

The stabilisation can be explained by MacroModel calculations, which confirm a possible structure where the pyrene moiety interacts with both nucleosides in the underlying base pair (Fig. 1).

DNA/INA duplex where a nucleotide is replaced by an intercalating pseudo-nucleotide

UV melting temperature measurements (Table 3) where a G nucleotide is replaced by the flexible, abasic linker ethylene

Table 2. Melting temperatures of self-complementary INAs with the 5′ modification

Duplex	T_m (°C)	ΔT_m (°C)
5′-CGCGCG 3′-GCGCGC	41.0	
5′-TCGCGCGA 3′-AGCGCGCT	46.9	
5′-XCGCGCG 3′-GCGCGCX	62.8	21.8
5′-XTCGCGCGA 3′-AGCGCGCTX	64.1	17.2

X = **5**.

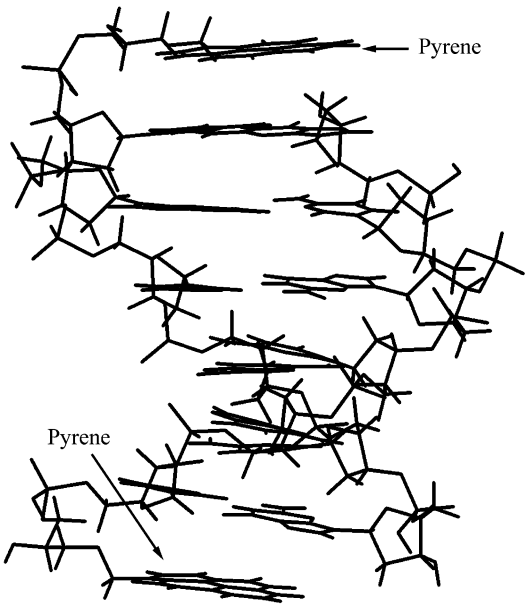


Figure 1. The structure of a self-complementary INA duplex with the sequence 5′-XCGCGCG-3′, X = **5**, found by calculations in MacroModel. The pyrene moiety is coaxially stacked with the underlying base pair.

glycol or 1,3-propandiol show a large decrease in duplex stability compared to the unmodified, fully complementary sequence (Table 3, entry 5 versus entries 1 and 2). The required DMT-protected cyanoethyl *N*,*N*-diisopropylphosphoramidites of ethylene glycol and 1,3-propandiol were synthesised by standard methods (34). The pyrene-containing IPN prepared from **5** was inserted in the same position of the hybrid instead of the abasic diols. This increased the melting temperature by 16.4–18.0°C, indicating that the pyrene is coaxially stacked with both sides of the duplex (Table 3, entry 3 versus entries 1 and 2), as the stabilisation per modification exceeds the effect of placing the pyrene IPN at the end of a duplex (Table 2).

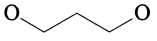
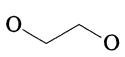
MacroModel calculations showed that IPN only makes a minor distortion of the double helix when intercalated into the duplex, having interaction with nucleobases both to the 5′ side and to the 3′ side of the intercalation site (Fig. 2). The stabilisation of the duplex by coaxial stacking of the pyrene moiety is not large enough to compensate for the loss in binding affinity due to the reduced number of hydrogen bonds by substitution of G with the IPN, the duplex with the INA being less stable than the unmodified fully complementary duplex by 8.6°C (Table 3, entry 3 versus entry 5). The same trend is found for DNA/RNA duplexes, although these have lower melting temperatures in general than the corresponding DNA/DNA duplexes. The stabilisation of the pyrene moiety is only 8.2°C for the DNA/RNA duplex when compared with ethylene glycol whereas the stabilisation is 16.4°C for the DNA/DNA duplex. Thus the pyrene insertion results in an improved discrimination between ssDNA and ssRNA giving a 9.0°C difference in the melting temperatures of their corresponding duplexes (Table 3).

