Pergamon

Tetrahedron Letters 42 (2001) 9005–9010

TETRAHEDRON LETTERS

Synthesis and binding properties of perylene-oligo-2′-deoxyribonucleotide conjugates

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Received 4 August 2001; accepted 23 October 2001

Abstract—Perylene has been covalently linked, via polymethylene tethers, to the 5′- and the 3′-ends of an oligopyrimidine sequence. The presence of the polycyclic ligand stabilizes the duplexes and the triplexes formed by the modified oligonucleotides and their single- and double-stranded DNA targets as compared to those formed with the parent unmodified oligonucleotide used as reference. Stabilization of the triplex is at its highest when perylene is linked to the 5′-end of the oligonucleotide via a nine-atom size linker. Stabilization of the duplexes is nearly equivalent whatever the position of the substitution (5′ or 3′) and the linker size used to tether both entities.

1. Introduction

Due to the ability of oligonucleotides to specifically regulate gene expression via 'antisense' and 'antigene' strategies, the development of analogues with improved properties has been the focus of intense research.[1,2] To be efficient, modified oligonucleotides must efficiently cross the cell membranes, be resistant to nuclease, and specifically and strongly hybridize to their targets on mRNA or double-stranded DNA.

Modifications developed to increase the affinity of 'antisense' and 'antigene' oligonucleotides for their targets inside cells include that of oligonucleotides and the covalent linking of ligands such as DNA minor groove binders,[3–5] polyamines,[6,7] peptides,[8] and intercalating agents.[9–17] The double helix intercalating agents involve molecules possessing a planar aromatic chromophore with dimensions similar to those of a standard Watson–Crick base pair corresponding to about three fused six-membered rings. The search for triple helix intercalating agents is based on the same principle with the use of molecules corresponding to about four or five six-membered rings able to interact with adjacent base-triplets of the triple-stranded structure.

A 3,4,9,10 di-anhydride perylene derivative has been used to bridge the two pyrimidine strands of DNA in a pyrimidine–purine–pyrimidine triplex.[18,19] 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 linked it to the 5′- and the 3′-ends of a pyrimidine decamer. Linkers involving five or nine atom lengths have been used to attach the dye to the 5′-end, while a longer linker involving 11 atoms was used for linking to the 3′-end of the oligonucleotide. Studies carried out with oligonucleotide–acridine conjugates showed that stabilization of triplex structures by a 3′-linked intercalator requires the use of a longer linker as compared to that observed with a 5′-linked one.[20] We report here the synthesis and binding properties of these perylene-2′-deoxyribonucleotide conjugates.

2. Synthesis

The synthesis of modified oligonucleotides was performed as outlined in Schemes 1 and 2. The perylene-linker derivatives **3**–**5** were obtained following a two-step procedure adapted from the literature,[21] as described in Scheme 1. First, the preparation of the aldehyde derivative **2** was achieved by reaction of the perylene **1**, in 1,2-dichloroethane, with $SnCl_4$ (2 equiv.) and then with 1,1-dichlorooxymethylether (1.2 equiv.) followed by hydrolysis.[22] Then, the synthesis of the perylene-linker derivatives **3**–**5** was performed by reac-

Keywords: oligonucleotide–perylene conjugates; attachment to 5′-end; attachment to 3′-end; absorption spectroscopy; T_m values.

