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Synthesis and hybridization properties of oligonucleotide–perylene conjugates: influence of the conjugation parameters on triplex and duplex stabilities†

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Received 15th July 2004, Accepted 4th October 2004
First published as an Advance Article on the web 3rd November 2004

We report here the synthesis of oligo-2′-deoxyribonucleotides (ODNs) conjugated with perylene. Introduction of perylene, coupled either directly or *via* a propyl linker to the anomeric position of a 2′-deoxyribose residue, induces the formation of two anomers. Single incorporations of each pure anomer of these sugar–perylene units have been performed at either the 5′-end or an internal position of a pyrimidic pentadecamer. The binding properties of these modified ODNs with their single- and double-stranded DNA targets were studied by absorption spectroscopy. Double incorporations of the sugar–perylene unit most efficient at stabilizing the triplex and duplex structures (the β-anomer involving the propyl linker) have been performed at both the 5′-end and at an internal position (or both the 5′- and 3′-ends) of the ODN chain. Comparison has been made with ODN–perylene conjugates involving either one or two perylenes attached *via* a longer polymethylene chain to either the 5′- or 3′- (or both the 5′-and 3′-) terminal phosphate groups. The ODNs involving two perylenes are more efficient at stabilizing the triplex and the duplex structures than the ODNs involving only one perylene and, among these, the ODN–perylene conjugate involving two sugar–perylene units attached at both termini is the most efficient. The results of the fluorescence studies have shown an important increase in the intensity of the fluorescent signal upon hybridization of the ODNs involving two perylenes with either the single- or the double-stranded targets. This increase in the intensity of the fluorescent signal could be used as proof of the hybridization.

Introduction

Synthetic oligonucleotides (ODNs), which are widely used in a great variety of areas such as biotechnology and diagnostics, offer interesting prospects for the regulation of gene expression.[1–5] Progress made in the genomic sequencing of an increasing number of organisms, including the human genome, opens a new application for modified ODNs as tools for functional genomics and for validating targets.[6,7] To be efficient in regulating gene expression, antisense and anti-gene ODNs must efficiently cross cell membranes, be resistant to nucleases and specifically and strongly hybridize to their targets on mRNA or double-stranded DNA. Numerous ODN analogs involving a modified backbone or nucleic acid bases conjugated, or not, to various ligands have been engineered in order to fulfill these requirements.[8–12] Despite these efforts, the stability of triplexes has needed further improvement in many cases. One way to stabilize the triplexes stems from the covalent attachment of intercalators.[13–21] A perylene derivative has been used to bridge the two pyrimidine strands of DNA in a pyrimidine–purine–pyrimidine triplex.[22] The planar linker involving seven rings provides interactions with all three base residues. To test the ability of the perylene derivative involving five fused six-membered rings to stabilize duplex and triplex structures, we previously linked it to the 5′- and the 3′-ends of a pyrimidine decamer *via* the terminal phosphates.[23] The results showed strong stabilization of the triplex when the perylene was attached to the 5′-end of the sequence. Since a survey of the literature together with the results of our previous work clearly showed that the properties of ODN–intercalator conjugates were dependent on the parameters of the linkage between both entities,[16–18,21,24] we chose to prepare other ODN–perylene conjugates by covalently linking perylene to the anomeric position of a 2′-deoxyribose, that could be incorporated at various positions inside the sequence. In a preliminary report we showed that when linked to the anomeric position of a 2′-deoxyribose *via* a propynyl linker, the perylene stabilizes both the duplex and the triplex structures.[25] In order to further explore the possibility of increasing the duplex and triplex stabilities, we chose to incorporate new sugar–perylene units, involving the perylene attached either directly or *via* a propyl linker to the anomeric position of the sugar residue, into a pyrimidic pentadecamer and to compare the binding properties of these new ODNs with those of the corresponding ODNs involving the perylene attached *via* a longer polymethylene linker to the terminal phosphodiester groups. The influence of the incorporation of two perylene units per ODN on the duplex and triplex stabilities was also studied. We report here the preparation of these new ODN–perylene conjugates, as well as their binding properties with complementary single- and double-stranded DNA target sequences which were studied using absorption and fluorescence spectroscopies.

Results and discussion

Perylene was covalently linked to a 15-mer oligopyrimidic sequence d-$^{5'}$TTCTTTTTCTTCTCT$^{3'}$ *via* the anomeric position of a 2′-deoxyribose unit without a linker, or by the intermediate of a trimethylene linker. Parameters such as the anomeric configuration and the incorporation position of the sugar–perylene units inside the sequence were investigated. To study the influence of the parameters of the linkage between the ODN and the perylene, the latter was also linked to the terminal phosphates of the ODN *via* polymethylene linkers. Double incorporations of the perylene were also performed. We report here the synthesis of these ODN–perylene conjugates and the results of their binding properties with both their double- and single-stranded DNA

† Electronic supplementary information (ESI) available: ^{1}H NMR data for fully deprotected compounds **28**$_\alpha$, **28**$_\beta$, **33**$_\alpha$ and **33**$_\beta$; and ^{1}H NMR data for *H*-phosphonate derivatives **30**$_\alpha$, **30**$_\beta$, **35**$_\alpha$ and **35**$_\beta$. See http://www.rsc.org/suppdata/ob/b4/b410695d/

DOI: 10.1039/b410695d

targets, evaluated by absorption and fluorescence spectroscopy. The structures of the modified ODNs **4**–**18** and the ODNs used as targets **1** and **2** and reference **3** are depicted in Fig. 1.

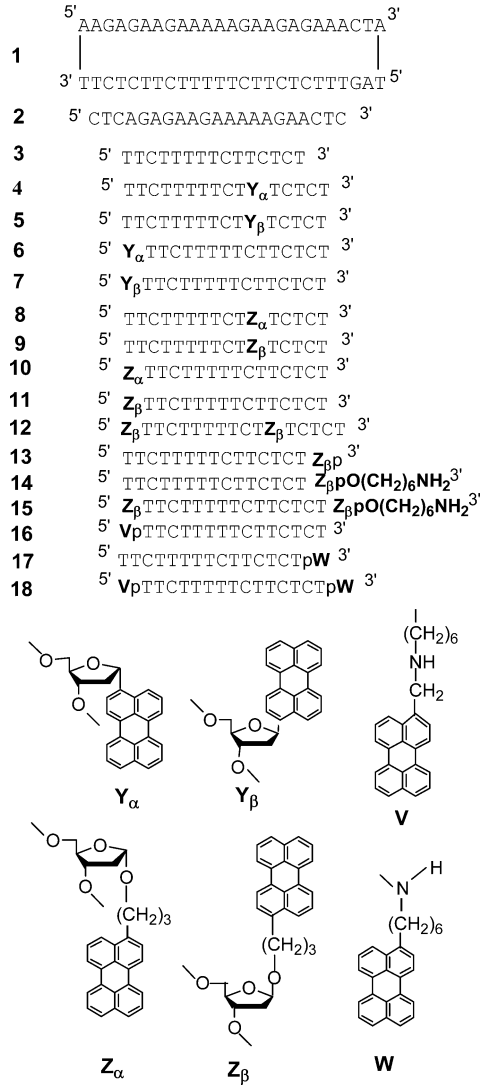


Fig. 1 Chemical structures of the ODNs **1**–**18**.

Synthesis

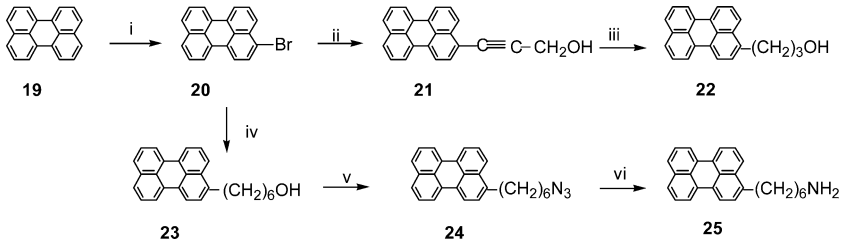
Perylene linkers derivatives. The perylene linker derivative **22**, involving a propyl linker, was obtained following a three-step procedure (Scheme 1). A Sonogashira coupling reaction between 3-bromoperylene **20**, obtained from perylene **19** following a reported procedure,[26,27] and propargyl alcohol led to the 3-(3-hydroxy-1-propynyl)perylene **21**. The 3-(3-hydroxypropyl)perylene **22** was then obtained by catalytic hydrogenation of **21**. The perylene linker **25** was obtained following a two-step procedure starting from the 3-(6-hydroxyhexyl)perylene **23** (Scheme 1). The latter was obtained following a procedure adapted from that of Schlichting *et al*.[27] The azido-derivative **24** was obtained by treatment of **23** with LiN_3, CBr_4 and triphenylphosphine, after which the 3-(6-aminohexyl)perylene **25** was obtained by catalytic hydrogenation of compound **24**.