INAs with intercalating pseudo-nucleosides forming bulges when hybridised

Normally the introduction of a bulge into the double helix decreases the melting temperature (35). This is also observed here when inserting a flexible, abasic linker into one of the strands (Table 4, entries 3–7). If the pyrene IPN, however, is

Table 3. DNA/INA and RNA/INA duplexes where a G nucleotide is replaced with an intercalating pseudo-nucleoside **5**

UU/ UU/
5'-AGCTTGCCTTGAG
3'-TCGAACXGAACTC

Entry	X	DNA T_m (°C)	RNA T_m (°C)	Discrimination $\Delta T_{m,\ DNA\text{-}RNA}$ (°C)
1		26.0	25.8	0.2
2		27.6	26.8	0.8
3	**5**	44.0	35.0	9.0
4	- (12-mer)	35.2	29.6	5.6
5	G (13-mer)	52.6	47.2	5.4

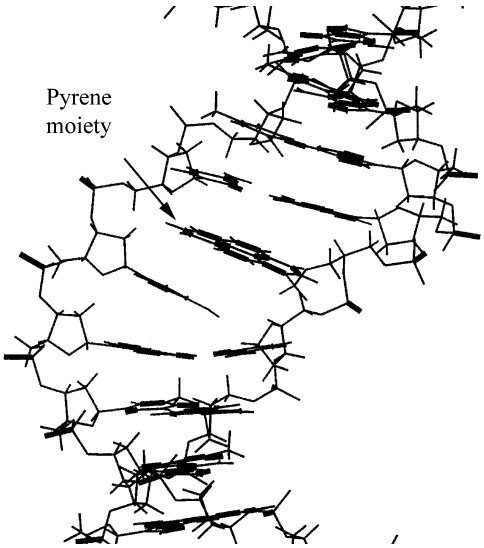


Figure 2. A slice of the calculated structure of the DNA/INA duplex 5′-AGCTTGCCTTGAG-3′ + 5′-CTCAAGXCAAGCT-3′, X = **5**. The pyrene is coaxially stacked with both the upper and lower neighbouring nucleobases of the opposite strand.

inserted as the bulge, the melting temperature of the DNA hybrid is increased by 3°C. Insertions of non-Watson–Crick binding intercalators normally tend to cause a decrease in the melting temperature, but Ossipov *et al*. found that introduction of a bulge in such a case could prevent a large destabilisation (12). Insertion of **5** as a bulge stabilises the duplex by 11.2°C compared to the flexible ethylene glycol linker, indicating that the pyrene moiety is intercalated into the duplex (Table 4, entry 3).

The difference in melting temperature between the DNA/INA duplex and the corresponding RNA/INA duplex is 12.6°C when **5** is inserted as a bulge. This difference is 7.4°C larger than in the unmodified duplexes and much larger than the differences between the duplexes containing a natural nucleotide or a flexible ethylene glycol as a bulge (Table 4). This means that the pyrene moiety is selective and only able to stabilise DNA/INA duplexes and not RNA/INA duplexes. The latter duplex has nearly the same melting temperature regardless of whether the bulge insertion contains the pyrene moiety or not, indicating that the pyrene does not intercalate in the RNA/INA duplex.

Structural calculation (MacroModel) of the pyrene-modified DNA/INA structure (Fig. 3) shows that the pyrene moiety makes only a minor distortion in the duplex and that the linker introduces enough flexibility in the backbone to have a distance of 3.4 Å between the pyrene moiety and the nucleobases of the same strand. The nucleobases in the opposite strand have a little shorter spacing between the pyrene moiety and the nucleobases than the optimum 3.4 Å (36).

To investigate the discrimination and stabilisation phenomena further, some INAs with two pyrene insertions were prepared. The results (Table 4, entries 4–7) show that, depending on the distance between two insertions and their neighbouring base pairs, it is possible to stabilise the double inserted pyrene DNA/INA duplex by up to 13.4°C (6.7°C per

Table 4. Melting temperatures of duplexes with bulging INAs hybridised to either DNA or RNA

Entry	Oligo	Target DNA 5'-AGCTTGCTTGAG				Target RNA 5'-AGCUUGCUUGAG				Discrimination T_m (DNA) - T_m (RNA)	
		T_m (°C)	ΔT_m (°C)			T_m (°C)	ΔT_m (°C)			ΔT_m (°C)	
1	3'-TCGAACGAACTC	47.4	-			42.2	-			5.2	
2	3'-TCGAAC G GAACTC	34.6	-12.8			37.8	-4.4			-3.2	
	X =			**5**				**5**			**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)
3	3'-TCGAAC X GAACTC	39.2	-8.2	50.4	3.0	37.8	-4.4	37.8	-4.4	1.4	12.6
4	3'-TCGAAC X-X GAACTC	33.2	-14.2	42.2	-5.2	33.0	-9.2	30.8	-11.4	0.2	11.4
5	3'-TCGAAC G X X AACTC	29.0	-18.4	51.4	4.0	31.6	-10.6	34.2	-8.0	-2.6	17.2
6	3'-TCGAAC GA X X ACTC	31.8	-15.6	55.6	8.2	33.2	-9.0	32.6	-9.6	-1.4	23.0
7	3'-TCGA ACGA X X ACTC	29.4	-18.0	60.8	13.4	29.0	-13.2	35.0	-7.2	0.4	25.8