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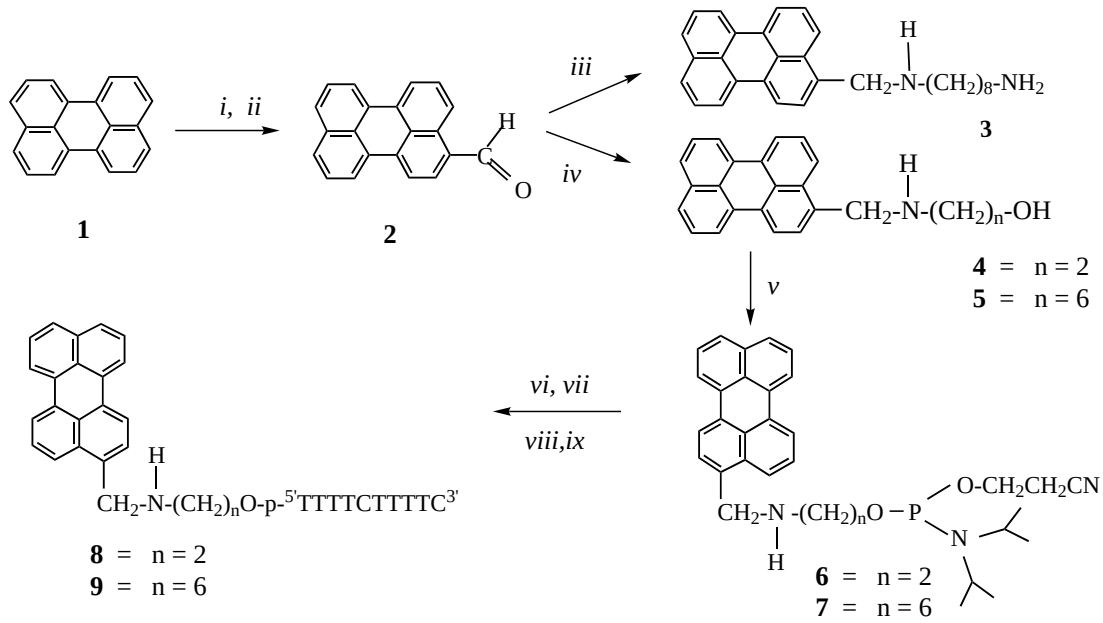
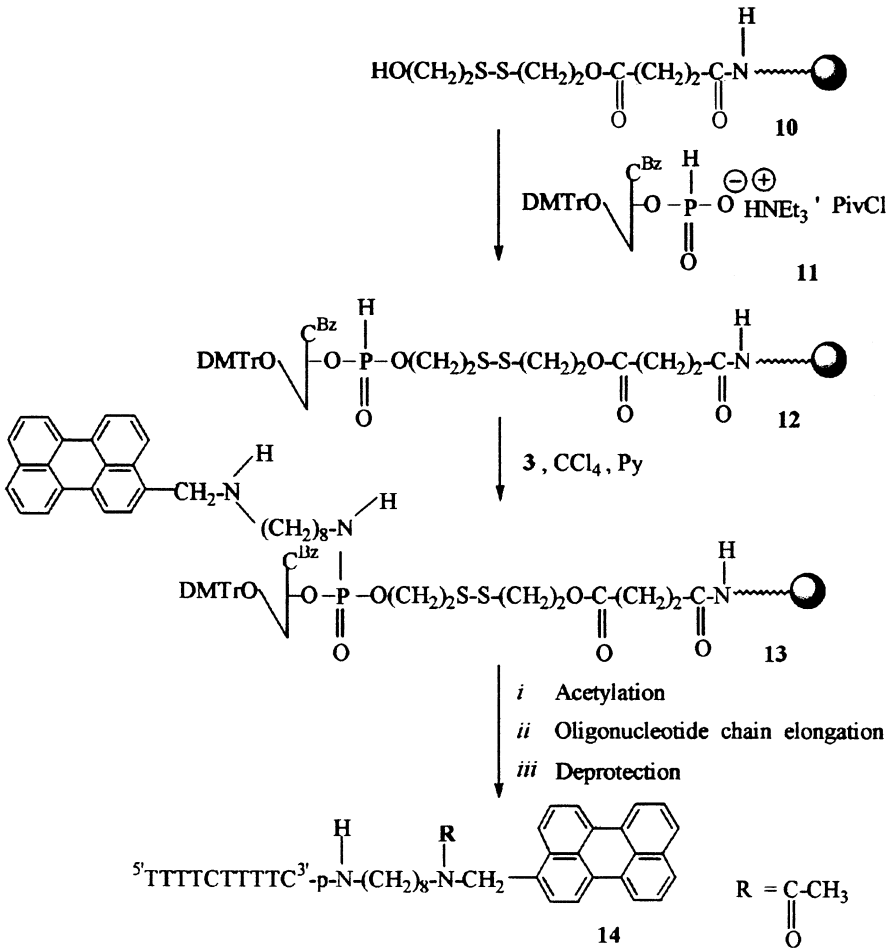
PII: S0040-4039(01)02010-X