Sugar–perylene units. The protected sugar–perylene derivatives $\mathbf{27}_\alpha$ and $\mathbf{27}_\beta$ were obtained by glycosylation of perylene **19** with 1-*o*-methyl-2-deoxy-3,5-di(*p*-toluoyl)-*erythro*-pentafuranose **26**[28] in the presence of $SnCl_4$ (Scheme 2). A mixture of anomers was obtained and separated by HPLC using a silica column with 43% yield and a β : α, 73 : 27 ratio. The anomeric configuration was assigned by 2D NMR (NOE) experiments performed on the fully deprotected compounds (*vide infra*). The protected sugar–perylene derivatives $\mathbf{32}_\alpha$ and $\mathbf{32}_\beta$ involving the propyl linker were obtained by glycosylation between 3-(3-hydroxypropyl)-perylene **22** and 1-α-chloro-2-deoxy-3,5-di(*p*-toluoyl)-*erythro*-pentafuranose **31**[29], in the presence of $ZnBr_2$, $NaHCO_3$ and 4 Å molecular sieves (Scheme 2). A mixture of anomers was obtained and separated by semi-preparative chromatography on silica plates with 78% yield and a β : α, 62 : 38 ratio (*vide infra*). The 5′- and 3′-hydroxyl functions of the fully protected sugar–perylene units $\mathbf{27}_\alpha$, $\mathbf{27}_\beta$, $\mathbf{32}_\alpha$ and $\mathbf{32}_\beta$ were deprotected by treatment with a sodium methylate solution to give the unprotected sugar–perylene units $\mathbf{28}_\alpha$, $\mathbf{28}_\beta$, $\mathbf{33}_\alpha$ and $\mathbf{33}_\beta$. The latter were then dimethoxytritylated on their 5′-hydroxyl function, using a classical procedure, to give compounds $\mathbf{29}_\alpha$, $\mathbf{29}_\beta$, $\mathbf{34}_\alpha$ and $\mathbf{34}_\beta$, which were transformed into their 3′-phosphonate derivatives $\mathbf{30}_\alpha$, $\mathbf{30}_\beta$, $\mathbf{35}_\alpha$ and $\mathbf{35}_\beta$ by treatment with 2-chloro-4 H-1,3,2-benzodioxaphosphorin-4-one[30] using classical conditions.

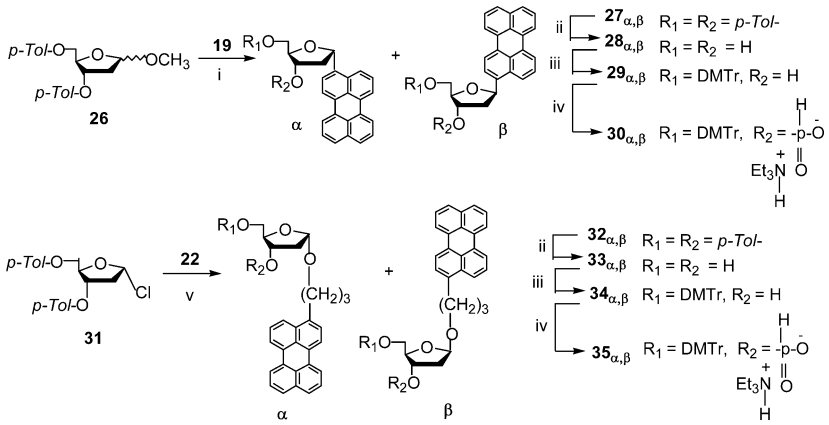
Structural assignments. Structural assignments of the anomeric configuration for sugar–perylene units $\mathbf{28}_\alpha$, $\mathbf{28}_\beta$, $\mathbf{33}_\alpha$ and $\mathbf{33}_\beta$ were made by 1H NOE studies. The method used is based on the separate irradiation of the H-2′ proton resonances and the observation of enhancements at vicinal 1′ and 3′ protons.

Examination of the structures of α and β sugar–perylene units shows that for α-anomers the 2′-β proton is in close proximity to both the 1′ and the 3′ protons, while the 2′-α proton is not near either of these protons. In the β-anomers, the 2′-α is only near the 1′ proton while the 2′-β proton is near only the 3′ proton. Thus, in an α-anomer separate irradiation of each of the 2′ protons should lead to two and zero enhancements at the vicinal protons, while in a β-anomer these two irradiations would lead to one significant enhancement for each irradiation. The results, indicated in Table 1, confirm that for one anomer (assigned to be in the α configuration) of each pair only one irradiation gives two strong enhancements of the vicinal protons, while for the other anomer (assigned to be in the β configuration) one significant enhancement is observed for each of the two irradiations.

ODN–perylene conjugates. In a series of syntheses, sugar–perylene units, involving the perylene attached *via* its 3′-position with or without a propyl linker to the anomeric position of the sugar, were incorporated either between the fifth and the sixth base of the sequence (starting from the 3′-end) or at the 5′-end of the sequence. The internal position was chosen to allow intercalation of the perylene unit between the TxAT



Scheme 1 Synthesis of the perylene derivatives **22** and **25**. *Reagents and conditions*: i: ref. 26, ii: propargyl alcohol, THF–piperidine, CuI, tetrakis(triphenylphosphine) palladium(0); iii: H_2, Pd/C, THF; iv: ref. 27; v: PPh_3, LiN_3, CBr_4, DMF; vi: H_2, Pd/C, THF.



Scheme 2 Synthesis of the sugar–perylene units **27**–**30** and **32**–**35**. *Reagents and conditions*: i: $C_2H_4Cl_2$, $SnCl_4$; ii: MeONa, CH_2Cl_2–MeOH (50 : 50, v/v); iii: DMTrCl, Py; iv: 2-chloro-4*H*-1,3,2-benzodioxaphosphorine-4-one, CH_2Cl_2–pyridine (4 : 6, v/v); v: $ZnBr_2$, $NaHCO_3$, 4 Å molecular sieves, THF.

Table 1 Proton NOE data for fully deprotected sugar–perylene units $\mathbf{28}_\alpha$, $\mathbf{28}_\beta$, $\mathbf{33}_\alpha$ and $\mathbf{33}_\beta$

		Irradiation at:	
Compounds	NOE observed	H2′-β (%)	H2′-α (%)
$\mathbf{28}_\alpha$	H1′	100.0	13.3
	H3′	67.6	18.1
$\mathbf{28}_\beta$	H1′	10.2	100.0
	H3′	103.3	34.1
$\mathbf{33}_\alpha$	H1′	103.4	35.1
	H3′	100.0	9.2
$\mathbf{33}_\beta$	H1′	20.0	100.0
	H3′	28.9	−2.4

base triplets. Coupling at the 5′-position of the sequence was also chosen because studies carried out with other intercalator–ODN conjugates showed that in the case of triple-stranded DNA structures the junction between the triplex and the overhanging double-stranded DNA target was a preferential binding site for the intercalators. The preparation of these ODNs was performed by manual incorporation of the sugar–perylene units *via* their *H*-phosphonate derivatives during the ODN chain elongation. After a standard deprotection step, purification was carried out by reversed-phase chromatography to give ODNs **4**–**12**. For the incorporation of the sugar–perylene unit at the 3′-end of the ODNs **14** and **15** we chose a support described for the preparation of 3′-aminooligonucleotides.[31] This choice resulted in higher yields than by using a previously described 3′-phosphate support[32] initially chosen for the incorporation of the sugar–perylene unit at the 3′-end (ODN **13**). Deprotection and purification steps were performed as for ODNs **4**–**12**. The ODN **16**, bearing the perylene *via* the 5′-terminal phosphate, was obtained using our previously reported perylene phosphoramidite derivative.[23] ODN **17** was obtained by reacting the perylene derivative **25** with an *H*-phosphonate group, obtained after coupling of the *H*-phosphonate derivative of the 3′-terminal nucleoside, as previously reported,[23] followed by the full length sequence assembly.