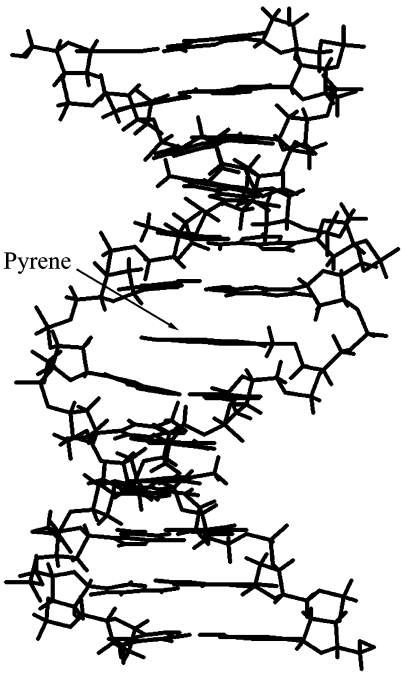


Figure 3. A slice of the calculated structure in MacroModel of a 12mer/13mer DNA/INA duplex with the sequence 5′-AGCTTGCTTGAG-3′ + 5′-CTCAAGXCAAGCT-3′, X = **5**. The pyrene moiety is able to interact with both the upper and lower neighbouring nucleobases in the opposite strand.

modification) compared to the natural DNA duplex. Both the stabilisation of the DNA/INA duplex and the destabilisation of the RNA/INA duplex is somewhat additive, so that the difference in melting temperature between the DNA/INA duplex and the RNA/INA duplex is up to 25.8°C when two pyrene moieties are inserted. For both stabilisation and discrimination the best results are obtained when the insertions are separated by 4 bp (Table 4, entry 7). When two insertions of the pyrene moieties are placed next to each other in the INA there is a decrease in melting temperature of 5.2°C compared to the unmodified DNA/DNA duplex and a decrease of 8.2°C compared to the mono modified DNA/INA duplex (Table 4, entry 4 versus entry 3). It is noteworthy that only one nucleobase pair between two IPN insertions in the DNA/INA duplex is sufficient to improve stabilisation and discrimination between DNA and RNA, when compared with the duplex where the INA strand is only modified with one insertion (Table 4, entry 5 versus entry 3).

Fluorescence of INAs

A decreased fluorescence emission upon excitation at 340 nm of the monopyrene-modified INA strands upon binding to the complementary strands indicates pyrene intercalation into the double helix (Fig. 4) (37,38). Double pyrene insertions in the oligonucleotides give the same results for all of the different INAs investigated (data not shown). The quenching effect upon hybridisation is more pronounced when the INA is hybridised with complementary ssDNA than when hybridised with complementary ssRNA (Figs 4 and 5), indicating less intercalation of pyrene into the RNA/INA duplexes. This supports the conclusion from the thermal melting experiments about the failure of pyrene intercalation into the bulged RNA/INA duplexes. The latter was deduced from the nearly identical melting temperatures with the abasic flexible ethylene glycol linker and pyrene-containing bulges when hybridised to ssRNA (Table 4).

Two pyrene moieties separated by only one nucleotide generates a third peak at 480 nm, due to excimer formation of the pyrene residues (39). However, this band is almost extinguished when this type of INA hybridises to a complementary DNA strand (Fig. 5). This indicates intercalation around an intact base pair preventing the two pyrene moieties being placed in the physical distance of ~3.4 nm needed for excimer formation. When a double pyrene IPN-modified INA hybridises to a complementary RNA, the two pyrene moieties are still able to interact since a substantial excimer band is found.