Scheme 1. (i) CH_2Cl_2, $SnCl_4$; (ii) 1,1-dichlorooxymethylether, H_2O; (iii) H_2N-$(CH_2)_8$-NH_2, toluene, $NaBH_4$; (iv) H_2N-$(CH_2)_n$-OH with $n=2$ or 6, toluene, NaBH4; (v) 2-cyanoethyldiisopropylchlorophosphoramidite, diisopropylethylamine, dichloromethane; (vi) support bound trityl-off oligonucleotides; (vii) tetrazole; (viii) oxidation step; (ix) conc. NH_4OH.



Scheme 2.  : CPG-Long chain alkylamine; DMTrCl: 4,4′-dimethoxytritylchloride; (i) acetic anhydride, *N*-methylimidazole; (iii) H^+, conc. NH_4OH.

tion of the aldehyde **2** with 1,8-diaminooctane, 2-aminoethanol or 6-aminohexanol (10 equiv.) in the presence of paratoluene sulfonic acid (0.03 equiv.), using toluene as a solvent, followed by $NaBH_4$ (2 equiv.) treatment.[23] Compounds **4** and **5** were phosphitylated by reaction of 2-cyanoethyl-diisopropyl-chlorophosphoramidite (1 equiv.) in the presence of diisopropylethylamine (2.5 equiv.) in dichloromethane to give the phosphoramidites derivatives **6** and **7** with 85% yield after purification on a silica gel column using ethyl acetate/hexane/NEt_3, 50:50:4, v/v/v as eluent ($R_{f6}=0.74$ and $R_{f4}=0.10$; $R_{f7}=0.80$, and $R_{f5}=0.13$) on analytical TLC silica plates using the same eluent as above.[24,25]

The modified support **13** was obtained following a two-step procedure, as described in Scheme 2. First, the modified support **10**[26] was reacted with the N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribocytidine-3′-*H*-phosphonate **11**[27] (24 equiv.) and pivaloyl chloride (75 equiv.) in pyridine/acetonitrile for 2 min to give the modified support **12**. Then after removal of the excess reagent, the latter was reacted with the perylene derivative **3** (30 equiv.) in a pyridine/CCl_4 (50:50, v/v) mixture (2 mL) for 1 h to give the modified support **13**. After washing, the support was acetylated for 2 min, using standard solutions, to block the unreacted hydroxyl functions. Trityl assay indicated a loading of 30 μmol/g of N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribocytidine on the support **13**. The yellow color of the support indicated the presence of perylene.

Incorporation of the perylene to the 5′-end of the oligonucleotide was performed manually by using 10 equiv. of the phosphoramidite derivatives **6** and **7** and a coupling time of 10 min (Scheme 1). After removal of the amidite excess, the oxidation step was performed for 30 s by using a standard solution used on the synthesizer. The synthesis of the oligonucleotide bearing the perylene to the 3′-end was performed via the phosphoramidite chemistry using the modified support **13** involving the perylene and the 3′-terminal nucleoside (Scheme 2). The chain assembly was completed using a standard coupling cycle with good efficiency as ascertained by trityl measurements. The deprotection step was performed by treatment at rt of the oligonucleotide–perylene conjugates bound to the support with a concentrated NH_4OH solution overnight. After removal of the ammonia, extraction of the organic impurities and filtration, the crude oligonucleotide–perylene conjugates **8**, **9** and **14**, were analyzed and purified by reversed-phase chromatography. Retention times are given in Table 1.