The perylene derivative **25**, with a hexamethylene linker, was used in place of our previously reported perylene derivative involving a linker containing a secondary amino function.[23] This change was made to prevent acetylation of the amino function during the ODN chain assembly that led to a weak stabilization of the complexes involving the acetylated ODNs owing to steric hindrance.[23] ODN **18** was obtained as described for the preparation of ODN **17** and by coupling the second perylene to the 5′-end of the sequence *via* the phosphoramidite derivative of perylene.[23] Purification of the ODNs was performed by reversed-phase chromatography. The retention times (Table 2) for the ODNs **5**, **7**, **9** and **11**, involving the perylene at the β-anomeric position of the sugar, were a little higher than those of the corresponding ODNs **4**, **6**, **8**, and **10**, involving the perylene at the α-anomeric position of the sugar. Retention times of ODNs involving two perylene derivatives **12**, **15** and **18** were higher than those of the ODNs involving only one perylene (ODNs **4**–**11**, **13**, **14**, **16** and **17**). These results indicate the formation of more lipophilic compounds. The UV-visible spectra of the ODN–perylene ODNs were recorded between $\lambda = 230$ nm and $\lambda = 500$ nm. The spectra contains two main absorption bands in the visible region between $\lambda = 350$ nm and $\lambda = 500$ nm (where only the perylene absorbs light) with $\lambda_{\text{max vis}} \approx 448$–450 nm and $\lambda_{\text{max vis}} \approx 420$–423 nm, with a shoulder at $\lambda \approx 395$ nm, for all the ODNs. The other absorption band in the UV range corresponded to the absorbance of the ODN and the perylene with a $\lambda_{\text{max UV}} \approx 259$–267 nm for all the ODNs. The comparison of the two series of ODNs involving a single sugar–perylene unit incorporation showed that in each series the positions of the main absorption bands were identical; independent of the position of incorporation or the anomeric configuration of the sugar–perylene unit. Only the UV : visible absorbance ratio was slightly different inside each series (UV : visible absorbance ratio ≈ 4.25–4.50 for ODNs **4**–**7** and ≈ 4.40–4.65 for ODNs **8**–**11**). The UV-visible spectra of ODNs **13** and **14**, involving a β-sugar-perylene unit with a propyl linker at the 3′-end of the sequence, and those of the ODNs **16** and **17**, involving a polymethylene linker to connect the perylene to the ODN *via* a phosphodiester linkage, were nearly identical to that of the ODN **11**, involving a β-sugar–perylene unit with a propyl linker at the 5′-end of the sequence. In the case of the ODNs **12**, **15** and **18**, involving two perylenes, the $\lambda_{\text{max vis}} \approx 449$ and ≈ 423 nm were unchanged, while a slight blue-shift (≈ 7–8 nm) of the $\lambda_{\text{max UV}}$ was observed as compared to the $\lambda_{\text{max UV}}$ (265–267 nm) of the corresponding ODNs involving only one perylene. The main change concerns the UV : visible absorbance ratio which is lower in the case of the ODNs involving two perylenes **12**, **15** and **18** (3 to 3.4), as compared to those observed for the corresponding ODNs involving only one perylene (*vide supra*). The UV-visible spectra of the ODN–perylene ODNs **11** and **15** are shown in Fig. 2. All the modified ODNs were characterized by electrospray mass spectrometry (Table 2).

Thermal denaturation studies and fluorescence measurements

Thermal denaturation studies. Experiments were followed by absorption spectroscopy. One transition was observed in the melting profile of each duplex while two transitions were observed in the melting of each triplex (data not shown). The double-stranded target has previously been circularised through the use of two hexaethylene linkers in order to increase its

Table 2 Characterizations for ODNs **1**, **3**–**18**. *T*ms and fluorescence data. Column 1: Retention times obtained by reversed-phase HPLC analyses for the ODNs **1** and **3**–**18** performed on a Lichrospher RP 18 (5 μm) column (125 mm × 4 mm) from Merck using a linear gradient of CH_3CN (12.5% to 42.5% over 40 min) in 0.1 M aqueous TEAA, pH 7, with a flow rate of 1 cm^3 min^{-1}. Column 2: Mass analysis data for ODNs **1** and **3**–**18**. Column 3: *T*m values for triplexes formed between ODNs **3**–**18** and the double-stranded target **1** in a 10 mM sodium cacodylate, pH 7, buffer containing 140 mM KCl and 5 mM $MgCl_2$. ODN concentrations were 1 μM of the circularized duplex target **1** and 1.5 μM of the third strand. Column 4: *T*m values for duplexes formed between ODNs **3**–**18** and the single-stranded DNA target **2** in a 10 mM sodium cacodylate, pH 7, buffer containing 100 mM NaCl. ODN concentrations were 1 μM (each strand). Column 5: Relative fluorescence intensity between duplexes and ODNs **4**–**18**, at 16 °C, in the same buffer as used for the melting studies. Column 6: Relative fluorescence intensity between triplexes and ODNs **9**, **11**, **12**, **15** and **18**, at 4 °C, in the same buffer as used for the melting studies

		Mass analysis		Triplexes		Duplexes			
ODNs	Reversed-phase Rt/min	Calculated	Found	*T*m/°C	Δ*T*m	*T*m/°C	Δ*T*m	F_{duplex}/F_{ODNs}	$F_{triplex}/F_{ODNs}$
1	—	$C_{517}H_{668}N_{182}O_{318}P_{52} = 16130.66$	16134.41	—	—	—	—	—	—
3	6 min 20 sec	$C_{146}H_{192}N_{34}O_{99}P_{14} = 4440.93$	4440.6	17.0	—	38.0	—	—	—
4	9 min 46 sec	$C_{171}H_{211}N_{34}O_{104}P_{15} = 4871.33$	4871.06	18.5	+1.5	41.0	+3.0	1.20	—
5	10 min 23 sec	$C_{171}H_{211}N_{34}O_{104}P_{15} = 4871.33$	4870.97	16.0	−1.0	40.0	+2.0	0.91	—
6	15 min 11 sec	$C_{171}H_{211}N_{34}O_{104}P_{15} = 4871.33$	4871.01	19.0	+2.0	44.0	+6.0	1.03	—
7	18 min 47 sec	$C_{171}H_{211}N_{34}O_{104}P_{15} = 4871.33$	4871.33	25.5	+8.5	45.0	+7.0	0.97	—
8	15 min 05 sec	$C_{174}H_{217}N_{34}O_{105}P_{15} = 4929.41$	4929.38	25.0	+8.0	43.0	+5.0	1.63	—
9	15 min 31 sec	$C_{174}H_{217}N_{34}O_{105}P_{15} = 4929.41$	4929.10	25.0	+8.0	40.0	+2.0	1.73	0.50
10	24 min 20 sec	$C_{174}H_{217}N_{34}O_{105}P_{15} = 4929.41$	4929.22	27.0	+10.0	45.0	+7.0	1.22	—
11	26 min 53 sec	$C_{174}H_{217}N_{34}O_{105}P_{15} = 4929.41$	4929.30	30.0	+13.0	45.0	+7.0	1.19	1.06
12	30 min 05 sec	$C_{202}H_{242}N_{34}O_{111}P_{16} = 5417.89$	5416.81	36.0	+19.0	44.0	+6.0	3.71	1.46
13	21 min 45 sec	$C_{174}H_{218}N_{34}O_{108}P_{16} = 5009.39$	5008.52	25.0	+8.0	44.0	+6.0	1.10	—
14	20 min 18 sec	$C_{180}H_{231}N_{35}O_{108}P_{16} = 5108.57$	5107.93	25.0	+8.0	44.5	+6.5	1.05	—
15	34 min 08 sec	$C_{208}H_{256}N_{35}O_{114}P_{17} = 5597.04$	5595.84	39.0	+22.0	51.0	+13.0	3.0	2.12
16	15 min 25 sec	$C_{173}H_{218}N_{35}O_{102}P_{15} = 4884.42$	4883.97	30.0	+13.0	44.0	+6.0	0.97	—
17	28 min 32 sec	$C_{172}H_{216}N_{35}O_{101}P_{15} = 4854.39$	4850.95	25.0	+8.0	44.0	+6.0	1.05	—
18	33 min 37 sec	$C_{199}H_{242}N_{36}O_{104}P_{16} = 5297.84$	5292.14	37.0	+20.0	46.0	+8.0	2.90	2.76