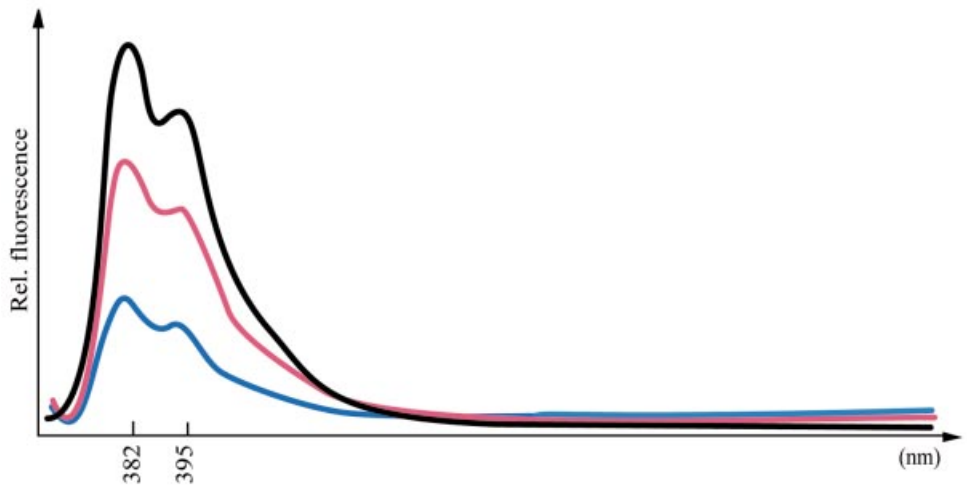


Figure 4. Fluorescence measurements of a 13mer, mono pyrene IPN-modified ssINA (black), its duplex with complementary 12mer RNA (red) and its duplex with complementary 12mer DNA (blue). The sequences are as shown in Table 4, entry 3.

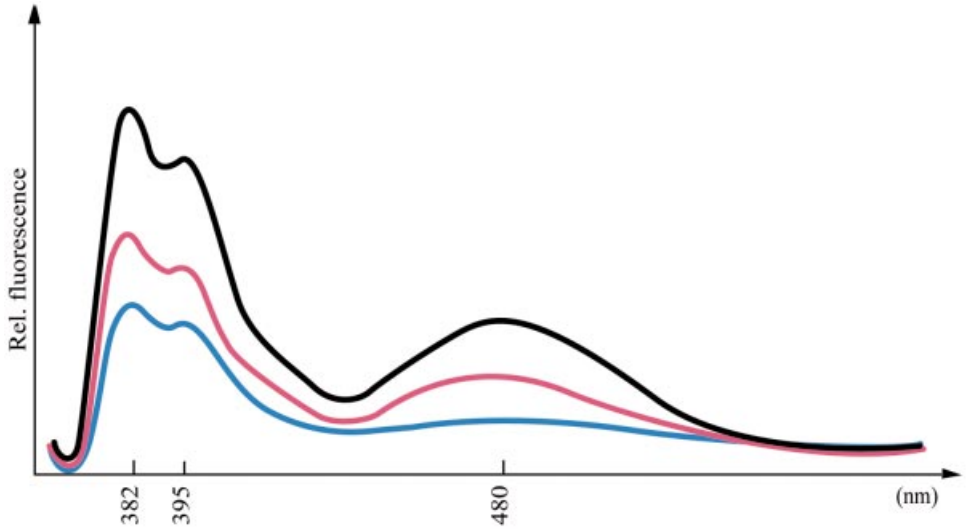


Figure 5. Fluorescence measurements of a 14mer ssINA with two pyrene IPN modifications separated by 1 nt (black), its duplex with complementary 12mer RNA (red) and its duplex with complementary 12mer DNA (blue). The sequences are as shown in Table 4, entry 5.

CONCLUSION

Here we have described the easy synthesis of an acyclic pyrene phosphoramidite **5**, which can be inserted as an IPN into oligonucleotides giving INAs in good yields using normal oligonucleotide synthesis procedures. INAs have a higher affinity for complementary ssDNA than unmodified DNA, and the duplex formed has a melting temperature that is up to 6.7°C higher per modification when the IPN is inserted internally in the formed duplex and up to 10.9°C when added as a dangling end. Furthermore, the pyrene-containing IPN introduces selectivity to INAs for complementary ssDNA over complementary ssRNA. Two appropriately placed pyrene IPNs increase the differences in melting temperature between the two types of duplexes in an additive manner, giving a difference in melting temperature for the DNA/INA duplex that is up to 25.8°C higher than for the corresponding RNA/INA duplex. Quenching of pyrene fluorescence is observed upon hybridisation as a consequence of pyrene intercalation. This phenomenon is most pronounced for the hybridisation to DNA when compared to the same sequence of RNA. The thermal melting data for duplexes support the idea of pyrene intercalation in the DNA/INA duplex and not in the RNA/INA duplex. It is believed that INAs can become an important tool for discrimination between DNA and RNA with identical sequences and that the INA technology can be used as a versatile tool in oligonucleotide applications when enhanced affinities towards DNA are required.

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