The mass values of the modified oligonucleotides **8** and **9** were confirmed by electrospray mass spectrometry analysis (Table 1). In the case of the oligonucleotide–perylene conjugate **14**, the result of mass analysis indicated a value superior by 42 Da to that expected. This could be due to the acetylation of the secondary amino function, present on the linker, during the capping steps performed along the sequence assembly.[28]

The UV–vis spectra of the oligonucleotide–perylene conjugates were recorded between $\lambda=220$ and 520 nm in 10 mM phosphate buffer, pH 7, containing 140 mM KCl and 5 mM $MgCl_2$. The spectra contain two absorption bands in the visible region between $\lambda=350$ and 520 nm (where only the perylene absorbs light) with $\lambda^{vis}_{max} \approx 446$ and $\lambda^{vis} \approx 420$ nm with a shoulder at $\lambda \approx 395$ nm. The other absorption band in the UV range corresponds to the absorbance of the oligonucleotide and the perylene with a $\lambda^{UV}_{max} \approx 257$ nm. The UV–vis spectra of the oligonucleotide–perylene conjugate **9** recorded between $\lambda=220$ and 520 nm in 10 mM phosphate buffer, pH 7, containing 140 mM KCl and 5 mM $MgCl_2$ is shown in Fig. 1.

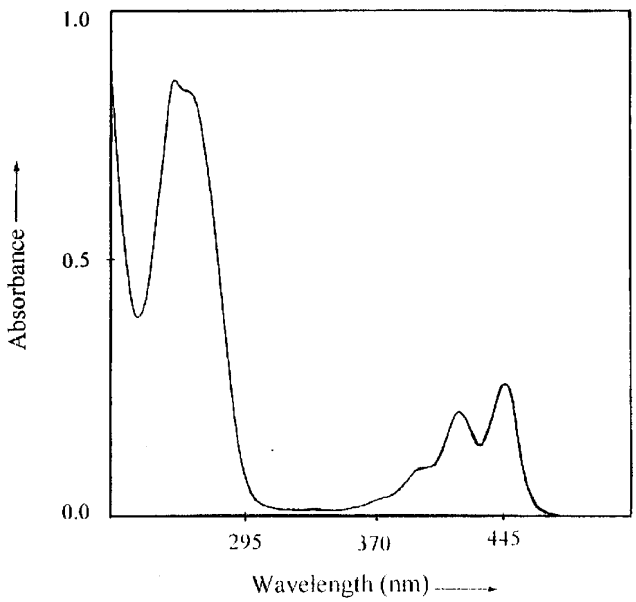


Figure 1. UV–vis spectra of the oligonucleotide–perylene conjugate **9** recorded between $\lambda=220$ and 520 nm in 10 mM phosphate buffer, pH 7, containing 140 mM KCl and 5 mM $MgCl_2$.

Table 1. Perylene-oligo-2′-deoxyribonucleotides conjugates

Oligonucleotides	Mass calculated	Mass observed	HPLC retention time[a]
8	3336.3	3337.2	27 min 3 s
9	3392.4	3393.3	31 min 13 s
14	3420.5	3462.1	44 min 20 s

[a] Analyses were performed on a reversed-phase RP 18 column (125×4 mm, Lichrospher 5 μM) from Merck, using a linear gradient of acetonitrile (5 to 50% over 60 min) in 0.1 M triethylammonium acetate buffer, pH 7, with a flow rate of 1 mL/min and detection at 260 nm.