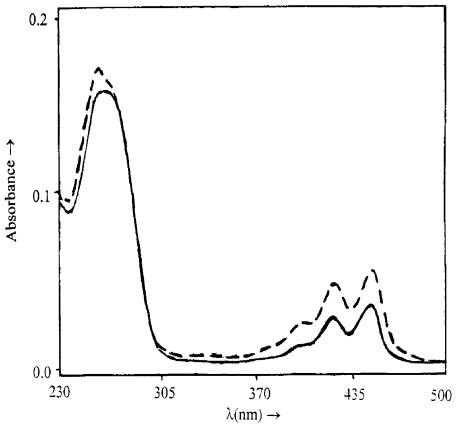


Fig. 2 Absorption spectra of the ODNs **11** (full line) and **15** (broken line) recorded between 230 and 500 nm in a 10 mM sodium cacodylate, pH 7, buffer containing 100 mM NaCl.

stability, allowing the dissociation of the third strand to be obtained before the melting of the duplex target.[33] The transition with the higher *T*ms corresponds to the melting of the target duplex (around 77 °C for all complexes, data not shown) and the transitions with lower *T*ms to the dissociation of the third strand. The *T*m values, listed in Table 2, indicate that the triplex and duplex structures formed between the modified ODNs **4**–**18** and the duplex **1** or single-stranded **2** targets were stabilized in most cases (except in the case of the hybridization of ODN **5** with the double-stranded DNA target), as compared to the corresponding complexes formed with the unmodified ODN **3** which was used as a reference.

ODNs involving one perylene. *Triplex stabilization*. The following observations can be made. Among the sugar–perylene units, those involving a linker (ODNs **8**–**11**) are the most efficient at stabilizing the triplex structures. When the incorporation was performed at internal positions of the sequence (ODNs **4**, **5**, **8** and **9**) the presence of a linker, between the perylene and the 2′-deoxyribose, was necessary to obtain a significant stabilization. In this case, an equivalent stabilization (Δ *T*m = +8 °C) was observed with both anomers (ODNs **8** and **9**). The absence of stabilization induced by the incorporation of the sugar–perylene unit, without the linker, at internal position of the ODN can be attributed to a problem of steric hindrance. When the incorporation was performed at the 5′-end of the ODN (ODNs **6**, **7**, **10** and **11**) a significant stabilization was obtained (Δ *T*m ≥ +8.5 °C), except in the case of ODN **6** involving the α-anomer of the sugar–perylene unit without the linker. However, the strongest stabilization (Δ *T*m = +13 °C) was obtained by the incorporation of the β-anomer of the sugar–perylene unit involving the propyl linker (ODN **11**). The stronger stabilization provided by the incorporation of the β-anomer (ODNs **7** and **11**) compared to that of the α-anomer (ODNs **6** and **10**) at the 5′-end of the third strand can be explained by the fact that in the case of the β-anomer the stacking of the perylene with the base triplet at the triplex–duplex junction is favoured. The stabilization observed in the case of the ODN **11** is equivalent to that obtained when the perylene was attached *via* a polymethylene linker to the 5′-terminal phosphate of the ODN (ODN **16**). A lower, but similar, stabilization (Δ *T*m = +8 °C) was obtained with the three ODNs **13**, **14** and **17** involving the perylene linked to the 3′-end. In the case of ODN **14**, the presence of an alkyl amino linker at the 3′-end did not increase the stability of the triplex as compared to that of the triplex involving the ODN **13**, ending with a phosphate group at the 3′-end. This can be due to unfavourable interactions between the linker and the 3′-end of the triplex structure.

Duplex stabilization. In the case of the duplex structure, the difference in the stability increase observed was less dependent on the position of the sugar–perylene unit than in the case of the triplex structure. Except in the case of the incorporation of the sugar–perylene at the internal position of the sequence (ODNs **4**, **5**, **8** and **9**), leading to a weaker stabilization, the maximum stabilization observed was +7 °C. These results indicate that the perylene is more efficient in stabilizing the triplex than the duplex structures as previously observed.[23,25]

ODNs involving two perylenes. *Triplex stabilization*. In all cases, the presence of a second perylene (ODNs **12**, **15**, **18**)

induced a stabilization increase of the triplex as compared to that obtained with the ODNs involving only one perylene (Fig. 3). The strongest stabilization was observed with ODN **15**, involving two sugar–perylene units at both the 5′- and the 3′-ends of the ODN (ΔTm = +22 °C). This ΔTm increase was nearly equal to the sum of the stability increase observed with ODNs **11** (ΔTm = +13 °C) and **14** (ΔTm = +8 °C) involving the sugar–perylene unit at the 5′- and the 3′-ends, respectively. When the perylenes were linked *via* an hexamethylene linker to the terminal phosphates (ODN **18**) a ΔTm = +20 °C was observed. The incorporation of sugar–perylene units at both the 5′-end and the internal position (ODN **12**) also led to an important stabilization of the triplex (ΔTm = +19 °C). This marked increase in the stability of the triplex structures provided by the presence of a second intercalator was also previously reported in the case of ODN–naphtalene conjugates.[20]

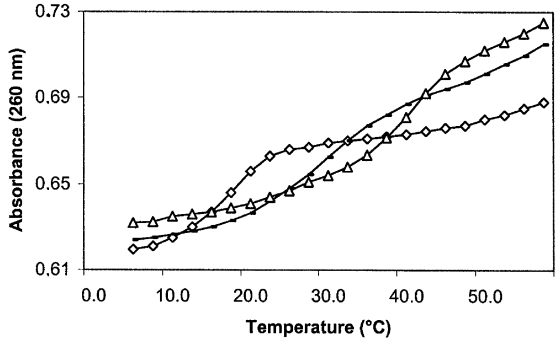


Fig. 3 UV melting profiles (recorded at $\lambda = 260$ nm) corresponding to the third strand dissociation of the triplex formed by ODNs **11** (–) and **15** (Δ) and by the unmodified ODN **3** (◊) in the presence of the circularised duplex **1**. Concentrations were 1 μM of the target and 1.5 μM of the third strands in a 10 mM sodium cacodylate buffer, pH 7, containing 140 mM KCl and 5 mM $MgCl_2$.

Duplex stabilization. In the case of the duplex, the stabilization provided by the presence of two perylene units was almost equivalent to that observed with ODNs involving only one perylene, except in the case of ODN **15** involving a sugar–perylene unit at each end of the sequence (ΔTm = +13 °C). This weak stability increase provided by the attachment of two intercalators (*via* terminal phosphates) to an ODN as compared to the stability of the duplexes formed with the same ODN linked to only one intercalator previously been observed with ODN–acridine conjugates.[24]

Fluorescence studies

Fluorescence emission spectra of ODNs **4**–**18** were compared with those of the duplexes and triplexes. The $\lambda_{max\ em}$ were almost equivalent for all the ODNs (451 nm for ODNs **4**–**7**; 453 nm for ODNs **8**–**11**, **13** and **14**; 454 nm for ODNs **12**, **15**, **16** and **18**; and 455 nm for ODN **17**). Upon hybridization with either the double-stranded **1** or the single-stranded target **2**, the $\lambda_{max\ em}$ did not change as compared to those observed for the ODNs. The main changes concerned the intensity of the fluorescent emission (Table 2).

ODNs involving one perylene. In the presence of the single-stranded target sequence **2**, two tendencies were clearly observed. In the case of the ODNs **4**–**7**, **13**, **14**, **16** and **17**, the intensity of the fluorescence emission was only slightly modified in the presence of the target. More important changes, (a little more than 50% of the intensity increase) were observed in the case of ODNs **8**–**11**, involving the sugar–perylene unit with the propyl linker. The weak fluorescence increase observed for these ODNs, upon hybridization with the target sequence, was previously observed in the case of the linkage of a perylene derivative to the 3′-end of an ODN *via* a pseudonucleoside unit.[34] In the presence of the double-stranded target sequence **1**, a 50% decrease in the fluorescence intensity was observed for the ODN **9**, while no change was observed for the ODN **11**.