3. Binding studies

The experiments were performed, by absorption spectroscopy, in a 10 mM phosphate buffer, pH 6, containing 140 mM KCl and 5 mM $MgCl_2$. We have used a pH 6 buffer in order to facilitate the protonation of the cytosines contained in the sequence. Thus, protonation of the cytosines is a requirement to the formation of two hoogsteen bonds between the cytosines and the target guanines present on the purine strand of the double-stranded DNA target. Pyrimidine sequences containing cytosines form triple helices with weak stability at pH 7. Molar extinction coefficients of the oligonucleotide–perylene conjugates **8**, **9** and **14** were determined by titration of conjugate solutions at 3°C with a solution of single-stranded complementary sequence **16**. Molar extinction coefficients of the unmodified oligonucleotides were determined according to the literature.[29] The mixing of the oligonucleotide–perylene conjugates **8**, **9** and **14** with either the single-stranded **16** or the double-stranded **17**/**18** DNA targets (see Fig. 2 for sequences) at 3°C induces a red shift of the λ^{vis}_{max} of the perylene spectra ($\approx$3 nm in the case of compounds **8** and **9**, and in the case of conjugate **14**, 5 nm for the duplex and 4 nm for the triplex, respectively). The spectral modifications reverse when the temperature is increased to 50°C. Duplex and triplex stabilities were determined by thermal denaturation. Melting temperatures (T_{ms}) and conditions used are given in Table 2. One transition was observed in the melting profile of each duplex, while two transitions were observed in the melting of each triplex. The transition with the higher T_m corresponds to the melting of the target duplex (around 61°C for all complexes) and the transition with lower T_m to the dissociation of the third strand.

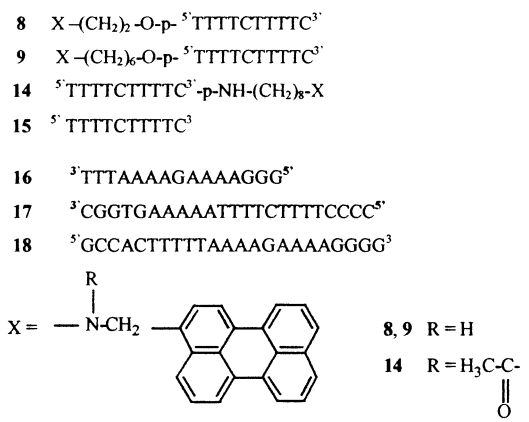


Figure 2. Structures of the oligonucleotides used in the melting studies

Table 2. Melting temperatures for duplexes and triplexes

Duplexes	T_m (°C) ($\pm$1°C)	Triplexes	T_m (°C) ($\pm$1°C)
8:**16**	34.4	**8**:**17**/**18**	29.4
9:**16**	34.4	**9**:**17**/**18**	30.4
14:**16**	31	**14**:**17**/**18**	23
15:**16**	21.8	**15**:**17**/**18**	$\leq$14

The experiments were conducted with 1 μM oligonucleotide concentrations (each strand) in a 10 mM phosphate buffer, pH 6, containing 140 mM KCl and 5 mM $MgCl_2$.

The duplexes formed by the oligonucleotide–perylene conjugates **8**:**16**, **9**:**16**, and **14**:**16** are more stable than the unmodified duplex **15**:**16** used as reference (Table 2). The presence of the perylene led to a T_m increase of 12.6°C when linked to the 5′-end of the oligonucleotide (identical with both linkers) and a T_m increase of only 9.2°C when linked to the 3′-end of the oligonucleotide. It must be noted that in the latter case the acetylation of the linker could prevent the ligand from adopting the better position for intercalation. The triple helices formed by the oligonucleotide–ligand conjugates and the double-stranded DNA target **8**:**17**/**18**, **9**:**17**/**18**, and **14**:**17**/**18** were also more stable than the unmodified triple helix **15**:**17**/**18** used as reference. Once again when the perylene was linked to the 5′-end of the oligonucleotide, the stabilization induced was nearly the same with the two different linkers ($\Delta T_m \geq 15.4$°C for conjugate **8** with short linker and $\Delta T_m \geq 16.4$°C in the case of conjugate **9** with a longer linker, respectively). When the perylene was linked to the 3′-end of the oligonucleotide the stabilization observed was weaker ($\Delta T_m \geq$ 9°C). This could be due to the acetylation of the linker as mentioned above. Another explanation could be that as in the case of oligonucleotide–acridine and oligonucleotide–ethidium conjugates, weaker stabilization of the triple helices was observed when the dye was coupled to the 3′-end of the third strand as compared to the stabilization observed when coupling was achieved at the 5′-end.[20] This question could be addressed by using different linkers without an amino function to prevent acetylation of the linker during the oligonucleotide chain assembly or post-synthetic coupling of the ligand. The stabilization observed for the triple helices is nearly equivalent to that provided by acridine[20] and naphthalene derivatives.[17]

4. Conclusions

This paper reports the synthesis and binding properties of oligopyrimidine–perylene conjugates. Perylene was covalently linked either to the 5′-end or the 3′-end of a decamer. The covalent attachment of the perylene to the 5′-end was performed via phosphoramidite chemistry using two linkers with different sizes. The coupling of the perylene to the 3′-end of the oligonucleotide was performed using a modified support involving the perylene. The binding studies performed by absorption spectroscopy showed that in any case the oligonucleotide–perylene conjugates formed more stable duplexes and triplexes than did the unmodified oligonucleotide used as reference.

Acknowledgements

This work was supported by funds from the Agence Nationale de Recherches sur le SIDA (ANRS). We

thank H. Labbé (Centre de Biophysique Moléculaire, Orléans) for recording NMR spectra and C. Buré (Centre de Biophysique Moléculaire, Orléans) for running the electrospray mass spectrometry.

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22. The perylene **1** (3 g, 11.88 mmol) was dissolved in 1,2-dichloroethane (100 mL) placed in a three-necked round-bottomed flask equipped with a magnetic stirrer, dropping funnel and nitrogen inlet tube with a bubbler. The mixture was cooled to 5°C with an ice-water bath and $SnCl_4$ (2.85 mL, 23.34 mmol) was added in one portion. Then, 1,1-dichlorooxymethylether (1.35 mL, 14.31 mmol) was added dropwise to the mixture over 1 h and the temperature kept for 1 h at $\leq$5°C. The resulting suspension was warmed to reflux for 2 h and further stirred for 15 h. HCl was emitted during the warming step. The reaction mixture was cooled to $\approx$10°C and hydrolyzed by addition of cold water (100 mL). After 3 h at room temperature the reaction mixture was extracted with dichloromethane (150 mL). The organic phase was washed with water (3×50 mL) dried over sodium sulfate, and concentrated to dryness. The residue was purified by silica gel chromatography using dichloromethane as eluent to give the aldehyde derivative **2** (2.4 g, yield 75%, $R_{f1}=0.97$ and $R_{f2}=0.65$ on analytical TLC silica plates using CH_2Cl_2 as eluent). ^{1}H NMR ($CDCl_3$): δ, 10.32–10.30 (m, 1H, C(O)-H), 9.18–9.15 (m, 1H, Ar), 8.30–8.27 (m, 4H, Ar), 7.94–7.70 (m, 4H, Ar), 7.56–7.54 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ, 126.11, 127.65, 128.26, 129.70, 130.14, 133.20, 133.36, 134.25, 134.86, 135.27, 135.63, 136.01, 136.32, 136.94, 137.72, 140.09, 142.88, 143.44, 197.
23. The aldehyde **2**, 1,8-diaminooctane (or 2-aminoethanol or 6-aminohexanol) (10 equiv.), paratoluene sulfonic acid (0.03 equiv.) and toluene were placed in a round-bottomed flask equipped with a magnetic stirring bar and a Dean–Stark trap. The reaction mixture was stirred at reflux for 4 h and then cooled, diluted with ethanol (half of the volume of toluene) and cooled to 0°C with an ice bath. Solid $NaBH_4$ (2 equiv.) was added to the stirred mixture while maintaining the temperature below 15°C and then the stirring was continued for 12 h at room temperature. After addition of water, the reaction mixture was extracted with dichloromethane. The organic phase was washed with water, concentrated to dryness and the residue purified on preparative TLC silica plates using successively CH_2Cl_2/MeOH (80:20, v/v), CH_2Cl_2/MeOH (50:50, v/v), and then CH_2Cl_2/MeOH/NH_4OH (50:50:5, v/v/v) as eluent for compound **3**, and successively CH_2Cl_2/MeOH (95:5, v/v) and CH_2Cl_2/MeOH (90:10, v/v) (three times) as eluent in the case of hydroxylated perylene-linker derivatives **4** and **5**.
Compound **3**. ^{1}H NMR (DMSO, d_6): δ, 8.37–8.28 (m, 4H, Ar), 8.04–8.01 (m, 1H, Ar), 7.78–7.75 (m, 2H, Ar), 7.55–7.52 (m, 5H, Ar), 4.8 (s, 1H), 4.05–4.04 (m, 2H, CH_2-Ar), 2.60–2.57 (m, 2H, CH_2), 2.49–2.43 (m, 2H, CH_2), 1.45–1.43 (m, 2H, CH_2), 1.28–1.13 (m, 12H). Mass analysis. ESI. Polarity positive. Calcd for $C_{29}H_{32}N_2$: M+H=408.5. Found: 409.4. Yield 50%.
Compound **4**. ^{1}H NMR ($CDCl_3$): δ, 8.25–8.15 (m, 5H, Ar), 7.97–7.95 (m, 1H, Ar), 7.71–7.69 (m, 2H, Ar), 7.57–7.48 (m, 5H, Ar), 4.22 (s, 2H, CH_2), 3.73 (t, 2H, $J=5.5$ Hz, CH_2), 2.96 (t, 2H, $J=6.5$ Hz, CH_2). Mass analysis. ESI. Polarity positive. Calcd for $C_{23}H_{19}NO$: $M=325$. Found: 326.1. Yield 65%.
Compound **5**. ^{1}H NMR (DMSO, d_6): δ, 8.22–8.13 (m, 5H, Ar), 7.94–7.92 (m, 1H, Ar), 7.69–7.68 (m, 2H, Ar), 7.67–7.66 (m, 2H, Ar), 7.55–7.48 (m, 5H, Ar), 4.16 (s,