ODNs involving two perylenes. In the case of the modified ODNs involving two perylene units **12**, **15** and **18**, a greater increase of the fluorescence intensity signal was observed upon hybridization with the single-stranded target **2** (3.71, 3.0 and 2.90 fold, respectively). Using the same conditions, no change in the fluorescence intensity signal was observed upon hybridization of ODN **15** with another single-stranded target sequence [d-$^{5'}$CCCCTTTTCTTTTAAAAAGTGGC$^{3'}$], indicating that the fluorescent signal modification was specific to the hybridization. The fluorescence intensities of the duplexes involving two perylenes differed little from those of the duplex involving only one perylene. But the fluorescence intensities of the modified ODNs involving two perylenes were lower than those of the modified ODNs involving only one perylene. This corresponds to a quenching of the fluorescence of the modified ODNs **12**, **15** and **18** with two perylenes. The most efficient quenching was observed with ODN **12**, involving the perylenes linked at both the 5′-position (*via* a sugar residue) and the internal position (after ten nucleotides). When the two perylenes are linked to the ends of the sequence, the distance is greater, but the intramolecular folding brings the two perylenes into closer proximity allowing the quenching.

Studies carried out with ODNs **12**, **15** and **18** and the double-stranded target **1** also indicated an increase in the fluorescence intensity signal (1.46, 2.12 and 2.76 fold, respectively).

Conclusions

We have reported the synthesis and the binding properties with double- and single-stranded targets, studied by absorption and fluorescence spectroscopy, of a series of pyrimidine sequences covalently linked to perylene. The linkage of the latter to the ODN sequence was performed *via* the anomeric position of a 2′-deoxyribose or *via* polymethylene linkers attached to the 5′- and 3′-terminal phosphates. In the case of the incorporation of the perylene by the intermediate of the sugar–perylene units, the polycyclic aromatic molecule was either directly connected to the anomeric position of the sugar residue or *via* a propyl linker. In each case, the sugar–perylene units were obtained as pure α- and β-anomers and incorporated separately into the ODNs. First, single incorporations were performed at either the 5′-end or an internal position of the sequence. The results of the binding studies indicated that the presence of a linker between the sugar residue and the perylene is necessary to obtain a significant stabilization of both the triplex and duplex structures. In the absence of the linker it is probable that, due to a problem of steric hindrance, the perylene cannot adopt a position favourable to afford a good stabilization. When a linker was used to connect the perylene and the sugar, in all cases stabilization was provided by the presence of the perylene and, in most examples, the perylene was more efficient at stabilizing the triplex than the duplex structures. However, the strongest stabilization was observed when the β-anomer was incorporated at the 5′-end of the sequence. In this case, the stabilization provided by the use of a sugar–perylene unit, involving the propyl linker, was equivalent to that obtained when the perylene was attached to the terminal phosphate of the ODN *via* a polymethylene linker. After the most efficient sugar–perylene unit was selected, it was incorporated twice at both the 5′-and the 3′-ends (or both the 5′-end and an internal position) of the ODN. Two perylenes were also attached to both the 5′- and the 3′- terminal phosphates of the ODNs *via* flexible linkers. In the case of triplex formation, the covalent attachment of two perylene units led to a higher stability increase than in the case of the linking of only one perylene unit to the same sequence (the ΔTm increase is the sum of the ΔTm increase observed for ODNs involving one perylene at either position). The best result was obtained

with the incorporation of two sugar–perylene units at each end of the ODNs, probably because the entropic factor was more favourable than with ODNs involving the perylenes attached *via* longer flexible linkers. These results confirm the paramount influence of the linkage parameters between the perylene and the ODNs. In addition, in the case of the ODNs involving two perylenes a significant intensity increase of their fluorescent signal was observed upon their hybridization with both their single-stranded and double-stranded targets that can be used as a proof of their binding.

Experimental

General methods

All solvents used were of the highest purity and did not contain more than 10 ppm H_2O. All chemicals were used as obtained unless otherwise stated. The reagents were dried for 15 h in a desiccator containing KOH and P_2O_5 under a reduced pressure (20 mm Hg). During all the synthesis and purification steps perylene derivatives were protected from light. Analytical thin-layer chromatography (TLC) was performed on precoated alumina plates (Merck silica gel 60F 254 ref. 5554) and preparative TLC on glass-backed silica plates (Merck silica gel 60 F254 ref. 5717). For flash chromatography, Merck silica gel 60 (40–63 μM) (ref. 9385 from Merck) was used. NMR spectroscopy was performed on a Varian Unity 500 spectrometer. ^{1}H Chemical shifts were referenced to either residual solvent peak DMSO (2.54 ppm) or Me_4Si. ^{31}P Chemical shifts were referenced to H_3PO_4 (external reference). ^{1}H NMR coupling constants are reported in Hz and refer to apparent multiplicities. Mass analysis was performed on a Quattro II (Micromas) instrument. ODNs were synthesized using cyanoethyl phosphoramidite chemistry and an Expedite Nucleic Acid Synthesis System 8909 from Perseptive Biosystems. Reversed-phase chromatography analysis and purification were performed on a 600 E System Controller equipped with a Waters 990 photodiode array detector. UV spectra were recorded on an Uvikon 860 spectrophotometer. Fluorescence spectra were recorded on a Fluoromax 2 (ISA-Jobin-Yvon) spectrofluorimeter in 0.5 cm path-length Suprasil quartz cuvettes (Hellma) with slits set at 0.5 mm (band pass = 2 nm).

Synthesis

Perylene-linker derivatives.

3-(3-Hydroxypropyl) perylene ***22*** *(Scheme 1).* 3-Bromoperylene **20** obtained as previously reported[26] (1.01 g, 3.05 mmol) was placed in a round-bottomed flask, dried in a dessicator and solubilized with a THF–piperidine (50 : 50, v/v) mixture (60 cm^3). Propargyl alcohol (342 mg, 6.10 mmol) and then tetrakis-(triphenylphosphine) palladium(0) (156 mg, 135 μmol) and copper iodide (32 mg, 168 μmol), previously dried over P_2O_5 in an oven at 60 °C for 4 h under a reduced pressure (20 mm Hg), were added under an argon atmosphere. The reaction mixture was refluxed at 70 °C for 2 h, poured into an ice–HCl (3 : 1, v/v) mixture (180 cm^3) and extracted with CH_2Cl_2 (300 cm^3 and then 150 cm^3 × 3). The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash chromatography (CH_2Cl_2) to give **21** as a yellow solid (615 mg, 66%). Rf_{21} 0.28, Rf_{20} 0.88 (CH_2Cl_2). δ_H (DMSO-*d*6) 4.5 (2H, s, CH_2), 7.50–7.90 (5 H, m, Ar-H), 8.15 (1H, d, *J* 9.0, Ar-H), 8.30–8-48 (5 H, m, Ar-H).

Compound **21** (940 mg, 3.07 mmol) was placed in a round-bottomed flask, solubilized with THF (90 cm^3) and 10% Pd/charcoal (280 mg) was added under argon. The flask was evacuated and flushed twice with argon, equipped with a balloon filled with hydrogen, evacuated and flushed twice. After 15 h of reaction, the mixture was filtered over Celite, concentrated and the residue was purified by flash chromatography (CH_2Cl_2) to give the perylene-linker derivative **22** as a yellow solid (800 mg, 84%). Rf_{22} 0.13 (CH_2Cl_2). δ_H (DMSO-*d*6) 1,76–1.85 (2H, m, CH_2), 3.02 (2H, t, *J* 7.8, Ar-CH_2), 3.46–3.54 (2H, m, CH_2O), 4.56 (1H, t, *J* 4.9, O*H*), 7.40 (1 H, d, *J* 7.8, Ar-H), 7.48–7.60 (3H, m, Ar-H), 7.75 (2 H, t, *J* 8.8, Ar-H), 7.94 (1 H, d, *J* 7.8, Ar-H), 8.22–8.38 (4H, m, Ar-H). ESI-MS: *m*/*z*, $C_{23}H_{18}O$ calc. 310.4, found 311.2 (M + H$^+$).

3-(6-Aminohexyl) perylene ***25*** *(Scheme 1).* The synthesis of the perylene linker derivative **23** was adapted from Schlichting *et al*.[27] Compound **23** (183 mg, 0.52 mmol), triphenylphosphine (207 mg, 0. 79 mmol) and LiN_3 (77 mg, 1.57 mmol) were dried in a dessicator. DMF (5 cm^3) was added and then a solution of CBr_4 (259 mg, 0.78 mmol) in DMF (2 cm^3) was added dropwise. After 2 h of reaction, MeOH (1 cm^3) was added and the solvents were removed by distillation under reduced pressure. The residue was purified by flash chromatography on silica gel (30% CH_2Cl_2 in cyclohexane v/v) to give **24** as a brown oil (192 mg, 98%). Rf_{24} = 0.55, Rf_{23} = 0 [cyclohexane : CH_2Cl_2 (50 : 50, v/v)]. Compound **24** (188 mg, 0.50 mmol) was solubilized with THF (25 cm^3), 10% Pd/charcoal (100mg) was added and hydrogenation was performed for 28 h as reported above for compound **22**. After Celite filtration, the mixture was concentrated and the residue purified on preparative silica plates (10% MeOH in CH_2Cl_2, v/v). The plates were eluted four times. Brown solid: (62 mg, 35%). Rf_{25} 0.46, [CH_2Cl_2 : MeOH (80 : 20, v/v) then CH_2Cl_2 : MeOH (50 : 50, v/v) then CH_2Cl_2 : MeOH : NH_4OH (50 : 50 : 5, v/v/v)]. δ_H (DMSO-*d*6) 1.30–1.47 (6H, m, 3 CH_2), 1.62–1.71 (2H, m, CH_2), 2.75–2.80 (2H, m, CH_2NH_2), 2.98 (2H, t, *J* 8.0, Ar-CH_2), 7.38 (1 H, d, *J* 6.5, Ar-H), 7.48–7.60 (3H, m, Ar-H), 7.75 (2 H, t, *J* 9.0, Ar-H), 7.91 (1 H, d, *J* 8.0, Ar-H), 8.24–8.39 (4H, m, Ar-H). ESI-MS: *m*/*z*, $C_{26}H_{25}N$ calc. 351.49, found 352.3 (M + H$^+$).

Sugar–perylene units ***27***$_\alpha$ *and* ***27***$_\beta$ *(Scheme 2).* Perylene **19** (252 mg, 1 mmol) and 1-*o*-methyl-2-deoxy-3,5-di(*p*-toluoyl)-*erythro*-penta-furanose **26** (384 mg, 1 mmol)[28] were dried in a dessicator and solubilized with 1,2-dichloroethane (100 cm^3) under an argon atmosphere. $SnCl_4$ (60 μl, 0.51 mmol) was added with a syringe to the mixture, under stirring, at room temperature. After 3 h of reaction, the mixture was washed with a 0.5 M aqueous $NaHCO_3$ solution (50 cm^3), then H_2O (50 cm^3) and with a 1 M aqueous NaCl solution (50 cm^3). The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness. The brown residue was purified twice by flash chromatography (50% cyclohexane in $CHCl_3$, v/v). Separation of the anomers was then performed by semi-preparative HPLC using a Hibar Lichrosorb column Si 60, 7 μm, (250 × 25 mm) from Merck (50% cyclohexane in $CHCl_3$, v/v) at a flow rate of 4 cm^3 min^{-1}. Before loading, the samples were filtered using Acrodisc Gelman CR, PTFE 0.45 μM, diameter 25 mm. Detection was performed at 460 nm. Yellow solids [**27**$_\alpha$ 70 mg; **27**$_\beta$ 190 mg, 43% (β + α) with a β : α ratio 73 : 27]. $Rf_{27\alpha}$ 0.27, $Rf_{27\beta}$ 0.34, Rf_{19} 0.87 and Rf_{26} 0.15 (cyclohexane : EtOAc, 80 : 20, v/v). δ_H ($CDCl_3$). **27**$_\alpha$: 2.32 (3H, s, CH_3), 2.40–2.48 (4H, m, H-2′, CH_3), 3.15–3.24 (1H, m, H-2″), 4.63–4.72 (2H, m, H-5′, H-5″), 4.86–4.90 (1H, m, H-4′), 5.66–5.71 (1H, m, H-3′), 6.00 (1H, pseudo t, *J* 5.6, *J* 7.7, H-1′), 7.09 (2H, d, *J* 7.7, Ar-H), 7.28 (2 H, d, *J* 8.1, Ar-H), 7.47–7.55 (3 H, m, Ar-H), 7.65–7.76 (5 H, m, Ar-H), 7.79 (1H, d, *J* 7.7, Ar-H), 8.03 (2 H, d, *J* 8.1, Ar-H), 8.19–8.25 (4 H, m, Ar-H). **27**$_\beta$: 2.31–2.40 (4H, m, H-2′, CH_3), 2.46 (3H, s, CH_3), 2.80–2.87 (1H, m, H-2″), 4.65–4.69 (1H, m, H-4′), 4.70–4.80 (2H, m, H-5′, H-5”), 5.67–5.71 (1H, m, H-3′), 5.91 (1H, dd, *J* 5.1, *J* 10.7, H-1′), 7.20 (2 H, d, *J* 8.1, Ar-H), 7.32 (2 H, d, *J* 8.1, Ar-H), 7.46–7.53 (3 H, m, Ar-H), 7.67–7.71 (2H, m, Ar-H), 7.77–7.86 (2 H, m, Ar-H), 7.95 (2 H, d, *J* 8.1, Ar-H), 8.05 (2H, d, *J* 8.6, Ar-H), 8.13–8.24 (4H, m, Ar-H). ESI-MS: *m*/*z* **27**$_\alpha$ and **27**$_\beta$, $C_{41}H_{32}O_5$ calc. 604.7, found **27**$_\alpha$ 627.5 (M + Na$^+$) and **27**$_\beta$ 627 (M + Na$^+$).

Sugar–perylene units ***32***$_\alpha$ *and* ***32***$_\beta$ *(Scheme 2).* Compounds **22** (664 mg, 2.14 mmol) and **31**[29] (1.248 g, 3.21 mmol), $ZnBr_2$ (hygroscopic) (133 mg, 0.58 mmol), $NaHCO_3$ (360 mg, 4.29 mmol) and activated molecular sieves 4 Å (3.3 g) were dried in a dessicator. THF (40 cm^3) was added with a syringe