2H, CH_2), 3.63 (t, 2H, $J=6.5$ Hz, CH_2), 2.75 (t, 2 H, $J=7$ Hz, CH_2), 1.58–1.39 (m, 4H, CH_2), 1.38–1.37 (m, 4H, CH_2). ^{13}C NMR ($CDCl_3$): δ, 31.67, 33.00, 35.75, 38.72, 55.47, 57.04, 66.83, 126.44, 126.76, 130.42, 132.76, 133.04, 133.75, 133.97, 134.34, 135.49, 136.83, 138.84, 140.42, 142.88. Mass analysis. ESI. Polarity positive. Calcd for $C_{27}H_{27}NO$: M+H = 381. Found: 382.3. Yield 68%.

24. Compound **6**. ^{31}P NMR [Ref. $OP(OMe)_3$] ($CDCl_3$): δ ppm: 142.83. Mass analysis. ESI. Polarity positive. Calcd for $C_{32}H_{36}N_3O_2P$: $M=525.4$. Found: 526.4.
25. Compound **7**. ^{31}P NMR [Ref. $OP(OMe)_3$] ($CDCl_3$): δ ppm: 142.84. Mass analysis. ESI. Polarity positive. Calcd for $C_{36}H_{44}N_3O_2P$: $M=581.18$. Found: 582.3.
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28. Treatment of the perylene-linker derivative **5** with anhydride acetic (10 equiv.) in anhydrous pyridine for 10 min (corresponding to the cumulative capping time during the sequence assembly) leads to the formation of two new products identified by ^1H NMR and electrospray mass analysis as the mono- and the bis-acetylated derivatives. This result confirmed that the oligonucleotide–perylene conjugate **14** was probably acetylated.
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