under an argon atmosphere, and the reaction mixture was stirred at room temperature. After a 4 h reaction, the mixture was filtered and concentrated to dryness. The yellow residue was solubilized with CH_2Cl_2 (130 cm^3). The organic phase was washed with H_2O (50 cm^3), dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash chromatography (CH_2Cl_2). Separation of the anomers was performed on preparative silica plates (30% cyclohexane in CH_2Cl_2, v/v). The plates were eluted eight times. Two bands corresponding to each pure anomers of the sugar–perylene unit **32** were separated and extracted with CH_2Cl_2 to give yellow solids corresponding to each isomer [$\mathbf{32}_\alpha$ 417 mg, $\mathbf{32}_\beta$ 685 mg, 78% (β + α) with a β : α ratio 62 : 38]. $Rf_{32\alpha}$ 0.44, $Rf_{32\beta}$ 0.51, Rf_{22} 0.13 and Rf_{31} 0.02 (CH_2Cl_2). δ_H ($CDCl_3$) $\mathbf{32}_\alpha$: 2.02–2.12 (2H, m,CH_2), 2.28–2.36 (4H, m, H-2′, CH_3), 2.38 (3H, s, CH_3), 2.52–2.60 (1H, m, H-2″), 3.06–3.22 (2H, m, Ar-CH_2), 3.54–3.60 (1H, m, CH_2O), 3.87–3.93 (1H, m, CH_2O), 4.52–4.58 (1H, m, H-4′), 4.60–4.68 (2 H, m, H-5′, H-5″), 5.35 (1H, d, *J* 5.1, H-1′), 5.44–5.48 (1H, m, H-3′), 7.16 (2H, d, *J* 7.7, Ar-H), 7.20 (2H, d, *J* 8.1, Ar-H), 7.33 (1H, d, *J* 7.7, Ar-H), 7.40–7.50 (3H, m, Ar-H), 7.67 (2H, t, *J* 8.1, Ar-H), 7.86 (1H, d, *J* 8.6, Ar-H), 7.91 (2H, d, *J* 8.1, Ar-H), 7.97 (2H, d, *J* 8.1, Ar-H), 8.08 (1 H, d, *J* 7.7, Ar-H), 8.14 (1 H, d, *J* 6.8, Ar-H), 8.17–8.21 (2H, m, Ar-H). $\mathbf{32}_\beta$: 1.96–2.04 (2H, m, CH_2), 2.32 (3H, s, CH_3), 2.37–2.46 (4H, m, H-2′, CH_3), 2.60–2.67 (1H, m, H-2″), 2.98–3.10 (2H, m, Ar-CH_2), 3.46–3.53 (1H, m, CH_2O), 3.84–3.91 (1H, m, CH_2O), 4.49–4.53 (1H, m, H-4′), 4.54–4.58 (1H, m, H-5′), 4.59–4.64 (1H, m, H-5″), 5.38 (1 H, dd, *J* 2.6, *J* 5.6, H-1′), 5.62–5.66 (1H, m, H-3′), 7.15 (2H, d, *J* 8.1, Ar-H), 7.25 (2 H, d, *J* 8.1, Ar-H), 7.30 (1H, d, *J* 7.7, Ar-H), 7.44–7.53, (3H, m, Ar-H), 7.66 (2H, t, *J* 7.9, Ar-H), 7.85 (1H, d, *J* 8.1, Ar-H), 7.91–7.98 (4H, m, Ar-H), 8.09 (1H, d, *J* 7.7, Ar-H), 8.14 (1H, d, *J* 7.3, Ar-H), 8.17–8.22 (2H, m, Ar-H). ESI-MS: *m/z* $\mathbf{32}_\alpha$ and $\mathbf{32}_\beta$, $C_{44}H_{38}O_6$ calc. 662.7, found $\mathbf{32}_\alpha$ 663.2 (M + H^+) and $\mathbf{32}_\beta$ 663.2 (M + H^+).

Deprotection of compounds $\mathbf{27}_\alpha$, $\mathbf{27}_\beta$, $\mathbf{32}_\alpha$ *and* $\mathbf{32}_\beta$. Compounds $\mathbf{27}_\alpha$, $\mathbf{27}_\beta$, $\mathbf{32}_\alpha$ and $\mathbf{32}_\beta$ were solubilized separately with a CH_2Cl_2–MeOH mixture (50 : 50, v/v) and deprotected by an excess of sodium methylate (5 eq.) in methanol. A precipitate appeared during the process. The starting materials ($Rf_{27\alpha}$ 0.90, $Rf_{27\beta}$ 0.90, $Rf_{32\alpha}$ 0.92 and $Rf_{32\beta}$ 0.94) were transformed into new yellow products with lower *R*f values ($Rf_{28\alpha}$ 0.26, $Rf_{28\beta}$ 0.23, $Rf_{32\alpha}$ 0.34 and $Rf_{32\beta}$ 0.33) (EtOAc). After a 15 h reaction, the mixtures were neutralized by addition of a 18 M solution of acetic acid. The yellow solids were filtered, washed with a MeOH–H_2O mixture (1 : 1, v/v) and dried. $\mathbf{28}_\alpha$ (105 mg, 86%). $\mathbf{28}_\beta$ (260 mg, 86%). $\mathbf{33}_\alpha$ (221 mg, 89%). $\mathbf{33}_\beta$ (361 mg, 92%). ^{1}H-NMR data are given as supplementary material†. ESI-MS: *m/z* $\mathbf{28}_\alpha$ and $\mathbf{28}_\beta$, $C_{25}H_{20}O_3$ calc. 368.4, found $\mathbf{28}_\alpha$ 369.3 (M + H^+) and $\mathbf{28}_\beta$ 369.3 (M + H^+). $\mathbf{33}_\alpha$ and $\mathbf{33}_\beta$, $C_{28}H_{26}O_4$ calc. 426.5, found $\mathbf{33}_\alpha$ 444.4 (M + H_2O)$^+$ and $\mathbf{33}_\beta$ 426.3 (M + H^+).

Tritylation of compounds $\mathbf{28}_\alpha$, $\mathbf{28}_\beta$, $\mathbf{33}_\alpha$ *and* $\mathbf{33}_\beta$. Compounds $\mathbf{28}_\alpha$, $\mathbf{28}_\beta$, $\mathbf{33}_\alpha$ and $\mathbf{33}_\beta$ were dried separately by co-evaporation with pyridine, solubilized with pyridine and 4,4′-dimethoxytrityl chloride (1.1 eq.) was added. After a 21 h reaction, the mixture was diluted with CH_2Cl_2, washed with a 0.5 M aqueous $NaHCO_3$ solution, dried over Na_2SO_4, filtered and concentrated to dryness. The orange-colored oil was purified by flash chromatography [3% EtOAc in CH_2Cl_2 containing 1% NEt_3 (v/v/v)] to give the tritylated compounds $\mathbf{29}_\alpha$ (71 mg, 37%), $\mathbf{29}_\beta$ (360 mg, 82%), $\mathbf{34}_\alpha$ (239 mg, 74%) and $\mathbf{34}_\beta$ (364 mg, 85%). $Rf_{29\alpha}$ 0.48, $Rf_{29\beta}$ 0.29, $Rf_{34\alpha}$ 0.55 and $Rf_{34\beta}$ 0.13, using the same eluent. ESI-MS: *m/z* $\mathbf{29}_\alpha$ and $\mathbf{29}_\beta$, $C_{46}H_{38}O_5$ calc. 670.8, found $\mathbf{29}_\alpha$ 670.2 (M + H^+) and $\mathbf{29}_\beta$ 671.3 (M + H^+). $\mathbf{34}_\alpha$ and $\mathbf{34}_\beta$, $C_{49}H_{44}O_6$ calc. 728.9, found $\mathbf{34}_\alpha$ 729.3 (M + H^+) and $\mathbf{34}_\beta$ 729.3 (M + H^+). Compounds $\mathbf{29}_\alpha$, $\mathbf{29}_\beta$, $\mathbf{34}_\alpha$ and $\mathbf{34}_\beta$ were used in the next synthesis step without additional characterization.

H-phosphonate derivatives $\mathbf{30}_\alpha$, $\mathbf{30}_\beta$, $\mathbf{35}_\alpha$ *and* $\mathbf{35}_\beta$. Compounds $\mathbf{29}_\alpha$, $\mathbf{29}_\beta$, $\mathbf{34}_\alpha$ and $\mathbf{34}_\beta$ were dried separately by co-evaporation with pyridine, dried in a dessicator and solubilized with a CH_2Cl_2–pyridine mixture (50 : 50, v/v), under an argon atmosphere, to obtain a 50 mM solution. Then, 1.2 eq. of 2-chloro-4*H*-1,3,2-benzodio-xaphosphorin-4-one[30] [100 mM solution in a CH_2Cl_2–pyridine mixture (1 : 5, v/v)] was added to the reaction mixture at 0 °C. After 1.5 h, water containing TEA (20% by volume) was added to the reaction mixture and vigorous stirring was maintained for 30 min. The crude product was extracted with CH_2Cl_2, the organic phase dried over Na_2SO_4 and concentrated under a reduced pressure to give a brown oil. The residue was purified by flash chromatography [5% MeOH in CH_2Cl_2 containing 0.5% NEt_3 (v/v/v)] to give $\mathbf{30}_\alpha$ (83 mg, 95%), $\mathbf{30}_\beta$ (260 mg, 60%), $\mathbf{35}_\alpha$ (154 mg, 58%), $\mathbf{35}_\beta$ (250 mg, 62%). $Rf_{30\alpha}$ 0.12, $Rf_{30\beta}$ 0.07, $Rf_{35\alpha}$ 0.09 and $Rf_{35\beta}$ 0.15, using the same eluent. ^{1}H-NMR data are given as supplementary material†. δ_p($CDCl_3$) $\mathbf{30}_\alpha$ 4.10, $\mathbf{30}_\beta$ 3.69, $\mathbf{35}_\alpha$ 4.16 and $\mathbf{35}_\beta$ 3.29. ESI-MS: *m/z* $\mathbf{30}_\alpha$ and $\mathbf{30}_\beta$: $C_{46}H_{39}O_7P$ calc 734.8, found $\mathbf{30}_\alpha$ 735.1 (M + H^+) and $\mathbf{30}_\beta$ 735.2 (M + H^+). $\mathbf{35}_\alpha$ and $\mathbf{35}_\beta$: calculated mass, $C_{49}H_{45}O_8P$ = 792.9; found $\mathbf{35}_\alpha$ 995.7 (M + H^+ + 2 TEA); found $\mathbf{35}_\beta$ 995.5 (M + H^+ + 2 TEA).

ODN–perylene conjugates ***4–18***. The ODNs **4**–**15** were assembled using classical phosphoramidite chemistry on a CPG support at a one μmol scale. To introduce the perylene–sugar residue into the ODNs, a manual coupling step was used for the *H*-phosphonate derivatives $\mathbf{30}_\alpha$, $\mathbf{30}_\beta$, $\mathbf{35}_\alpha$ and $\mathbf{35}_\beta$. The syntheses were performed as follows. At the position chosen for the incorporation of the sugar–perylene unit an additional detritylation step was performed. Then, after drying of the support, the selected *H*-phosphonate derivatives $\mathbf{30}_\alpha$, $\mathbf{30}_\beta$, $\mathbf{35}_\alpha$ or $\mathbf{35}_\beta$, 15.2 eq. [in a pyridine–CH_3CN mixture (50 : 50, v/v) (0.38 cm^3 of a 40 mM solution) dried overnight on 3 Å and 4 Å molecular sieves] and pivaloyl chloride 49.4 eq. [in a pyridine–CH_3CN mixture (50 : 50, v/v) (0.38 cm^3 of a 130 mM solution), prepared one hour before use] were added simultaneously to either the support or to the ODN chain bound to the support. After 2.5 min of reaction the solution was removed and the support washed with an anhydrous pyridine–CH_3CN mixture (50 : 50, v/v) (1 cm^3 × 6). The coupling yield was monitored by a trityl cation assay. Then, the *H*-phosphonate derivative was transformed into the 2-cyanoethyl phosphotriester group by a 15 min treatment of the ODN bound to the support with a 10% solution (1 cm^3) of 3-hydroxypropionitrile in a CCl_4–CH_2Cl_2–CH_3CN–NEt_3–NMI mixture (90 : 10 : 10 : 5 : 5, v/v/v/v/v) dried overnight on 3 Å and 4 Å molecular sieves following a procedure adapted from a literature report.[35] Finally, the residual *H*-phosphonate linkage was oxidized by a 1 h treatment with 0.1 M iodine solution in pyridine–H_2O mixture (98 : 2, v/v). After removal of the solution, the support was washed with an anhydrous pyridine–CH_3CN mixture (50 : 50, v/v) (3 × 1 cm^3) and then with anhydrous CH_3CN (1 cm^3). Except in the case of the 5′-terminal addition of the *H*-phosphonate derivative, the support was treated with a mixture of capping solutions used on the synthesizer (0.5 cm^3 each) for 10 min, washed with CH_3CN (4 × 1 cm^3) and dried. The ODN chain assemblies were completed *via* phosporamidite chemistry to give the fully protected ODNs. ODN **16** was obtained by using the previously reported perylenyl phosphoramidite.[23] ODN **17** was obtained by using the perylene derivative **25** and proceeding as described in our previous report,[23] followed by the full length sequence assembly. ODN **18** was obtained as described for the preparation of ODN **17** and by coupling the second perylene to the 5′-end of the sequence using our previously reported phosphoramidite derivative.[23]

The deprotection step was performed by a 28% aqueous ammonia treatment for 18 h at 20 °C, either alone or in the presence of DTT in the case of the preparation of ODNs **13**, **17** and **18** involving the use of a modified support containing a disulfide bridge.[32] For ODNs **4**–**15** and **17** the detritylation step was performed by a treatment with a 80% acetic acid solution for 30 min before the purification step. Analyses and purifications by reversed-phase chromatography were performed on a

Lichrospher 100 RP18 column (5 μM, 125 mm × 4 mm) from Merck with a linear gradient of CH_3CN (12.5% to 42.5% over 40 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1cm^3 min^{-1} and detection at $\lambda = 260$ nm. The mass values of the modified ODNs were confirmed by electrospray mass spectrometry analysis (Table 2). The overall yield after the synthesis and purification steps was 11–32% for ODNs **4**–**12** and **16**, while lower yields were obtained for ODNs **13** (3%), **14** (7%), **15** (5%), **17** (1%) and **18** (1%), involving the incorporation of a perylene unit at the 3′-end of the ODNs by reaction of the perylene derivatives on modified supports.

Hybridization studies

Melting studies. Experiments were carried out by absorption spectroscopy using the double-stranded DNA target d-$^{5'}$TAGTTTCTCTTCTTTTTCTTCTCTT$^{3'}$/$^{3'}$ATCAAAGAGA-AGAAA-AAGAAGAGAA$^{5'}$ **1** [circularized using two hexaethylene linkers[33] in order to increase its stability] and the single-stranded complementary DNA target d-$^{5'}$CTCAGAGA-AGAAAAAGAACTC$^{3'}$ **2**. Concentrations of the circularized double-stranded ODN **1**, as well as those of the unmodified ODNs **2** and **3**, were calculated using molar extinction coefficients at 260 nm determined using the nearest-neighbour model[36] (ODN **1**: $\varepsilon_{260} = 497\,700$ M^{-1} cm^{-1}, ODN **2**: $\varepsilon_{260} = 222\,800$ M^{-1} cm^{-1}, ODN **3**: $\varepsilon_{260} = 118\,500$ M^{-1} cm^{-1}). The molar extinction coefficients (ε) for the ODN–perylene ODNs **5**, **11** and **15** were determined by titration of the ODN solutions in a 10 mM sodium cacodylate, pH 7, buffer containing 100 mM NaCl at 3 °C with a solution of single-stranded complementary sequence **2** (ODN **5**: $\varepsilon_{260} = 154\,400$ M^{-1} cm^{-}1, ODN **11**: $\varepsilon_{260} = 154\,100$ M^{-1} cm^{-1} and ODN **15**: $\varepsilon_{260} = 165\,800$ M^{-1} cm^{-1}). Considering the minor differences observed for the UV-visible spectra corresponding to each series of ODNs, the ε value obtained for ODN **5** was used for ODNs **4**, **6** and **7**. The ε value obtained for ODN **11** was used for ODNs **8**–**10**, **13**, **14**, **16** and **17**. The ε value obtained for the ODN **15** was used for ODNs **12** and **18**. All concentrations are given on a per strand basis. In the case of the duplexes, a 1 μM ODNs (each strand) was used in a 10 mM sodium cacodylate buffer, pH 7, containing 100 mM NaCl. In the case of the triplexes, the experiments were performed with a 1 μM concentration in the circularized double-stranded target and a 1.5 μM concentration in the third ODN strand in a 10 mM cacodylate buffer, pH 7, containing 140 mM KCl and 5 mM $MgCl_2$. Duplex and triplex stabilities were determined by thermal denaturation. Results are given in Table 2. The uncertainty in the *T*m values reported ± 0.5 °C.

Fluorescence studies. Fluorescence studies were performed using the same buffer conditions as used for the binding studies. The emission spectra of 1 μM concentrations of the ODNs were first recorded and then a small volume of the target sequences **1** or **2** (1 eq.) was added. The mixtures were allowed to hybridize in the dark at 16 °C for the duplexes (30 min) and at 4 °C for the triplexes (overnight) to ensure complete hybridization. The emission spectra of the duplex and triplex structures were recorded between $\lambda = 450$ and 550 nm using the same λ_{exc} as for the ODN–perylene conjugates ($\lambda_{exc} = 446$ nm for ODNs **4**–**7**, **13** and **18**; $\lambda_{exc} = 448$ nm for ODNs **8**–**11**, **16** and **17**; and $\lambda_{exc} = 449$ nm for ODNs **12**, **14** and **15**).

Acknowledgements

We thank H. Meudal for recording NMR spectra and C. Buré for running the electrospray mass spectrometer.

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